Environmental chemicals and their effects on female reproductive health: Searching for molecular mechanisms and effect biomarkers

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Environmental chemicals and their effects on female reproductive health: Searching for molecular mechanisms and effect biomarkers

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Photo
Julie Boberg (left, heart-shaped follicle), Hanna KL Johansson (right, word cloud).

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Preface

The work included in this PhD thesis was carried out at the National Food Institute, Technical University of Denmark and in the Fowler research group at the Institute of Medical Sciences, Aberdeen University, Scotland.

I have had great support and guidance from my supervisors: Rie Vinggaard who from the very beginning believed in me and gave me the opportunity to conduct this work, Julie Boberg who have had great patience with me and has been my "female-expert-to-go-to", and Terje Svingen who have come by my office to check up on me during dark times, and challenged my linguistic skills, gently nudging me in the "right" direction with loads of red text.

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Hanna KL Johansson

31 August 2016

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Summary

Incorrect developmental programming of the female reproductive tract can lead to compromised reproductive fitness later in life. It has been suggested that exposure to endocrine disrupting chemicals (EDCs) *in utero* can disrupt ovarian programming in humans, which is supported by several animal studies. However, it remains unclear which specific processes during development are affected, and if there are particular sensitive developmental windows. Most of the etiological evidence derives from rodent studies, whereas cause-effect relationships in humans are extremely difficult to obtain, not least due to the fact that there is a significant lag time between exposure during fetal life and disease symptoms in adulthood. Furthermore, humans are typically exposed to chemicals at a much lower dose than those of experimental studies, but exposed to a large number of different chemicals. This may lead to combination or mixture effects, where chemicals present at doses that would not cause effects on their own, can add up and cause an effect. The aim of the PhD project was to identify early biomarkers and sensitive windows for late life effects on the ovary after chemical exposure to mixtures of EDCs during early development.

A comprehensive literature review was synthesized to obtain an overview over current knowledge on the effects environmental chemicals can have on the developing ovary. This work identified four potentially sensitive windows of reproductive programming in females; i) primordial germ cell migration and gonadal sex determination, ii) meiosis, iii) follicle assembly, and iv) early folliculogenesis. For the experimental work, which aimed at identifying potential early biomarkers for late life diseases, two general approaches were adopted; a targeted approach looking at specific endpoints and a selection of effect biomarkers, and a more open-ended screening approach looking for potentially novel biomarkers. In the targeted approach, endpoints known to be important for reproductive function and ovary health were investigated at the molecular and morphological levels in neonatal, pre-pubertal and adult rat ovaries exposed to mixtures of EDCs during development. In the screening approach, a proteomics screen was performed to investigate differentially expressed proteins in the rat ovary after developmental exposure to mixtures of EDCs.

In the initial targeted approach, rat dams were exposed to a mixture of phthalates, pesticides, UV-filters, bisphenol A, butyl-paraben, as well as the mild analgesic paracetamol (PM). The compounds were tested all together (Totalmix) or in subgroups with anti-androgenic (AAmix) or estrogenic (Emix) properties. PM was tested separately. Reproductive endpoints were investigated in offspring at pre-puberty (PD22) and adulthood (approx. 1 year of age). In pre-pubertal animals a significant reduction in primordial follicle numbers was seen after AAmix and PM exposure, whereas in the 1 year old animals reduced ovary weights were seen in Totalmix-, AAmix-, and PM-groups. Finally, animals in the Totalmix group showed a higher incidence rate of irregular estrous cycles than control animals.

The reduction in primordial follicles after AAmix exposure was suspected to be caused by interruption to follicle assembly. Thus, a small pilot study, exposing explanted neonatal ovaries to AAmix, submixtures (pesticide mix (PEmix), phthtalate mix (PHmix)), and mono(2-ethylhexyl)phthalate (MEHP), was conducted. No significant effects were seen on gene expression, but histological evaluation showed that primordial follicles were reduced in the PEmix exposed ovaries.

For the proteomics screening study, a shotgun proteomics approach was performed on PD17 ovaries from offspring corresponding to those of the initial targeted study. Protein extracts were analyzed by LC-MS/MS, and evaluation of the data for potential effect biomarkers showed that three proteins, Trimethyllysine dioxygenase (TMLH), Keratin, type II cytoskeletal 8 (KRT8), and anti-Müllerian hormone (AMH) were dysregulated in all exposure groups. Also, ingenuity pathway analysis revealed canonical pathways known to be involved in ovary function, such as mTOR and HIPPO signaling, to be affected in all exposure groups.

In conclusion, the studies conducted for this PhD revealed that follicle count in pre-pubertal rats can potentially be used as a marker for early life affected ovary development caused by EDC mixture exposure, leading to reproductive senescence later in life. Furthermore, three proteins were identified as possible biomarkers for effects on the developing ovary, and potentially for late life adverse effects.

Dansk Resumé

Forringet reproduktiv evne i det voksne liv kan opstå på grund af fejlagtig programmering i udviklingen af de kvindelige reproduktive organer. Det er blevet foreslået at eksponering til hormonforstyrrende kemikalier *in utero* kan forstyrre æggestokkenes udvikling i mennesker, hvilket understøttes af adskillige studier i dyr. Det er stadig uklart, hvilke specifikke processer i udviklingen er påvirket og om der findes særligt følsomme stadier. De fleste ætiologiske beviser hentes fra studier i gnavere, mens årsag-effekt sammenhænge i mennesker er ekstremt svære at opnå, ikke mindst på grund af den betydelige forsinkelse mellem eksponering i fosterstadiet og symptomer på sygdomme i voksenlivet. Desuden er mennesker typisk eksponeret for meget lavere doser af kemikalier, end de doser der anvendes i eksperimentelle studier, men er til gengæld eksponeret for et stort antal forskellige kemikalier. Dette kan føre til kombinations- eller blandingseffekter, hvor kemikalier til stede i doser der ikke fører til effekter alene, i samspillet med andre kemikalier tilsammen fører til en effekt. Formålet med dette PhD projekt var at identificere biomarkører og følsomme stadier i den tidlige udvikling, hvor æggestokkene kan blive påvirket af eksponering til hormonforstyrrende kemikalier, hvilket kan give skadelige effekter senere i livet.

Et omfattende litteraturstudie blev gennemført for at få overblik over den nuværende viden om miljøkemikaliers effekter på æggestokke under udvikling. Studiet identificerede fire potentielle følsomme stadier i den reproduktive programmering i kvinder; i) migrationen af primordiale kønsceller og gonadernes kønsbestemmelse, ii) meiose, iii) follikeldannelse og iv) tidlig follikeludvikling. Det eksperimentelle arbejde til denne PhD havde til formål at identificere potentielle tidlige biomarkører for sygdomme senere i livet og to tilgange blev brugt; en målrettet tilgang, hvor specifikke effektmål og et udvalg af effektmarkører blev undersøgt og en mere åben screeningtilgang, hvor potentielle nye biomarkører blev undersøgt. I den målrettede tilgang blev effektmål, som er vigtige for reproduktiv funktionalitet og æggestokkenes sundhed undersøgt. Disse blev undersøgt på molekylært og morfologisk plan i neonatale, præpubertære og voksne rotters æggestokke efter eksponering til blandinger af hormonforstyrrende kemikalier under udviklingen. I screeningtilgangen blev en screening af proteomet udført, for at undersøge forskelligt udtrykte proteiner i rotternes æggestokke efter eksponering til blandinger af hormonforstyrrende kemikalier under udviklingen.

I den målrettede tilgang blev hunrotter eksponeret for en blanding af phthalater, pesticider, UV-filtre, bisfenol A, butyl-paraben og det milde smertestillende præparat paracetamol (PM). Kemikalierne blev undersøgt sammen (Totalmix) eller i undergrupper med antiandrogene (AAmix) eller østrogene (Emix) egenskaber. PM blev undersøgt separat. Reproduktive effektmål blev undersøgt i præpubertært afkom (PD22) og som voksne (ca. 1 år gamle). I præpubertære dyr sås en signifikant reduktion i antallet af primordiale follikler efter eksponering til AAmix og PM mens der sås reduceret vægt af æggestokke i de 1 år gamle dyr, perinatalt eksponeret til Totalmix, AAmix og PM. Dyrene eksponeret for Totalmix havde desuden en større forekomst af uregelmæssig østrus cyklus end kontroller.

Reduktion af primordiale follikler efter AAmix eksponering blev mistænkt for at være forskyldt af forstyrrelse af follikeldannelse. Derfor blev et lille pilotstudie udført, hvor eksplanterede neonatale æggestokke blev eksponeret for AAmix og undergrupper af blandinger (pesticidblanding (PEmix), phthalateblanding (PHmix) og Mono(2-ethylhexyl)phthalate (MEHP)). Ingen signifikante effekter blev fundet på genekspression mens histologiske undersøgelser viste at antallet af primordiale follikler var reduceret i æggestokkene eksponeret for PEmix.

I screeningtilgangen blev shotgun proteomics anvendt på PD17 æggestokke fra afkom tilsvarende dem i den målrettede tilgang. Proteinekstrakter blev analyseret med LC-MS/MS og evaluering af data med henblik på at identificere potentielle biomarkører viste, at tre proteiner, Trimethyllysine dioxygenase (TMLH), Keratin, type II cytoskeletal 8 (KRT8) og anti-Müllerian hormone (AMH) var fejlreguleret i alle eksponerede grupper. Ingenuity pathway analysis viste at grundlæggende proteinsammenhænge, kendt for at være involveret i æggestokkenes funktion, såsom mTOR og HIPPO signallering, var påvirket i alle eksponerede grupper.

Som konklusion viste eksperimenterne udført i denne PhD, at antal follikler i præpubertære rotter potentielt kan anvendes som biomarkør for tidlig overgangsalder forårsaget af eksponering til en blanding af hormonforstyrrende kemikalier. Desuden blev tre proteiner identificeret som mulige biomarkører for effekter på æggestokke under udvikling og potentielt for effekter senere i livet.

List of Manuscripts

Johansson HK, Svingen T, Fowler PA, Vinggaard AM, Boberg J. *Chemical exposure and ovarian dysgenesis: Sensitive developmental windows* (Accepted for publication in Nat Rev Endocrinol 17/11/16)

Johansson HK, Jacobsen PR, Hass U, Svingen T, Vinggaard AM, Isling LK, Axelstad M, Christiansen S, Boberg J. *Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging*. Reprod Toxicol 2016, Jun; 61: 186-94

Johansson HK, Boberg J, Vinggaard AM, Fowler PA, Stead D, Damholt ZBV, Hägglund P, Filis P. *Mixtures of endocrine disrupting chemicals alter the rat ovary proteome: a search for early biomarkers of late life adverse effects*. Manuscript in preparation.

Additional Publications

Boberg J, **Johansson HK**, Hadrup N, Dreisig K, Berthelsen L, Almstrup K, Vinggaard AM, Hass U. *Perinatal exposure to mixtures of anti-androgenic chemicals causes proliferative lesions in rat prostate*. Prostate 2015, Feb; 75(2):126-40

Mandrup KR, **Johansson HK**, Boberg J, Pedersen AS, Mortensen MS, Jørgensen JS, Vinggaard AM, Hass U. *Mixtures of environmentally relevant endocrine disrupting chemicals affectmammary gland development in female and male rats*. Reprod Toxicol 2015, Jul; 54: 47-57

Abbreviations

4-MBC	4-methyl-benzylidene camphor
ACTH	adrenocorticotropic hormone
AGD	anogenital distance
AMH	müllerian inhibiting factor
ANOVA	analysis of variance
AR	androgen receptor
BDNF	brain-derived neurotrophic factor
BPA	bisphenol A
CL	corpora lutea
CONTAMED	contaminant mixtures and human reproductive health – novel strategies for health impact and risk assessment of endocrine disrupters
DBP	di-n-butyl phthalate
DEHP	di-(2-ethylhexyl) phthalate
DES	diethylstilbestrol
dpc	days post coitum
dpp	days post partum
EDCs	endocrine disrupting chemicals
EE	ethinyl estradiol
Emix	estrogen mixture
ER	estrogen receptor
FDR	false discovry rate
FSH	follicle stimulating hormone
GD	gestation day
GH	growth hormone
LC-MS/MS	liquid chromatography mass spectrometry/mass spectrometry
LH	luteinizing hormone
MEHP	mono-(2-ethylhexyl) phthalate
ODS	ovarian dysgenesis syndrome
OECD	organisation for economic co-operation and development
OMC	octyl methoxycinnamate
p,p'-DDE	dichlorodiphenyl-dichloroethylene
p,p-DDT	dichlorodiphenyltrichloroethane)
PCOS	polycystic ovarian syndrome
PD	pup day
PEmix	pesticide mixture
PHmix	phthtalate mixture
PM	paracetamol
PND	postnatal day
PRL	prolactin
TDS	testicular dysgenesis syndrome
Totalmix	total mixture
TSH	thyroid stimulating hormone
UV-filter	ultra violet filter
VO	vaginal opening
AAmix	anti-androgen mixture

Chapter 1: Prelude & Overview

1.1 Female Reproductive Health and Endocrine Disrupting Chemicals

Over the last few decades, an increase in several female reproductive problems such as premature delivery, pre-eclampsia, gestational diabetes, and precocious puberty has been observed. In addition the number of women reporting difficulties conceiving and maintaining pregnancy has increased, especially among those below 25 years of age (Woodruff 2011; Woodruff et al. 2010). As these changes have occurred over a relatively short period of time, genetic adaptation can be excluded as the driving force, leaving epigenetic changes, environmental factors or sociological forces more likely. In many parts of the world, postponement of planned pregnancies is becoming more common amongst women, which can have major consequences for both their fertility and ability to complete pregnancy (Aitken 2013). Also environmental factors, including endocrine disrupting chemicals (EDCs), have been proposed to contribute (Diamanti-Kandarakis et al. 2009; Woodruff and Walker 2008; Woodruff et al. 2008). It remains uncertain, however, to what degree environmental chemicals can affect female reproductive health and if so, by what modes or mechanisms. It is this knowledge gap that formed the basis for this PhD project.

Several pathologies that present in adulthood are believed to originate from incorrect developmental programming during early embryogenesis through fetal morphogenesis and prenatal life. For instance, the impact of fetal environment on later life cardiovascular disease was presented many years ago (Barker et al. 1989, 1993); a concept which since has been applied to numerous late life disease manifestations, including compromised female reproductive fitness, collectively phrased as the ovarian dysgenesis syndrome (ODS) (Buck Louis et al. 2011).

EDCs have been proposed to contribute to ODS in humans (Buck Louis et al. 2011; Fowler et al. 2012), which is supported by animal studies showing that both pre- and perinatal exposures to EDCs can result in disrupted ovary-related functions later in life (Chao et al. 2012; Fernández et al. 2010; Gao et al. 2015; Rodríguez et al. 2010; Susiarjo et al. 2007; Wang et al. 2014; Zhang et al. 2013). Unfortunately, study guidelines for examination of reproductive toxicity in developmentally exposed offspring only include investigations in early adulthood and not later in life (OECD 2001, 2011). Therefore, effects on the reproductive system that occur with ageing may be overlooked. There are two ways of addressing this shortcoming. One is to extend the study guidelines to also include examination of aged animals, at a great additional cost. Another is to pinpoint sensitive biomarkers that can be detected early on, but predict late life effects, which would be the most cost-effective approach.

Biomarkers are widely used to diagnose different types of human cancers, including breast cancer (Couch et al. 2014; Dai et al. 2016), prostate cancer (Gaudreau et al. 2016), testis cancer (Rajpert-De Meyts et al. 2015), and ovary cancer (Bottoni and Scatena 2015; Couch et al. 2014). In relation to female reproduction, biomarkers such as AMH (Broer et al. 2014), FSH and inhibin B (Roudebush et al. 2008) are used for evaluation of female reproductive status. Finding an early biomarker(s) that can be used in young individuals to predict later life effects on reproductive endpoints would be of great interest, both in terms of animal experiments used for regulation of chemicals and for clinical use. Search for such an early biomarker(s) was also a key motivation for this PhD project.

Mixture effects are another important aspect that needs to be considered with respect to potential adverse effects of chemical exposure. In everyday life, humans are simultaneously exposed to a large number of different chemicals with endocrine disrupting properties (Svingen and Vinggaard 2016). Individually the human exposure levels to each chemical may not be of concern, but *in vitro* studies as well as *in vivo* studies (Kortenkamp 2014) have shown that single chemicals at doses not causing effects individually can add up to cause an overall effect, usually referred to as a combination, or mixture effect. Presently, when evaluating toxicity of chemicals, the general requirement is to assess one chemical at a time, which means that conventional risk assessment potentially underestimates the human hazard from low-dose exposures (Kortenkamp 2014; Svingen and Vinggaard 2016). Thus, there is a need for evaluating the toxicity of mixtures rather than single compounds (Backhaus et al. 2010; Hass et al. 2007; Kortenkamp 2007), a last point that was addressed during the PhD project.

1.2 Aim and Hypotheses

The overall aim was to identify sensitive processes and early biomarkers for late life effects on the female reproductive system after chemical mixture exposure during development. Two specific hypotheses were formulated:

1) Exposure to a mixture of EDCs during perinatal life will lead to adverse effects on reproductive endpoints in the adult female. These effects can be predicted in pre-pubertal rats by histological or molecular assessments.

2) Differentially regulated proteins in the ovary of pre-pubertal rats exposed to a mixture of EDCs during perinatal life can function as biomarkers for disrupted ovary development, and potentially predict late life effects on female reproductive endpoints.

1.3 Methodology

Experiments on intact animals and explanted ovaries were conducted. Ovaries were analyzed by histological and molecular methods.

To address the hypotheses listed above, two general approaches were implemented:

1) **Targeted approach**. Based on sensitive processes during ovary development, and previous studies investigating effects on the ovary following chemical exposure during development, morphological and molecular biomarkers were investigated (Chapter 3 and Chapter 5).

2) **Screening approach**. The ovarian proteome was analyzed for potential changes in expression levels of peptides that could serve as useful biomarkers of late life effects (Chapter 4).

1.4 Organization of this Thesis

The preceding section (Chapter 1) has introduced the topic of the PhD project and laid out the overall scope of the thesis. In the following sections, up-to-date literature reviews and specific research projects will be presented. Chapter 2 is an extensive literature review on the topic of developmental exposure to chemicals during sensitive time windows of ovarian development, and how this potentially causes late life effects. This chapter thus gives a general background for the overarching theme of the thesis: female reproduction and chemical exposure. Chapter 3 presents an initial study focusing on potential late life effects on the female reproductive system caused by exposure to EDC mixtures, including both young and aging rats and different combinations of chemicals. Chapter 4 presents an ovarian proteomics screen used to detect potentially novel biomarkers from pre-pubertal rats exposed to the same EDC mixtures as in Chapter 3. In Chapter 5 an organ culture approach using fetal and neonatal ovaries was used to further investigate the anti-androgen mixture, which showed most effects in the initial study (Chapter 3), aiming to elucidate some of the molecular mechanisms driving the overall effects. Finally, in Chapter 6, a short overview of the results from Chapters 2-5 and discussion are given, including general and future perspectives in relation to the combined results.

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Chapter 2: *Chemical exposure and ovarian dysgenesis: Sensitive developmental windows*

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ABSTRACT

The development of properly functioning ovaries is essential for a woman's reproductive health. Early disruption to ovarian programming can have long-lasting consequences, potentially manifesting as disease much later during adulthood. A growing body of evidence suggests that early life exposure to environmental chemicals can cause a range of late-life disorders, encompassed within the ovarian dysgenesis syndrome hypothesis. Here we describe four specific sensitive time windows where the ovary is particularly sensitive to disruption by exogenous insults: including gonadal sex determination, meiotic division, follicle assembly, and the first wave of follicular recruitment. To date, most evidence points towards the germ cell lineage being the most vulnerable, particularly meiotic division and follicle assembly. But the somatic cell lineages can also be affected by chemicals and pharmaceuticals, for instance bisphenols or mild analgesics. This review summarizes current knowledge pertaining to environmental chemicals and their potential contributions towards the ovarian dysgenesis syndrome, and further highlights knowledge gaps that need to be addressed to better safeguard female reproductive health.

INTRODUCTION

A woman's reproductive health and ability to have children impact directly on numerous aspects of her life, from personal well-being and socioeconomic standing, to morbidity and lifespan. It can further impact on society as a whole, affecting not only overall birth rates, but also represent a significant economic burden associated with the treatment of female reproductive disorders [1]. A better understanding of the underlying mechanisms leading to impaired female reproductive health is thus increasingly important, both from an individual and a societal viewpoint.

To a large degree, the biology of female reproductive health traces back to development of the ovaries during fetal life. From these early stages of life the ovaries harbor and nurse the oocytes, and later on, also produce female sex hormones that control pubertal progression, menstrual cycling, and menopausal onset. These are all connected and sometimes sequential processes, such that disruption of fetal ovarian development can result in reproductive dysfunction much later in life.

Disruption of normal ovarian development and function can result from various processes, ranging from genetic mutations to hormonal dysregulation. Exposure to endocrine disrupting compounds (EDCs) is increasingly associated with reproductive dysfunction. The WHO have defined an endocrine disruptor as: *an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations*" [2,3]. More than 15 years ago it was suggested that fetal exposure to environmental chemicals can contribute to a rise in male reproductive disorders and decline in male fertility, now widely known as the testicular dysgenesis syndrome. A corresponding relationship between early ovarian dysgenesis (Box 1) and late reproductive disorders in females was recently developed and termed the ovarian dysgenesis syndrome (ODS) (Box 2). This is defined as early alterations in ovarian structure or function that cause impairment of reproductive parameters in adulthood [4,5] (Fig. 1).

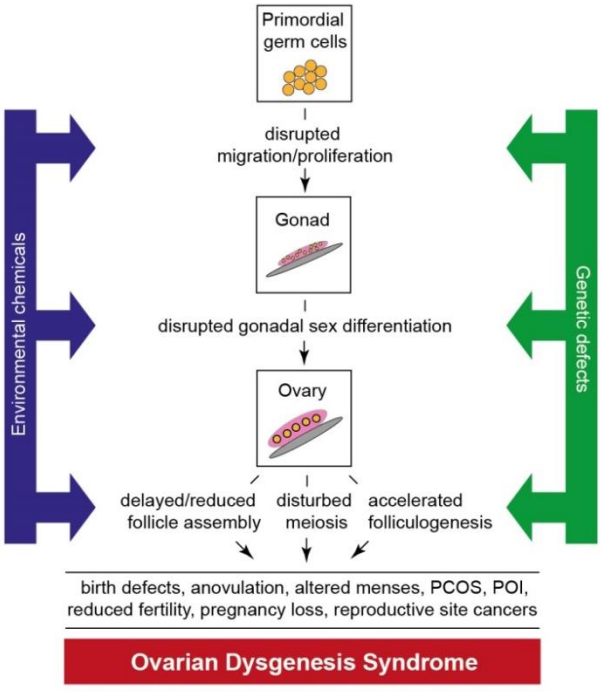

Fig. 1 Ovarian dysgenesis syndrome (ODS) hypothesis. ODS is based on the underpinning concept that early disruption of ovarian structure or function caused by either genetic or environmental factors, leads to impairment of reproductive function later in life. Several processes during development are sensitive to perturbation; primordial germ cell proliferation, gonadal sex differentiation, meiosis, follicle assembly, and early folliculogenesis are all processes shown to be disrupted after chemical exposure.

A growing body of evidence supports the view that disruption of ovarian development early in life, including intrauterine exposure to EDCs, can manifest as ODS later in life. [4,6,7]. Notably, because of the intrinsic difficulty in retrospectively obtaining exposure data for women presenting with reproductive disorders, studies reporting on clear associations between fetal EDC exposure and ODS are scarce. One well-known case exists, namely diethylstilbestrol (DES). DES is a synthestic estrogen that was used in the period between 1940 and 1970s to reduce the risk of complications associated with pregnancy. Unfortunately, fetal exposure to DES caused a rare form of vaginal cancers in girls and young women who had been exposed *in utero* [8]. Later studies also showed associations between *in utero* exposure to DES and impaired late-life fecundity [9], earlier age at menopause [10–12], increased incidence of uterine fibroids [13,14], endometriosis [15], and reproductive site cancers [16]. Besides DES, other *in utero* exposures have recently been associated with female reproductive health effects later in life. Examples include the associations between maternal smoking and reduced fecundity [17], perfluorooctanoic acid exposure with delayed menarche [18], and organochlorine pollutants such as dichlorodiphenyldichloroethylene and hexachlorobenzene with a reduced number of ovarian follicles [19]. Also, increased serum levels of bisphenol A (BPA) have been associated with PCOS, although the possible mechnisms by which BPA contribute to the pathogenessis are poorly understood [20]. In support of these associations in humans, animal studies have revealed comparable effects. For instance, developmental exposure to chemicals with estrogenic or anti-androgenic activity has been shown to reduce the follicle pool in rodents [21–24], and this can in turn lead to a shortened reproductive lifespan. This, extrapolated to the human, would not only affect a woman's ability to have children, but would also carry an increased risk of associated diseases such as osteoporosis and cardiovascular disease [25].

Box 1 Ovarian dysgenesis

Synonymous with gonadal dysgenesis, but specific for female reproduction and not including testicular conditions or gonadal dysgenesis where ovaries/testes fail to form altogether, such as streak gonads. Rather, ovarian dysgenesis refers to conditions where the ovaries are either underdeveloped or imperfectly formed, which compromises further reproductive

Box 2 The ovarian dysgenesis syndrome hypothesis

The ovarian dysgenesis syndrome (ODS) hypothesis proposes a common origin for a group of related female reproductive diseases and disorders such as reduced fertility, polycystic ovarian syndrome, premature ovarian insufficiency, and reproductive site cancers. The potential causative events can be many, but it is proposed that ovarian development and function is disrupted by genetic or environmental factors, ultimately leading to late-life reproductive health issues.

It is without question that disruption to fetal development can have dire consequences for postnatal life. In the case of late-onset female reproductive diseases such as subfertility, PCOS, or premature menopause, however, a causative link is difficult to establish since the initiating events occur much earlier than the adverse phenotypes are observed. It is particularly difficult to provide direct evidence for the ODS hypothesis in human subjects, but controlled animal experiments can be useful to clarify such relationships. For example, pre- and perinatal exposures to EDCs have been shown to result in disruption of endocrine-

sensitive tissues and organs in female offspring later in life [21,23,24,26–29], which largely phenocopy corresponding human disorders.

This review synthesizes current knowledge on possible endocrine disrupter actions on the developing ovary, with particular focus on four potentially sensitive windows during ovarian development: gonadal sex differentiation, meiosis, follicular assembly, and early folliculogenesis (Fig. 2). To pinpoint these sensitive windows, we have limited this review to mainly include studies with short exposure periods during specific developmental phases, and we have therefore omitted a large number of studies using longer exposure periods such as the entire gestational and lactational period. Some animal studies on chemical exposures have used rather high doses compared to average human exposure levels, which will be highlighted where relevant, but it should be kept in mind that during sensitive developmental window, even short-term exposure can have irreversible consequences.

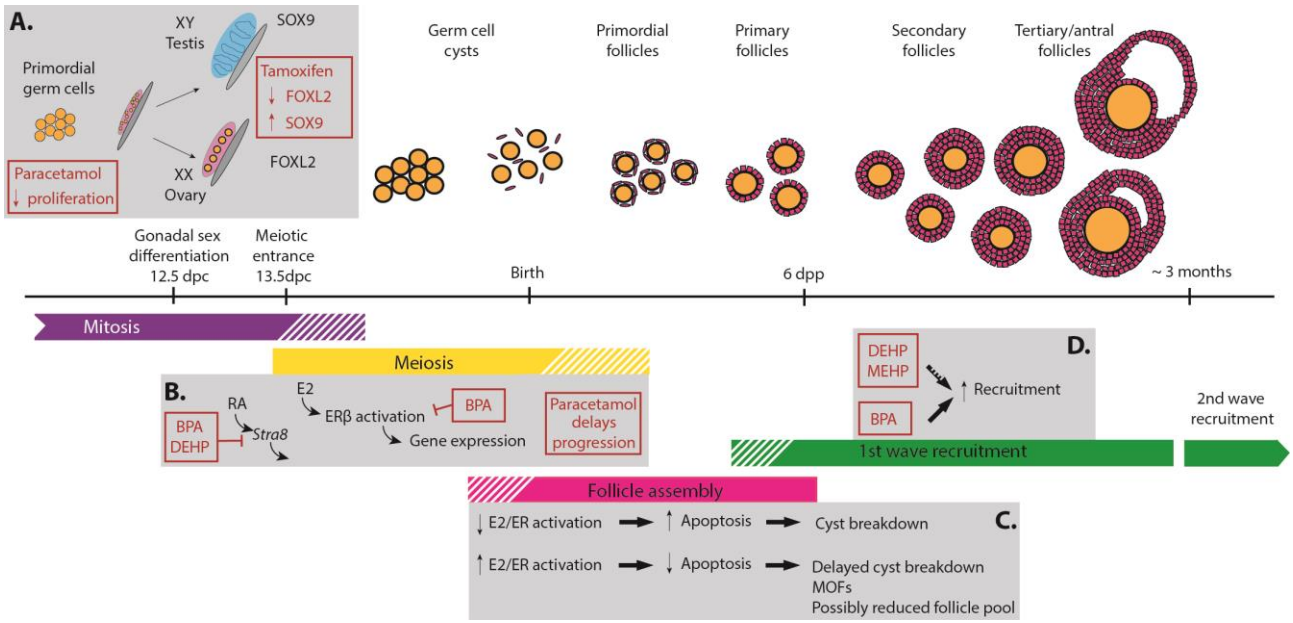

Fig. 2 Germ cell development and chemical disruption in the mouse. A) Primordial germ cells proliferate until initiation of meiosis at 13.5 dpc. The common analgesic paracetamol is associated with reduced germ cell proliferation which can cause a reduction in the number of oocytes in the adult ovaries. Gonadal sex differentiation is also sensitive to perturbation, and the pharmaceutical tamoxifen can disturb the balance between male and female factors expressed in the XX gonad (future ovary). B) Meiotic progression is delayed following BPA or DEHP exposure, which may be explained by reduced expression of *Stra8* mRNA (BPA, DEHP) and protein (DEHP). BPA may also antagonize ERβ, thereby leading to synaptic aberrations and increased levels of recombination. Furthermore, paracetamol can delay meiotic progression. C) Follicle assembly is critical for establishing the primordial follicle pool. BPA, DEHP and parabens can all interfere with this process, possibly by disrupting apoptotic signaling pathways. D) The first wave of follicular recruitment is initiated immediately after the primordial follicle pool has been established and BPA, DEHP, or the DEHP-metabolite MEHP, can accelerate the rate of follicular recruitment.

EARLY GONADAL DEVELOPMENT

The primordial germ cells originate far from the testes or ovaries and thus have to migrate through the developing fetus before taking up residence in the primitive gonadal structures[30]. At this time of development the gonadal ridges are still bipotential until gonadal sex determination pushes the XY or XX gonads down a male or female trajectory culminating in the differentiation of either testes or ovaries, respectively. This occurs during the first trimester in humans, and about halfway through gestation in rats and mice [31]. The divergence in development between the two sexes is triggered by the expression of the Y chromosome-specific gene *Sry* in XY gonadal ridges [32]. In the absence of a Y chromosome, or more specifically *Sry*, the XX gonadal ridges differentiate into ovaries. The early differentiation of testes and ovaries are chiefly under genetic control and involves several antagonizing morphogenetic pathways, as extensively reviewed by others [31,33–35]. Nevertheless, a potential vulnerability of this process towards exogenous perturbation has been suggested [36].

Studies investigating potential effects of chemicals on primordial germ cells and gonadal sex differentiation are scarce, but some exist (Table 1). In a recent study [37], intrauterine exposure to the pharmaceutical paracetamol (also known as acetaminophen. See Box 3) from 7 days *post coitum* (dpc) was shown to reduce the number of germ cells in female mouse fetuses as early as 13.5 dpc, likely caused by perturbed proliferation of the primordial germ cells (Fig. 2A). In mice exposed from 7 dpc until birth, the effects persisted into adulthood where the ovarian follicle reserves and total number of follicles were significantly reduced, finally resulting in subfertility in ten months old animals [37]. By exposing mouse embryonic stem cells, the same study showed an apparent decrease in mitotic activity, which could point towards a mechanism by which paracetamol can limit oocyte numbers at birth. Of further interest is that fetal germ cells express Cyclooxygenase 2 (COX2) and Prostaglandin E2 (PGE2) receptor [38,39], both of which are targets of paracetamol. In fact, Dean and co-workers [39] showed that intrauterine exposure of rats to paracetamol can delay meiotic entry of germ cells in the developing ovaries. However, exactly how paracetamol interfere with the germ cells, either directly at the molecular level, or indirectly by affecting supporting somatic cells, remains largely unknown [40]. Appropriate mechanistic studies are therefore important avenues to pursue in future studies.

A recent study showed that exposure to the ER-modulating pharmaceutical Tamoxifen at 10.5 dpc caused disruption of gonadal sex differentiation by inducing ectopic upregulation of *Sox9*, and down regulation of *Foxl2* in the XX gonads at 13.5

Box 3 Tyleonol, paracetamol and acetaminophen

Acetaminophen and paracetamol are essentially the same, mild analgesic and antipyretic drug, but distributed under different names in North America and the rest of the world, respectively. They are both generic names of the main active ingredient in the original brand drug Tylenol, which derives from N-acetyl-para-aminophenol. Panadol is another brand name.

dpc [41]. *Sox9* is the key factor transactivated by SRY in the XY gonadal ridges and is both necessary and sufficient to ensure proper testis differentiation [31]. One way in which SOX9 ensures testis differentiation, apart from transactivating pro-testis genes, is by also suppressing pro-ovary factors such as *Foxl2* within the pre-Sertoli cell population [42,43]. Disrupting the balance between these opposing factors can lead to sex-reversal phenotypes, and also cause reprogramming of the cell types in adulthood [44–47]. Such sexual reprogramming of the gonadal supporting cells will adversely affect the germ cell population, ultimately leading to infertility. Notably, *FOXL2* mutations in humans cause premature ovarian insufficiency [48], emphasizing the importance of this protein in female reproduction. Interestingly, in adult mice the repressive activity of FOXL2 towards *Sox9* expression is synergistically increased in the presence of ERα [46] and *Esr1/Esr2* double knock-out mice display a phenotype similar to that of *Foxl2* mutations [49]. This implies an important role of estrogen receptors in sustaining the integrity of the ovary during fetal life, since they are expressed in the human (*ESR2* only) and rodent fetal ovary [50,51]. Whether or not the mechanism of action causing the effects seen by Yu and co-workers [41] was due to interference with estrogen receptors, however, remains unanswered.

Table 1 Effects of exposure to chemicals during primordial germ cell mitosis and gonadal sex differentiation.

Species	Exposure	Effects of Exposure	Reference
Mouse (C57BL/6JBom)	Paracetamol (50 or 159 mg/kg/day) by gavage, 7 dpc-birth, killed at 7 weeks	↓ primordial follicles, ↓ growing follicles (primary and secondary), ↔ preantral, antral or atretic antral follicles, ↓ total follicles, ↓ fertility at 6 and 10 months (only 50mg/kg/day tested)	37
	Paracetamol (50 mg/kg/day) by gavage, 7-13.5 dpc	↓ *Mvh* expression (indicating reduction in germ cell numbers)	
	Paracetamol (100 µM) in ovary culture 12.5-15.5 dpc	↔ *Mvh, Stra8, Scp3, Oct4, Sox2, Nanog* (indicating no effect on germ cell numbers, meiotic entry nor expression of pluripotency markers)	
Nanog GFP reporter mouse embryonic stem cells (C57/BL6 mouse)	Paracetamol (50, 100, or 150 µM) to low passage stem cells for 72h	↓ total number of cells (indicating inhibited proliferation)	
Mouse (ICR)	Tamoxifen (1 mg/kg bw) i.p., 10.5 and 13.5 dpc pups	↑ *Pdgra* in XX gonads (ectopical expression), ↑ Sox9 (gene and protein) in XX gonads, ↓ Foxl2 (gene and protein) in XX gonads	41
	Tamoxifen (0.1 or 1.0 µM) to ovary culture 13.5-16.5 dpc	↑ *Pdgfra* and *Fgf9* in XX gonad, ↓ 17B-estradiol and progesterone, ↑ testosterone, ↓ estradiol/testosterone ratio	
Embryonic stem cells (ICR mice)	BPA (50µM), to embryonic stem cells	↓*Sox9*, *Fgf9* ↑*Foxl2, Wnt4*	52

Interestingly, the industrial chemical BPA, widely reported to possess estrogenic and other endocrine disrupting properties, has also been shown to down-regulate the testis-promoting genes *Sox9* and *Fgf9*, and upregulate the ovary-promoting genes *Wnt4* and *Foxl2* in mouse embryonic stem cells during differentiation through embryoid body formation [52]. This could suggest that BPA exposure favors ovary differentiation, but as these findings were seen *in vitro*, it remains to be seen whether it can directly affect gonadal development *in vivo* and at human relevant dose levels.

As paracetamol and tamoxifen are pharmaceuticals, humans may be exposed to high doses over short periods throughout life, and it is not unlikely that pregnant women may be exposed to paracetamol in doses comparable to those affecting gonad development in mice [37]. Tamoxifen use (for breast cancer treatment) is not recommended during pregnancy due to risk of congenital malformations, but in the context of explaining risk factors for ovarian dysgenesis, it here serves as a model compound having an estrogen receptor modulating mode of action. Nevertheless, it appears that early chemical exposure can result in adverse effects on the future oocyte reservoir and reproductive lifespan, a worrying scenario that warrants far more attention than it presently receives.

MEIOSIS

Meiotic division is unique to the germ cells and occurs at different developmental stages in males and females. In males, meiosis is initiated at puberty and continues throughout adulthood, whereas in females meiosis is initiated during fetal life, but arrested in prophase I until puberty [53]. In female mice, meiosis is initiated at 13.5 dpc through the action of retinoic acid [54,55]. This triggers a chain of events whereby the germ cells are primed for oogenesis by entering the first phase of meiosis [56]. Disruption of germ cells at this stage can have long-lasting consequences for reproductive health parameters, for instance by reducing the number of oocytes through defective recombination [57], loss of oocytes due to lack of REC8 – an important component of the Cohesin complex [58]; or oocyte loss and creation of aneuploid gametes due to disturbance of the synaptonemal complex protein SYCP3 expression [59]. There are indications that EDCs can interfere with some of these processes (Table 2), for instance by interfering with estrogenic activity [28], which in turn can have adverse consequences for the quality and quantity of germ cells in the adult female, ultimately affecting fertility and reproductive lifespan.

Chemical exposure affects meiotic progression

Little more than a decade ago, members of the Hunt laboratory at Washington State University noted a spontaneous increase in meiotic spindle misalignments and an increased rate of aneuploidy in control female

mice; that is, the expected normal rate was inexplicably increased. After much effort in tracking down the source of this abnormal phenotype in what should be healthy control animals, it was discovered that damaged cages and water bottles were leaching BPA [60]. This finding spurred on additional investigations and there are now data suggesting that BPA can disturb meiotic division in both mice and monkeys [28,61]. It is thought that this effect is caused by BPA antagonizing estrogen receptor-beta (ERβ), since ERβ knock-out mice display virtually identical defects as BPA-exposed mice [28] (Fig. 2B). It is pertinent that the human fetal ovary only expresses ERβ, not ERα [62].

BPA exposure has also been associated with a delay in prophase I at 17.5 dpc in mice, which could be explained by a reduced expression of *Stra8*, possibly due to increased methylation [63]. Subtle changes in other genes involved in meiosis have also been seen after *in vivo* exposure to a low dose of BPA [64], which warrants further investigations.

There are also some indications that the phthalate DEHP can affect meiotic progression. For instance, Zhang *et al* [65] showed delayed meiotic progression at 17.5 dpc in mouse fetuses exposed to DEHP *in utero*. This likely relates to delayed initiation of meiosis, since the mice displayed significantly reduced expression of STRA8 at 13.5 dpc, both at the mRNA and protein levels (Fig. 2B).

There is strong evidence to suggest that the mild analgesics paracetamol and indomethacin can interfere with meiosis. Exposure of female rat fetuses to paracetamol (350 mg/kg/day) or indomethacin (0.8 mg/kg/day) resulted in a reduction in the number of germ cells at 21.5 dpc, likely caused by a delay in meiotic entry or progression, culminating in reduced ovary weight and fertility in adulthood [39]. The idea that analgesics can have endocrine disrupting properties and potentially affect reproductive development of fetuses exposed via their mother has been around for the better part of two decades, but the focus has very much been on male development [40]. The fact that the germ cells may be susceptible to perturbation by analgesics at human-relevant doses, however, adds another level of concern for the often indiscriminate use of over-the-counter mild analgesics.

Taken together, it appears clear that both industrial chemicals and pharmaceuticals can adversely affect early oocyte development in mammals, for instance by disrupting meiotic recombination and synapsis [28,57] or delaying meiotic progression [65], which ultimately will impact on the offspring's reproductive capacity (Table 2). Further studies aiming to decipher the human relevance of these findings are warranted, but at least the dose levels of BPA and DEHP affecting meiosis in mouse and monkey is low [61,64,65] and within the human exposure range [66–68]. Effects from paracetamol are seen at much higher doses, but also here comparable to human exposure during short-term use. Interestingly, xenobiotics can also affect female reproductive parameters later during development, as will be discussed in the following.

Table 2 Effects of exposure to chemicals during meiosis.

Species	Exposure	Effects of Exposure	Reference
Mouse (C57BL/6J)	BPA (released 400ng/day pellet implant, GD 11.5-18.5, fetuses removed and killed at GD 18.5	↔ progression through prophase, ↑ synaptic abnormalities (mainly incomplete synapsis and end-to-end associations), ↑ MLH1 foci counts (effects on recombination)	28
	BPA (released 400ng/day) pellet implant, GD 11.5-term, pups fostered by untreated females, killed at 4-5 weeks of age	↑ average number of chiasmata per cell (4 weeks), ↑ frequency of univalents (4 weeks), ↑ hyperploid eggs, ↔ aneuploidy in embryos (from super-ovulated 4-5 week old females mated with wild-type males)	
Rat (Wistar)	Indomethacin (0.8 mg/kg/day) s.c., 15.5-18.5dpc, killed 15.5, 16.5, 17.5, 18.5, and 21.5dpc. 25 dpp and 90 dpp (adult)	F1: ↓ germ cell number, 21.5dpc, ↑ *Stra8, Dmrt1, Lin28* (17.5 and 18.5dpc) indicating delay in meiotic entry ↓ ovary weight and fertility (adult) F2: ↓ovary weight (25 dpp and adult), ↓ primordial follicles and total follicles (25 dpp), ↑ serum AMH (adult)	39
	Paracetamol (350mg/kg) by gavage, 13.5-21.5dpc, killed 15.5, 16.5, 17.5, 18.5, and 21.5 dpc. 25 dpp and 90dpp (adult)	F1: ↓ germ cell number, 21.5dpc, ↑ *Stra8, Dmrt1, Lin28* (17.5 and 18.5 dpc) indicating delay in meiotic entry ↓ ovary weight and fertility (adult) F2: ↓ovary weight (25 dpp and adult), ↓ primordial follicles and antral follicles (25 dpp), ↑ serum AMH (adult)	
Mouse (various strains and genotypes)	BPA (intentional damage of water bottles comprised of BPA only) via water bottle	↑ congression failure	60
	BPA (20, 40, 100 ng/g bw/day oral, beginning at 20-22dpn lasting for 6-8 days	↑ congression failure	
Rhesus macaques	BPA (400µg/kg bw/day) oral, GD50-100, fetus killed at GD100	Due to technical problems a meaningful analysis of MLH1 foci was not possible, ↔ centromere associations (synaptic effects)	61
	BPA (serum levels 2.2-3.3ng/mL) silastic tubing implant (continuous exposure), GD50-100, fetus removed and killed at GD100	↑ MLH1 foci/cell (effects on recombination), ↑ centromere associations (synaptic effects)	
Mouse (CD-1)	BPA (0.02, 0.04, 0.08 mg/kg) oral, 12.5-18.5 dpc, pups killed on 15.5, 17.5, and 19.5 dpc	15.5-19.5dpc: delay in prophase I, 17.5dpc: ↓ *Stra8,* ↑ methylation of *Stra8*	63
Mouse (C57BL/6J)	BPA (20ng/g bw/day)oral, initiated at 11dpc and fetuses removed and killed on 12, 12.5, 13.5 and 14.5 dpc	↑ genes affected from 12-14.5dpc (most changes on 14.5dpc: 2661 genes), subtle changes (less than 2 fold for almost all transcripts), 30 genes were ↑↓ regulated at three successive time points, out of these 23 ↑ and 7↓ expression.	64
Mouse (CD-1)	DEHP (40µg/kg/day) oral, 0.5dpc-18.5dpc, fetuses removed and killed on 13.5 and 17.5dpc	↓ Stra8 expression (gene and protein, 13.5dpc) ↑ oocytes in leptotene and zygotene phases, ↓ oocytes in pachytene and diplotene phases (indicating delay in meiotic progression, 17.5dpc),	65

FOLLICLE ASSEMBLY

After colonizing the gonads during fetal life, female germ cells quickly arrange into germ cell cysts [69]. Towards the end of gestation in mice, 2-3 days before birth, germ cell cysts within the medullary region of the ovary break down, resulting in a reduction in the number of oocytes and the beginning of primordial follicle formation [70]. In contrast, germ cell cyst breakdown within the ovarian cortex initiates shortly after birth [71]. Although the exact mechanisms involved are not known, somatic pre-granulosa cells contribute by intruding between closely associated germ cells, thereby establishing single cells surrounded by pre-granulosa cells, called primordial follicles [71]. Concurrently, a large proportion of the germ cells undergo programmed cell death, where both autophagy and apoptosis are involved [72,73]. By 4 days *post partum* (dpp), approximately 1/3 of the original oocyte reserve remains [70,71]. In rodents, follicle assembly continues until 6 dpp, after which almost all the remaining oocytes are enclosed within primordial follicles [71].

In humans the timing of primordial follicle assembly is very different from that of rodents, with follicle assembly occuring mid-gestation (Fig. 3). The processes involved – cyst breakdown, germ cell death and formation of primordial follicles – are similar [74], although less spatially organized. However, the initiating mechanisms might be different, especially considering the different environmental conditions that exist between fetal and postnatal life. By extension, these differences must be considered when extrapolating data from one species to another, as when evaluating studies on rodent exposure to environmental chemicals and potential effects on germ cell development.

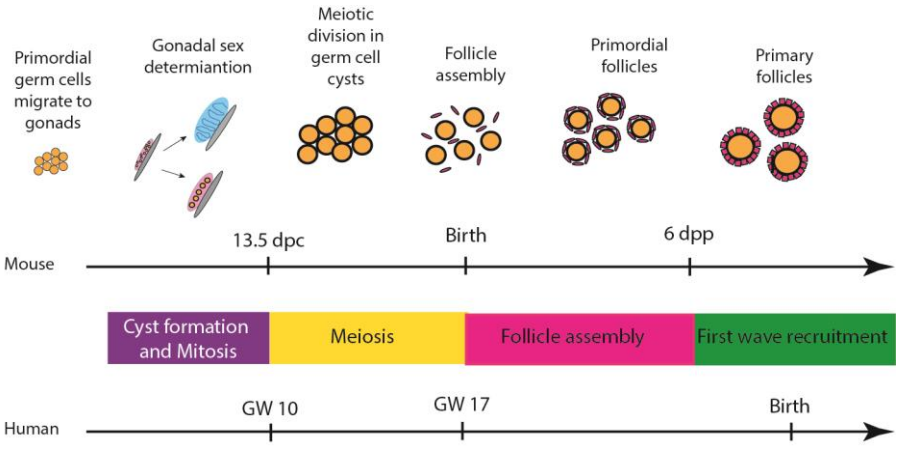

Fig. 3 Temporal comparison between human and mouse germ cell development. Germ cell development, from primordial germ cell migration to the gonadal anlagen during embryogenesis through to folliculogenesis in the developed ovaries, is comparable in mice and humans, but differs in terms of the developmental stage when the processes take place.

Chemical exposure affects follicle assembly

The mechanisms controlling follicular assembly are not well understood. In mouse fetuses, however, high maternal levels of estradiol and progesterone have been suggested to inhibit follicular assembly, whereas a reduction in steroid hormone levels around the time of birth proposedly initiates the process [75–77]. In non-human primates, where follicle assembly occurs during gestation as in humans, the situation is opposite; reduction in estrogen levels inhibits follicle assembly [78]. Furthermore, the human fetal ovary expresses steroidogenesis enzymes and steroid hormone receptors, especially ESR2 (ERβ), during the second trimester, which allows estrogenic, progestogenic and androgenic signaling to occur [62,79]. This indicates an important role for estrogen in this process, but also highlights the potential susceptibility to disruption by estrogenic compounds interfering with normal estrogen signal transduction. Indeed, maternal cigarette smoking, which lowers adult fecundity, is associated both with increased estrogen in the human fetus and onset of early primordial follicle formation [80]. Such effects of estrogen also suggests that EDCs can affect the germ cells differently depending on what species are exposed (Table 3). Nevertheless, in rats and mice estrogens possibly delays initiation of follicular assembly, which is supported by several studies. For instance, subcutaneous exposure of neonatal mice to the phytoestrogen genistein inhibits cyst breakdown and disrupts primordial follicle assembly [76]. Similar effects are seen after subcutaneous exposure of neonatal mice from 1-5 dpp to the synthetic estrogens diethylstilbestrol (DES), ethinyl estradiol (EE), and BPA, which all inhibit cyst breakdown, increase the total number of oocytes, and increase the percentage of primordial follicles, when measured at 5 dpp [81]. An opposite effect, reduction in primordial follicles at 8 dpp, was seen in a study by Rodríguez *et al* [23] where neonatal rat pups had been exposed to DES (0.2 and 20μg/kg) and BPA (20mg/kg) at 1, 3, 5, and 7 dpp. This suggests that mice and rats are affected differentley, supported by a study showing a reduced oocyte pool at 3 and 6 dpp in rat pups treated with estrogen between 0-2 dpp [82]. Finally, in the hamster it seems that estrogen stimulates follicle assembly rather than oppose it as is the case in mice [83].

Table 3 Effects of exposure to chemicals during follicle assembly.

Species	Exposure	Effects of Exposure	References
Rat (Wistar)	DES (0.2 and 20 μg/kg) s.c., 1, 3, 5, and 7 dpp, killed on 8 dpp	↓ primordial follicles, ↑ recruited follicles, ↑ MOFs (20mg/kg)	23
	BPA (0.05 and 20 mg/kg) s.c., 1, 3, 5, and 7 dpp, killed on 8 dpp	↓ primordial follicles (20mg/kg), ↑ recruited follicles (20mg/kg), ↔ MOFs	
Mouse (FVB)	BPA (0.5, 20, and 50 μg/kg/day) oral, 11dpc-birth, pups killed on 4 dpp	↑ germ cells in nests, ↓ primordial follicles (0.5 and 50 μg/kg/day), ↔ primary follicles, ↑ in anti-apoptotic factors *Bcl2* (50 μg/kg/day) and *Bcl2l1* (20 and 50 μg/kg/day), ↓ pro-apoptotic factors *Bax* (0.5, and 20 μg/kg/day) and *Bak1* (20 and 50 μg/kg/day)	29

Table 3 continued

Species	Exposure	Effects of Exposure	References
Rhesus macaques	BPA (400µg/kg bw/day) oral, GD100-term, fetus removed and killed at GD100	↑ MOFs	61
	BPA (serum levels 2.2-3.3ng/mL) silastic tubing implant (continuous exposure), GD100-term, fetus removed and killed at GD100	↔ MOFs, phenotype: ↑ small, unenclosed oocytes (possibly due to delayed meiosis)	
Mouse (CD-1)	BPA (0.02, 0.04, 0.08 mg/kg) oral, 12.5-18.5 dpc, pups killed 15.5, 17.5, and 19.5 dpc ,and 3,5, and 7 dpp	15.5-19.5dpc: delay in prophase I, 17.5dpc: ↓ expression of *Stra8,* ↑ methylation of *Stra8*, 3 dpp: ↑ oocytes in cysts (0.08mg/kg), ↓ primordial follicles (0.08 mg/kg), ↑ oocytes/section (0.08mg/kg), 5 and 7 dpp: ↔ oocytes	63
Mouse (CD-1)	DEHP (40µg/kg/day) oral, 0.5dpc-18.5dpc, killed after birth	Large regions of germ cells cysts, rare follicles, ↑ number of germ cells in ovarian cysts	65
Mouse (CD-1)	Genistein (10-9 - 10-4 M) organ culture, 1-7 dpp collected after 2, 3, 4, 5, 6, and 7 days of culture	↓ single oocytes (10-8 M and higher), ↔ total number of oocytes /section, ↑ unassembled follicles	75
Mouse (CD-1)	Genistein (50mg/kg/day) s.c., 1-5 dpp, pups killed on 2-6 dpp	↑unassembled oocytes (4 dpp), ↓ primordial and primary follicles (4 dpp), ↓ single oocytes (4,5, and 6 dpp), ↑ (0.5%) intracellular bridges, ↑ total no of oocytes (4 and 6 dpp), ↑ apoptosis (2 dpp), ↓apoptosis (3 dpp)	76
Mouse (CD-1)	DES (5mg/kg/day, 50mg/kg/day) s.c. on 1-4 dpp, pups killed on 3 and 5 dpp	3 dpp (only 50mg/kg/day tested): ↓ apoptosis, 5 dpp: ↓ single oocytes, ↑ oocytes/section (50mg/kg/day), ↑ primordial follicles (50mg/kg/day)	81
	EE (5mg/kg/day, 50mg/kg/day) s.c. on 1-4 dpp, pups killed on 3 and 5 dpp	3 dpp (only 50mg/kg/day tested): ↓ apoptosis, 5 dpp: ↓ single oocytes, ↑ oocytes/section (50mg/kg/day), ↑ primordial follicles (50mg/kg/day)	
	BPA (5mg/kg/day, 50mg/kg/day) s.c. on 1-4 dpp, pups killed on 3 and 5 dpp	3 dpp (only 50mg/kg/day tested): ↓ apoptosis, 5 dpp: ↓ single oocytes (50mg/kg/day), ↑ oocytes/section, ↑ primordial follicles, ↓primary follicles (50mg/kg/day)	
Mouse (BalB/C)	DEHP (2.5, 5, 10 µg/g bw/ day) i.p., 0-4 dpp, killed on 5 dpp	↓ primordial follicles, ↔ total oocyte number,	84
	DEHP (25, 50, 100 µM) organ culture, 16.5-3 dpp	↓ primordial follicles, ↔ total oocyte number,	
	DEHP (50 µM) organ culture, 16.5-3 dpp	↓ single oocytes	
	DMSO + ICI 182,780 (10µM) organ culture, 16.5-3 dpp	↔ single oocytes	
	DEHP (50 µM)+ICI 182,780 (1µM), DEHP (50 µM)+ICI 182,780 (10µM) organ culture, 16.5-3 dpp	↑ single oocytes compared to DEHP alone	
	DEHP (50 µM) organ culture, 16.5-1dpp	↓ *Erα, Erβ, Pr, Jag1, Notch2, Hes1, Hey2* mRNA, ↓ ERβ, PR, JAG1, NOTHC2, HEY2 protein	
	DEHP (50µM) + ICI 182,780 (10µM), organ culture, 16.5dpc-1dpp	↓ *Jag1, Hes1, Hey2* mRNA	
Rat (Sprague-Dawley)	methyl-(MP), propyl-(PP), and butyl-paraben (BP) (62.5, 250, 1000 mg/kg/day, E2 (40µg/kg/day) s.c. on 1-7 dpp, killed 8 dpp	↑ primordial follicles (E2, high-dose PP and BP), ↓ early primary (E2, high-dose PP and BP),	85

Table 3 continued

Species	Exposure	Effects of Exposure	References
Mouse (CD-1)	BPA (10µM and 100 µM) organ culture on 1-3 dpp, collected on day 3	↑ oocytes in nests, ↓ primordial follicles, ↑ apoptotic cells (100 µM), ↑ gene expression of the pro-apoptotic *Bax* (100 µM), ↔ gene expression of the anti-apoptotic *Bcl2*	86
	DEHP (10µM and 100µM) organ culture, 1-3 dpp, collected on day 3	↑ oocytes in nests, ↓ primordial follicles, ↑ apoptotic cells, ↑ gene expression of the pro-apoptotic *Bax* (100 µM), ↔ gene expression of the anti-apoptotic *Bcl2*	
Mouse (CD-1)	BPA (0.44, 4.4, 22, and 44 µM) organ culture, 0-8 dpp, collected on day 1, 2, 4 and 8	1 dpp: ↔ expression of Bad, Bax and Bok, ↑ Bcl2 (4.4 and 22µM) 2 dpp: ↔ oocytes, ↑ expression of *Bad* (4.4 and 44 µM), ↔ expression of *Bax* and *Bok,* ↑expression of *Bcl2 (22 and 44µM) and Bclxl* 4 dpp: ↑ germ cells, ↓ primordial follides, ↔ primary follicles, ↔ total oocyte count, ↔ expression of *Bad, Bax and Bok*, ↑ *Bcl2* (44µM), 8 dpp: ↑ germ cells (except 0.44µM), ↓ primordial follicles (except 0.44µM), ↓ primary follicles (except 0.44µM), ↔ total oocyte count	87
Sheep (Texel)	Sewage sludge (2.25 metric tons of dry matter/ha) grazing, 0-80 dpc	↔ ovarian mass, ↔ follicular density (number/mm^2), ↓ healthy post-primordial transitory follicles, ↑ atretic post-primordial transitory follicles, ↑ follicles with intense nuclear staining, ↓ atretic primordial follicles, 4 differentially expressed transcripts	88
	Sewage sludge (2.25 metric tons of dry matter/ha) grazing, 30-110 dpc	↔ ovarian mass, ↔ follicular density (number/mm^2), ↓ healthy post-primordial transitory follicles, ↑ atretic post-primordial transitory follicles, ↑ follicles with intense nuclear staining, ↓ atretic primordial follicles, ↓ healthy secondary follicles, 99 differentially expressed transcripts	
	Sewage sludge (2.25 metric tons of dry matter/ha) grazing, 60-140 dpc	↔ ovarian mass, ↔ follicular density (number/mm^2), ↓ healthy post-primordial transitory follicles, ↑ atretic post-primordial transitory follicles, ↑ healthy primordial follicles, ↓ atretic primordial follicles, ↑ primary follicles, 120 differentially expressed transcripts	
	Sewage sludge (2.25 metric tons of dry matter/ha) grazing, 0-140 dpc	↔ ovarian mass, ↔ follicular density (number/mm^2), ↓ healthy post-primordial transitory follicles, ↑ atretic post-primordial transitory follicles, ↓ atretic primordial follicles, 33 differentially expressed transcripts	

Indirect exposure of fetuses to estrogenic chemicals via the pregnant dam also leads to effects in the offspring. Oral exposure of pregnant mice to 80 µg/kg BPA from 12.5 until 18.5 dpc resulted in increased percentage of oocytes in cysts, increase in total number of oocytes per section, and reduction in primordial follicle numbers at 3 dpp [63]. Similar effects were found after orally exposing rat dams to 0.5, 20 and 50 µg/kg/day BPA from 11 dpc until birth: increased number of oocytes in nests at all doses and decreased percentage of primordial follicles in the 0.5 and 50 µg/kg/day dose groups [29]. In a preliminary study of rhesus monkeys, fetal exposure to BPA disrupted follicle assembly, as daily oral exposure was associated with an increase in the number of multi-oocyte follicles, an increase in small unenclosed oocytes in the medulla, and different sized oocytes in some of the growing follicles [61].

As suggested by the above-mentioned studies, BPA can affect the oocytes in different species. In mice, the effects on cyst breakdown and total number of oocytes are the same regardless of whether exposure occurs during fetal or neonatal periods. In contrast, the two different exposure periods have opposite effects on the number of primordial follicles formed. Besides timing of exposure there can be other reasons for these diverging results, such as dose and the time at which the ovaries were examined, particularly since follicle assembly is a dynamic event and the distribution of oocytes/follicles may vary within a few days.

The phthalate DEHP causes effects similar to those of BPA, such as an increase in the number of germ cell cysts, and a reduced number of follicles in new-born mice after oral exposure of the pregnant mother [65,84]. Similarly, subcutaneous exposure to parabens neonatally (1-7 dpp) can affect follicle numbers, with increased numbers of primordial follicles, but decreased numbers of early primary follicles after exposure to propyl- and butylparaben [85]. In that study it was hypothesized that this was due to inhibition of initial recruitment, but since exposure started at 1 dpp, incorrect follicle assembly cannot be excluded; specifically, a general delay in follicular progression may be suspected.

Several *in vitro* culture studies using neonatal mouse ovaries have been conducted and typically show effects that resemble those observed after *in vivo* exposure, including increased number of germ cell cysts and effects on follicle numbers (Table 3). This was the case for genistein exposure from 1-7 dpp [75], BPA exposure from 0-3 dpp [86], BPA exposure from 0-8 dpp [87], DEHP exposure from 0-3 dpp [86], and DEHP exposure from 16.5 dpc-3 dpp [84]. The notion that the timing of follicle assembly is a particularly sensitive window for exposure to xenobiotics is further supported by an extensive study in sheep. By exposing pregnant ewes during early, mid, late or the whole gestation period to a mixture of environmental chemicals, including EDCs, disrupted fetal folliculogenesis was induced in all exposure groups, likely due to effects on follicle assembly. At the molecular level, however, the most pronounced changes to gene and protein expression was observed in fetuses having been exposed during mid-late gestation [88].

Taken together, these studies support the view that EDCs can have an inherent capacity to disrupt follicle assembly, potentially affecting both the quantity and quality of follicles later in life (Table 3), although it remans unkown whether such effects occur at human relevant dose levels.

Mechanisms affecting follicle assembly

One mechanism that may help explain the observed effects on follicle assembly following exposure to xenobiotics is apoptosis. The reduction in total number of oocytes by programmed cell death is an integral part of normal ovary development [89]. By disrupting this process, environmental chemicals can significantly impact on the subsequent ovarian function. Endogenous estrogen can reduce apoptosis in the adult ovary [90], and thus, it is easy to speculate that EDCs with estrogenic potential can prevent apoptosis at perinatal and

neonatal stages. Indeed, a number of studies showing that subcutaneous exposure of neonatal mouse pups to genistein, DES, EE, and BPA, can all reduce the number of oocytes undergoing apoptosis [76,81]. In addition, co-administration of an ER antagonist prevent the effects of DEHP exposure on follicle assembly [84]. Furthermore, exposure to BPA from 11 dpc until birth increases expression of anti-apoptotic factors, but decreases expression of pro-apoptotic factors [29], results that are largely recapitulated when exposing explanted ovaries to BPA [87] (Fig. 2C).

The opposite effect − increased numbers of apoptotic germ cells − has been seen in explanted ovary cultures exposed to BPA or DEHP for three days [86]. However, increased rates of apoptosis could be due to cytotoxicity since the effective dose was more than double the dose used by Zhou *et al* [87]. Reduced numbers of germ cells could also result from reduced proliferation, as has been observed in human fetal ovaries exposed to a bioactive component of cigarette smoke *in vitro* [91], or from a combination of disrupted apoptosis and proliferation. Further studies aimed at better delineating these processes in relation to xenobiotics would be of great interest.

In rodents, meiotic progression is thought to be related to primordial follicle assembly. When oocytes in the neonatal ovaries reach the end of prophase I (diplotene stage), follicular assembly is initiated [92]. Exposure to BPA seems to delay meiotic progression through prophase I, possibly reducing *Stra8* expression, which then could help explain the observed reduction in number of assembled primordial follicles shortly after birth [63]. It is difficult to pinpoint the mechanism underpinning the follicular phenotype based on reduction in *Stra8* expression alone, however, as this could indicate that: i) there are fewer oocytes overall, ii) fewer oocytes have entered meiosis or, iii) the majority of oocytes have already progressed through meiosis.

EARLY FOLLICLE RECRUITMENT

Exposure to xenobiotics such as BPA, paracetamol, or mixtures of environmental chemicals with anti-androgenic potentials during both fetal and postnatal life can adversely affect the follicle reserve, and even folliculogenesis itself [22,93,94]. However, because of the extensive differences in the developmental periods during which exposure to chemicals occurred in such studies, it is often difficult to deduce specific sensitive time windows. In this section we will focus on studies dealing with exposure occurring after follicle assembly and up to puberty (Table 4); in other words, during the first wave of folliculogenesis.

As opposed to the developing human ovary where primordial follicles typically localize in the cortex, mouse primordial follicles are formed in both the medulla and the cortex. Immediately after formation, primordial follicles of the medulla are activated and constitute the first wave of follicles. This first wave dominates the ovary up until three months *postpartum* and appears to be involved in the onset of puberty, activation of the

hypothalamic-pituitary-gonadal axis, and influence fertility during young adulthood. After three months of age, the second wave of follicles is recruited from the cortical region, and serves as the oocyte pool for the reminder of the reproductive lifespan [95].

Table 4 Exposure to chemicals during early folliculogenesis.

Species	Exposure	Effects of Exposure	References
Mouse (CD-1)	DEHP (20 and 40µg/kg bw) hypodermal on 7-14 dpp, killed on 15 dpp	↓ primordial follicles, ↑ antral follicles	24
	DEHP (20 and 40µg/kg bw) hypodermal on 5, 10, an 20 dpp, killed on 21 dpp	↓ primordial follicles, ↑ secondary and antral follicles,	
Mouse (CD-1)	DEHP (0.54, 5.4, and 54 µM) ovary culture on 4-10 dpp, collected at 10 dpp	↔ germ cells, primordial follicles, or primary follicles, ↔ PTEN and pAKT	96
	MEHP (0.68, 6.8, 68 µM) ovary culture, on 4-10 dpp, collected on 10 dpp	↓ germ cells, ↔ primordial follicles, ↑ primary follicles, ↓ PTEN positive cells/ovary (6.8, 68 µM), ↓ PTEN positive primordial follicles, ↔ pAKT positive cells/ovary, ↑ pAKT positive primordial follicles	
Sheep	BPA (50µg/kg/day) s.c. on 1-14 dpp, killed on 30 dpp	↓ ovary weight, ↓ primordial follicles, ↑ transitional and primary follides, ↔ total number of follicles	98

Effects following postnatal EDC exposure

DEHP and its main metabolite monoethylhexyl phthalate (MEHP) can stimulate follicle recruitment both *in vivo* and *in vitro*. A reduction in primordial follicle numbers, concomitant with an increase in secondary and antral follicle numbers, has been observed in 15 day old mice after exposure to DEHP from 7-14 dpp, as well as in 21 day old mice after single-day exposures at 5, 10, 15, or 20 dpp [24]. As exposure was initiated when follicle assembly was completed, the reduction in primordial follicles may have been caused by increased recruitment of first-wave follicles. However, direct effects on the oocytes causing increased death and thereby fewer oocytes cannot be excluded, as the total numbers of oocytes were not reported. A stimulatory effect of DEHP on follicle assembly or recruitment is supported by the finding that exposure of cultured mouse ovaries to MEHP from 4-10 dpp decreased the percentage of oocytes not encapsulated in a follicle, and increased the percentage of primary follicles without affecting the percentage of primordial follicles [96]. This stimulatory effect of DEHP/MEHP exposure may be caused by activation of the phosphatidylinositol 3-kinase (PI3K) pathway.

In oocytes, PI3K is negatively regulated by phosphatase and tensin homolog deleted on chromosome 10 (PTEN). In mice lacking PTEN, levels of pAKT are increased and the entire primordial follicle pool is activated, causing depletion of primordial follicles and resulting in premature ovarian failure [97]. Interestingly, Hannon *et al* [96] found a reduced percentage of PTEN-positive oocytes and an increased percentage of pAKT-positive oocytes in the MEHP-exposed ovary explants, indicating that the changes seen were indeed due to increased recruitment. It is surprising, however, that no effects were seen on the primordial follicle pool when the number of PTEN positive oocytes dropped. One explanation for this could be that an increased recruitment may not have reached the point of significantly influencing the number of primordial follicles at 10 dpp. Indeed, the reduction in primordial follicle number was not seen until 15 and 21 dpp in the study by Zhang *et al* [24], and a longer culture period than that employed by Hannon *et al* [96] might induce the expected increase in primordial follicle numbers.

BPA has also been investigated for its potential effects on early folliculogenesis. In sheep, follicle assembly is completed *in utero*. In a study by Rivera *et al* [98], where animals were exposed to BPA from 1-14 dpp, 30 day old lambs displayed reduced ovary weight, fewer primordial follicles, more transitional and primary follicles, but a stable total number of oocytes. This indicates that recruitment of primordial follicles was increased [98].

Taken together, these studies indicate that DEHP, as well as BPA, can intensify recruitment of follicles from the primordial follicle pool during early folliculogenesis (Fig. 2D). This can have significant consequences later in life, such as a shorter reproductive lifespan due to reduced number of primordial follicles, which is of major concern if observed at human exposure levels. There are only a few available studies investigating this specific time period, however, and more studies are needed to better understand the mechanisms behind the observed effects.

EDCs AND WOMEN'S REPRODUCTIVE HEALTH

Based on animal experiments, there is little doubt that exposure to EDCs can affect ovarian development and function. However, whether the same chemicals will cause similar effects in humans at doses relevant to real-life exposure, remains less clear. One of the main questions is whether EDCs can adversely affect human female fertility. Although controversial and sometimes disputed, there are studies indicating that over the past few decades, populations of industrialized countries all over the world have experienced a decline in total fertility rates, measured as live births per woman [99]. This, of course, can at least partly be explained by changing attitudes towards family planning and contraceptives, as well as other social changes, but changes to the biochemical environment may also contribute. Currently, a still increasing chemical burden may

contribute towards the increase in women reporting to have difficulties conceiving and maintain pregnancy, as now described for women of all ages [100,101].

Historical data show that chronological age is a determining factor for fertility, but of less importance for fertility until 35 years of age [102,103]. Accelerated biological aging of the ovary before the age of 35 can therefore not be explained by chronological age alone, and knowledge attained during assisted reproductive techniques indicates that a combination of environmental and genetic factors may contribute to reduced quality of the oocyte pool with age [104,105]. Chemicals such as p,p-DDT [106], fluorinated chemicals [107] and pesticides [108] have all been associated with decreased fecundability, measured as time to pregnancy. Furthermore, BPA and other EDCs have been associated with PCOS, one of the leading causes of subfertility[20].

An increase in prematurity, pre-eclampsia, gestational diabetes, and premature puberty has also been reported. This decline in reproductive health has occurred over a relatively short period of time, making genetic changes an unlikely explanation [100,101], whereas EDCs have been proposed as potential contributors [101].

Another aspect not covered in detail herein, are cancers of the reproductive organs and tissues. A clear causation between early exposure to EDCs and gynecological cancers are lacking, but a recent meta-analysis concluded that infertile women are at greater risk of developing endometrial cancer than the general female population. In contrast, data concerning a potential higher risk of developing breast or ovarian cancer are conflicting [109]. Interstingly, a very recent study showed an association between irregular menstrual cycling, in many cases caused by PCOS, and ovarian cancer [110]. Another class of cancers, malignant ovarian germ cell tumors (mOGCT), is of special interest in the light of EDC exposure, as they are believed to originate from fetal pluripotent germ cells [111]. These cancers share much of their etiology with their male counterparts, testicular germ cell tumors (TGCT), which arise from genetic aberrations, but most likely also influenced by environmental factors during early developmental stages [112]. In fact, patients with Disorders of Sex Development (DSD: congenital conditions where chromosomal, gonadal or anatomical sex is atypical [113,114]) have an increased risk of developing GCTs, attesting to the importance of the somatic environment, and not only intrinsic factors, in the regulation of germ cell development [115].

Human versus animal data

BPA has been measured in several biomonitoring studies, including in the USA and Europe where median Bisphenol A exposure ranges between 0.03 and 0.04 µg/kg bw/day for adults. However, approximately 5% of the population is considered to be exposed to more than 0.15 µg/kg bw/day [66–68]. As listed in Table 2 and 3, several studies have shown BPA effects on ovary development at doses around 20 µg/kg bw/day, and in

some studies even in the ng/kg bw/day range. In human risk assessment, a 100-fold difference between no-effect levels in animal studies and human exposure levels is generally required before the human exposure levels can be considered safe. With developmental effects on ovaries around, or below, 20 µg/kg bw/day, and therefore a no-effect level below this figure, the margin of safety could thus be below 100 for the most highly exposed persons.

DEHP metabolites, such as MEHP, are typically used as proxy to retrospectively estimate actual DEHP exposure. Calculation of such estimates is complex, but show that humans are exposed to about 1.5 µg/kg bw/day of DEHP in Europe and 4 µg/kg bw/day in the USA. For around 5% of the population the levels exceed 4.4 or 34 µg/kg bw/day in Europe or the USA, respectively [68,116]. Effects on ovary development have been observed following exposure to 20 and 40 µg/kg/day, indicating that the no-effect level is at least lower than 20 µg/kg/day (Tables 3 and 4). Thus, a very low safety margin is currently in place for a large section of the population if these findings reflect true adverse changes in ovary development at low doses of DEHP, or possibly other phthalates as well. Such safety evaluations should be based on a larger evidence base, however, and preferably include studies examining several concentrations to allow for dose response calculations.

The use of paracetamol has become widespread and more than 50% of women are estimated to use paracetamol at least some time during pregnancy [117]. In Denmark, everyday use during early pregnancy is seen for 0.2% of women, whereas 0.7% use paracetamol 1-2 times per week [118]. Additionally, exposure to paracetamol is almost ubiquitous in many populations, even when no use is reported. In Germany paracetamol has been detected in persons who had neither recently consumed paracetamol nor been occupationally exposed to its precursor aniline, indicating that other unknown exposure sources exist. Similar exposure data was recently shown in a Danish study, where mothers and their children had similar paracetamol levels in their blood despite no obvious exposure source [40]. So far, no clear associations between paracetamol exposure and human female reproductive health effects have been reported. It is, however, striking that the dose levels affecting ovary development in rats (50 mg/kg bw/day) are comparable to exposure levels of humans taking the maximal recommended dose of paracetamol, even when not correcting for differences in metabolism between rats and human [37].

Development of the fetus differs in timing relative to the gestational period between species. In humans, follicle assembly takes place during fetal life, whereas it in the rat is occurs neonatally [74]. Furthermore, since the first wave of folliculogenesis is initiated immediately after follicle assembly, this also differs between humans and rodents (Fig. 3). This ultimately means that for humans the most susceptible period for exposure with regards to these endpoints is the gestational period. What complicates the picture even further, is that humans are continuously exposed to a large number of chemicals at any given time [119], whereas controlled animal experiments typically involves the exposure of single chemicals during specific time windows. To

overcome this hurdle, a series of studies were conducted exposing sheep on pastures before and during pregnancy to a mixture of environmental chemicals [88,120,121]. The studies focusing on female fetuses found that constant maternal exposure to a low-concentration mixture of chemicals can disrupt ovary development [120], and that exposure during mid and late gestation was most detrimental [88]. It was also found that the pre-conception period is important in relation to effect outcomes on the offspring [121]. Finally, a change in exposure between the pre- and post-conception period seemed to have a greater impact on fetal ovary development than a continuous exposure [121].

To date, studies on mixture effects have focused on male reproduction, where certain EDCs have been shown to contribute to the mixture effects [122,123]. Comparable scenarios in females are likely, and mixture effects, mainly after exposure to phytoestrogens, have been shown [124–126]. Low doses of chemicals with endocrine modes of action on the ovary – including DEHP, BPA and paracetamol – may, therefore, produce mixture effects at exposure levels considered safe for individual compounds.

PERSPECTIVES AND CONCLUSION

For decades, the prevailing view of mammalian sex differentiation has been that male fetuses are critically dependent upon sex hormones for masculinization, whereas female fetuses develop more independently of these. As a result, studies aimed at elucidating effects of EDCs on fetal development, including long lasting consequences manifesting in adulthood, has primarily focused on males. There are strong indications, however, that females are far from protected from the potential harm caused by exposure to EDCs, even *in utero*. With this review we have aimed to illuminate potential links between developmental exposure to EDCs and late-life reproductive health effects with focus on ovarian development and function. It is clear from our synthesis that there are many questions that remains to be answered, but we would like to highlight four general points that we believe warrants particular focus:

- Early germ cell development and gonadal sex differentiation are processes not normally associated with subtle disruptions by xenobiotics. The pharmaceuticals paracetamol and tamoxifen, however, may interfere with these processes. Similarly, BPA may be capable of disturbing the molecular pathways governing the balance between the male and female cell fates in the testes and ovaries. Therefore, since disrupted sex differentiation can have far-reaching consequences for the individual, this area of research requires a much stronger emphasis, and to date very few studies have been conducted focusing on this early period of development.
- Meiosis is a process unique to the germ cells and correct progression is critical for the quality and quantity of germ cells in the adult female. The industrial chemicals BPA and DEHP, and the pharmaceuticals paracetamol and indomethacin have been shown to disturb meiosis. To enable

evaluation of a specific chemical for potential effects on meiosis elucidating the mechanism by which these chemicals act will be of great importance.

- Among the four potentially sensitive developmental windows presented in this review, follicle assembly is the most thoroughly investigated. Several studies have reported effects of exposure to BPA and DEHP, or its metabolite MEHP, but the specific mechanisms by which these chemicals impact the process is not clear. Mechanistic knowledge is important for inter-species extrapolation, and we encourage further studies to investigate mechanisms potentially sensitive to EDCs, especially apoptosis, proliferation and cell cycle stages as stepping stones.
- EDCs, including BPA and DEHP, may increase the size of the first follicle wave in the rodent postnatal period and may, ultimately, lead to earlier reproductive senescence. Studies investigating effects of exposure to other EDCs are therefore warranted.

When viewed together, many reproductive disorders in girls and young women – ranging from precocious puberty to PCOS and ovarian cancers – may in many instances have shared etiologies. There is mounting evidence to suggest that these disorders often can be manifestations of incorrect programming during fetal life such that together they would comprise an ovarian dysgenesis syndrome (ODS) similar to that proposed for male reproductive disorders two decades ago with the testicular dysgenesis syndrome (TDS) hypothesis. Further effort into studying the underlying causes of female reproductive disorders coupled with more solid human epidemiological data is therefore needed, and will contribute significantly towards safeguarding female reproductive health into the future.

To end; while results from animal studies can clarify relationships between early exposure and effects during adult life, associations from human epidemiological studies in this field are difficult to interpret due to the lag-time between developmental exposure and the appearance of adverse outcome in adulthood. Also, complex exposure patterns may distort the image, contributing with yet another layer of complexity. Nevertheless, an integrated evaluation of animal and human studies indicates that late-life female reproductive health can be compromised by environmental factors, including chemical exposures during the otherwise protected milieu of the womb.

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Chapter 3: *Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging*

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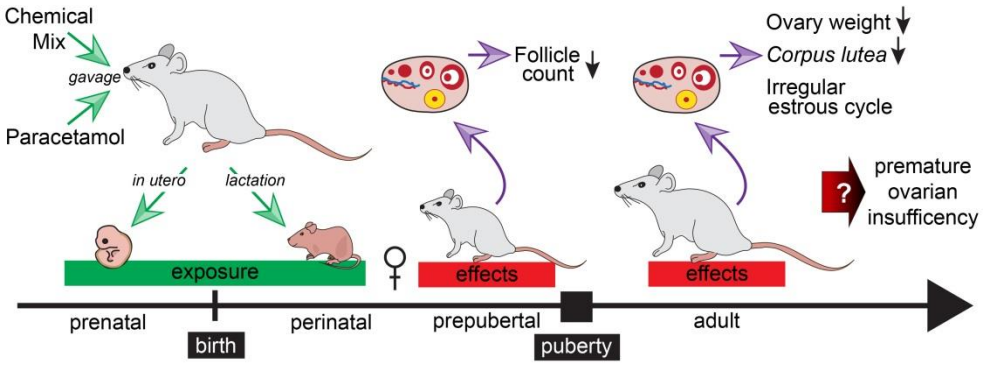
ABSTRACT

Exposure to endocrine disrupting compounds (EDCs) during development can have negative consequences later in life. In this study we investigated the effect of perinatal exposure to mixtures of human relevant EDCs on the female reproductive system. Rat dams were exposed to a mixture of phthalates, pesticides, UV-filters, bisphenol A, butyl-paraben, as well as paracetamol. The compounds were tested together (Totalmix) or in subgroups with anti-androgenic (AAmix) or estrogenic (Emix) potentials. Paracetamol was tested separately. In pre-pubertal rats, a significant reduction in primordial follicle numbers was seen in AAmix and PM groups, and reduced plasma levels of prolactin was seen in AAmix. In one-year-old animals, the incidence of irregular estrous cycles was higher after Totalmix-exposure and reduced ovary weights were seen in Totalmix, AAmix, and PM groups. These findings resemble premature ovarian insufficiency in humans, and raises concern regarding potential effects of mixtures of EDCs on female reproductive function.

Graphical abstract:



Key words: Endocrine disruption, anti-androgen, female reproductive system, ovarian toxicology, follicle, estrous cycle, reproductive senescence

1. INTRODUCTION

Exposure to endocrine disrupting compounds (EDCs) during development can have long-lasting consequences extending into adulthood, for instance compromised reproductive health (Isling et al. 2014; Schug et al. 2011). Numerous studies have investigated the link between early exposure to EDCs and late life effects on the male reproductive system, often referred to as the testicular dysgenesis syndrome (TDS) hypothesis, comprising a range of male disorders presumed to have a common developmental origin (Skakkebæk et al. 2001; Toppari et al. 2010). Studies addressing comparative questions in females, however, are far fewer. An ovarian dysgenesis syndrome (ODS) hypothesis has been proposed though. And as with males, pathologies presenting in adulthood; impaired placental function, early pregnancy loss, breast cancer, pubertal timing, and polycystic ovarian syndrome (PCOS), are suspected to share a common developmental origin (Buck Louis et al. 2011; Fowler et al. 2012).

The generally accepted view, although challenged by a few studies (reviewed in Virant-Klun (2015)), is that females are born with a set number of follicles that depletes throughout their reproductive lifespan, inevitably leading to infertility. Disturbed establishment of the follicle pool during development may therefore be detrimental to fertility in the adult female (McGee and Hsueh 2000). Rodent studies have indicated that oocyte meiosis, ovarian folliculogenesis, fertility, and the onset of reproductive senescence can be altered by environmental contaminants, including EDCs (Ahn et al. 2012; Chao et al. 2012; Rodríguez et al. 2010; Shi et al. 2007; Susiarjo et al. 2007; Wang et al. 2014; Zhang et al. 2013). This has led to an increased level of concern regarding female reproductive health, particularly since reduced fecundity has been associated with EDCs in humans (Caserta et al. 2011).

When evaluating toxicity of a chemical, the state-of-the-art has been to assess one chemical at a time. A more realistic scenario, however, is that humans are exposed to a mixture of different chemicals simultaneously. Thus, there is a need for evaluation of toxicity of mixtures rather than single compounds only (Backhaus et al. 2010; Hass et al. 2007; Kortenkamp 2007). The aim of this study was to address effects of perinatal exposure to mixtures of EDCs on female reproductive endpoints in a top-down approach starting from a human relevant mixture and subgroups of this mixture. Thirteen EDCs, including phthalates, pesticides, UV-filters, Bisphenol A, butylparaben, and the pharmaceutical drug paracetamol (PM) were tested in a mixture ratio based on high-end human exposure levels as previously described (Axelstad et al. 2014; Christiansen et al. 2012). The tested phthalates are known to influence steroid synthesis (reviewed in Hannon and Flaws (2015)) and indications of altered ovarian development have been seen in rodent studies (Zhang et al. 2013, 2014b). The tested pesticides are known to act mainly as androgen receptor antagonists (Kjærstad et al. 2010; Orton et al. 2011) and the UV-filters, Bisphenol A and butylparaben are known estrogen receptor agonists (Rosenmai et al. 2014; Routledge et al. 1998; Schreurs et al. 2002), but the compounds may also act via other modes of action (Kjærstad et al. 2010; Rosenmai et al. 2014). PM is a

prostaglandin synthesis inhibitor with possible anti-androgenic modes of action (Kristensen et al. 2011). To evaluate whether effects of the Totalmix could be attributed to one or more components of the mixture, we also tested subgroups chemicals with anti-androgenic or estrogenic potentials, and PM alone (Table 1). The mixtures were administered in doses 100 to 450 times high-end human exposure levels, as these doses were predicted to affect anti-androgenic endpoints in male offspring, whereas the PM dose was corresponding to human exposure levels, see also (Axelstad et al. 2014).

Effects of these EDC mixtures on early male reproductive development (Axelstad et al. 2014), and mammary gland development (Mandrup et al. 2015) have previously been published. In the current study we hypothesized that perinatal exposure to mixtures of EDCs adversely affects the ovary in young and senescent animals, and investigated the impact of perinatal EDC exposure on ovarian gene expression, number of follicles, pituitary hormone levels in plasma, sexual maturation, estrous cyclicity and ovarian histology in rat offspring. Together, the results from young and senescent animals indicated premature ovarian insufficiency after exposure to this human-relevant EDC mixture, and these effects were likely caused by the anti-androgen mixture components and PM.

2. MATERIALS AND METHODS

2.1 Test compounds

Test compounds were: di-n-butyl phthalate (DBP) (purity >99.0 %, CAS no. 84-74-2), di-(2-ethylhexyl) phthalate (DEHP) (purity >99.5 %, CAS no. 117-81-7), vinclozolin (purity >99.5 %, CAS no. 50471-44-8), prochloraz (purity >98.5 %, CAS no. 67747-09-5), procymidone (purity >99.5 %, CAS no. 32809-16-8), linuron (purity >99.0 %, CAS no. 330-55-2), epoxiconazole (purity >99.0 %, CAS no. 106325-08-8), octyl methoxycinnamate (OMC) (purity >98.0 %, CAS no. 5466-77-3), dichlorodiphenyl-dichloroethylene (p,p'-DDE) (purity >98.5 %, CAS no.72-55-9); all purchased from VWR - Bie & Berntsen (Herlev, Denmark). And: 4-methyl-benzylidene camphor (4-MBC) (purity >98.0 %, CAS no. 36861-47-9), bisphenol A (BPA) (purity >99.5 %, CAS no. 80-05-7), butyl paraben (purity >99.0 %, CAS no. 94-26-8) and paracetamol (PM) (purity >99.0 %, CAS no. 103-90-2); all purchased from Sigma-Aldrich (Brøndby, Denmark). Corn oil was used as a control compound and as vehicle; purchased from VWR - Bie & Berntsen (Herlev, Denmark).

2.2 Mixtures

The mixture compositions were based on high-end human exposure levels of 13 well-characterized endocrine disrupters, as previously described (Axelstad et al. 2014; Christiansen et al. 2012). Totalmix contained all 13 compounds; AAmix contained compounds with predominantly anti-androgenic modes of action; Emix contained compounds with predominantly estrogenic properties (Table 1). PM was included in the Totalmix, as well as tested on its own. Totalmix was given at 100-, 200- or 450-times human high-end exposure, the AAmix and Emix at 200- and 450-times human high-end exposure. PM was given at 350 mg/kg, which corresponds to the dose given in the Totalmix450 (Table 1).

Table 1 Mixture composition and dose for the tested mixtures in mg/kg per day. Design of the mixtures has previously been described (Axelstad et al. 2014; Christiansen et al. 2012).

	Mixture dose (mg/kg per day)							
Chemical	Totalmix-100	Totalmix-200	Totalmix-450	AAmix 200	AAmix 450	Emix 200	Emix 450	PM
DBP	1	2	4.5	2	4.5	0	0	0
DEHP	2	4	9	4	9	0	0	0
Vinclozolin	0.9	1.8	4.05	1.8	4.05	0	0	0
Prochloraz	1.4	2.8	6.3	2.8	6.3	0	0	0
Procymidone	1.5	3	6.75	3	6.75	0	0	0
Linuron	0.06	0.12	0.27	0.12	0.27	0	0	0
Epoxiconazole	1	2	4.5	2	4.5	0	0	0
p,p'-DDE	0.1	0.2	0.45	0.2	0.45	0	0	0
4-MBC	6	12	27	0	0	12	27	0
OMC	12	24	54	0	0	24	54	0
Bisphenol A	0.15	0.30	0.675	0	0	0.30	0.675	0
Butyl paraben	6	12	27	0	0	12	27	0
Paracetamol	80	160	360	0	0	0	0	360
Sum (mg/kg per day)	112	224	504	16	36	48	109	360

2.3 Animals and exposure

A detailed design of the animal study can be found in Axelstad et al. (2014). In short, time-mated nulliparous Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) 3 of pregnancy. The day vaginal plug was detected was designated as GD 1 and the expected day of delivery (GD23) was designated as pup day (PD) 1. The dams received vehicle (controls), or one of the eight mixtures (Table 1). Each dose group comprised 16-20 dams, with 14-20 viable litters obtained for each group. Rats were exposed by oral gavage from GD7-21, and again after birth from PD1-22. PM exposure

was from GD13-19 and PD14-22, both in mixtures and single dosing, to avoid possible effects on embryo implantation (Gupta et al. 1981) and problems during parturition. At PD22, 1-2 females per litter were weaned and kept until adulthood. One female pup per litter was killed at weaning (PD22) and one or two female pups per litter were killed at 13 months of age whilst in estrous or proestrous, evidenced by vaginal smears in the morning. Blood was collected for hormone analysis, and ovaries were weighed and prepared for histological examination and/or gene expression analysis. The study was performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee.

2.4 *In vivo* measurements

2.4.1 Anogenital distance and sexual maturation

Anogenital distance (AGD) was measured, by the same technician, in all offspring. The technician was blinded with regard to exposure group. Measurements were performed using an ocular stereomicroscope with unit markings on the ocular. Onset of puberty was defined as day of vaginal opening (VO) and assessed daily from PD28 until VO was detected in all female offspring. Age and body weights were recorded on the day when VO was first observed.

2.4.2 Estrous cyclicity

Vaginal smears were taken daily between 8 and 10 a.m., for 21 consecutive days at 3 and 12 months of age. A swab moistened in saline was inserted into the vaginal lumen and cells were transferred to a microscope glass slide to air dry. The smears were fixed in 96% ethanol and stained with Gill's hematoxylin, Orange G6 and eosin-azure 50 (VWR - Bie & Berntsen, Herlev, Denmark) according to the adapted Papanicolaou (PAP stain) procedure (Hubscher et al. 2005). The smears were mounted in Eukit (VWR - Bie & Berntsen, Herlev, Denmark) and examined by light microscopy under blinded conditions. Classification was done according to stages; estrous, metestrous, diestrous or proestrous, or transitions between stages (Goldman et al. 2007; OECD 2009).

The animals were categorized as either being regularly cycling (cycles lasting four to five days) or irregularly cycling (cycles lasting less than four days or more than five days) (Cooper and Goldman 1999). Episodes of three to four consecutive days of vaginal estrous and/or four to five days of diestrous were considered extended (Goldman et al. 2007).

2.5 Histological examination

Ovaries from one female per litter, alternately left and right, were examined at PD22 as follows: control, Totalmix450, AAmix450, Emix450, and PM (n = 12-16) ovaries were fixed in formalin, processed for paraffin embedding, sectioned (5µm sections at 90µm intervals, all sections were counted) and stained with

hematoxylin and eosin (H&E). In all sections, primordial (oocyte surrounded by flat pre-granulosa cells), primary (oocyte surrounded by one layer of cuboidal granulosa cells), secondary (oocyte surrounded by two or more layers of granulosa cells and theca cells), tertiary (presence of antrum), and atretic follicles (presence of condensed, dark cells) were counted, provided a nucleolus was visible.

At 13 months of age, histological evaluation was performed on one section per ovary (all groups), uterus and pituitary (control, Totalmix450, AAmix450, Emix450 and PM groups). The number of corpora lutea (CL) and the presence of follicular cysts (follicles devoid of oocytes, displaying a large antrum surrounded by 1-2 layers of flattened granulosa cells and a thecal cell layer) and cyst-like structures (follicles devoid of oocytes, displaying a large antrum surrounded by a few layers of granulosa cells) were investigated. In uterus the number and appearance of endometrial glands was evaluated, and the presence of squamous metaplasia, endometrial cysts and endometrial stromal polyps was registered. Pituitary glands were examined with emphasis on the presence of nodular hyperplasia and adenoma in pars distalis (MacKenzie and Boorman 1990).

2.6 Plasma hormone levels

On PD22, blood was collected in heparin-coated vials, centrifuged and plasma withdrawn. Plasma levels of adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF), follicle stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH), prolactin (PRL) and thyroid stimulating hormone (TSH) was measured using a Milliplex map Rat Pituitary Magnetic Bead Panel (Cat. No. RPTMAG-86K; Merck Life Science A/S, Hellerup, Denmark). The PM group was not included. Plasma levels of Inhibin A were measured by ELISA (Cat. No. CSB-E08239r, CUSABIO Biotech Co.) Measurements were conducted according to the manufacturer's instructions.

2.7 Gene expression

For all nine dose groups (n = 9-10 per group), the alternate left and right ovary was excised from one female per litter at PD22 and stored in RNAlater (Qiagen, Hilden, Germany) at -80 ºC. Relative gene expression was analyzed by RT-qPCR as previously described (Svingen et al. 2015). In short, total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified on a NanoDrop-1000 Spectrophotometer. RT-qPCR reactions were run in duplicates on a 7900HT qPCR system (Applied Biosystems, Foster City, CA) in 20 µl reactions including 3 µl diluted (1:20) cDNA and genespecific TaqMan assays (Life Technologies Europe BV, Naerum, Denmark: *Amh* (Rn01535314_g1), *Bmp15* (Rn00572320_m1), *Ddx4* (Rn01489814_m1*)*, *Fshr* (Rn01648507_m1), *Fst* (Rn00561225_m1), *Lhr* (Rn00564309_m1)). Intra-assay variability of technical replicates was <0.5 cycles. Data were analyzed by the comparative Ct-method normalized with the geometric mean of verified reference genes *Rps18* (Rn01428913_gH) and *Sdha* (Rn00590475_m1) (Svingen et al. 2015).

2.8 Statistical analysis

For all analyses, the alpha level was set at 0.05. Data from continuous endpoints were examined for normal distribution and homogeneity of variance and if required, logarithmic transformation was performed. For endpoints where ANOVA and Dunnett's post-test were used, data were compared as follows: Control versus Totalmix, Control versus AAmix, Control versus Emix. PM was compared to control by use of Student's t-test. For non-normally distributed data Kruskal-Wallis and Dunn's post-test or Mann-Whitney was used. Data from follicle count were investigated by t-test as only highest doses were used. Litter was used as an independent, random and nested factor when more than one pup from each litter was examined.

AGD data were analyzed using pups' birth weights as covariate and by the AGD-index, i.e. AGD divided by the cube root of body weight. Statistical analyses were adjusted using litter as an independent, random, and nested factor. Age and weight at sexual maturation was analyzed by ANOVA using body weight at PD22 as a covariate to compensate for size differences. Estrous cyclicity data were tested using logistic regression and tested for over-dispersion with Deviance and Pearson Goodness-of-Fit tests. Correction for over-dispersion due to litter effects was used when appropriate.
Organ weights were analyzed by ANOVA using body weight as a covariate. Histological data were evaluated using Fisher's Exact Test. Regression was used to investigate relationship between ovary weight and number of CL.
The statistical software SAS (SAS Enterprise Guide 4.3), R (R Core Team 2013), and GraphPad Prism 5 (GraphPad Software, San Diego California USA) were used for analysis.

3. RESULTS

3.1 Gene expression

No significant changes in overall expression were observed for any of the somatic markers (Suppl. Fig. S1), suggesting that the overall ratio of cell-specific populations were relatively unchanged. A significant reduction in *Ddx4* ($p = 0.03$) and trend to a reduction in *Bmp15* ($p = 0.07$) transcript levels were observed in the PM-exposed group (Fig. 1A and B). As this suggested a smaller number of oocytes, manual counting of follicles was performed.

3.2 Follicle count

Primordial follicles were significantly reduced (78.2% of control values, $p = 0.02$) in rats exposed to AAmix450, and a tendency towards reduction was seen in the PM-exposed group ($p = 0.06$) (Fig. 1C). There were no statistically significant differences in number of recruited follicles (pool of primary, secondary and

tertiary) between groups .Visual evaluation of the total number of follicles (total number in all sections counted), indicated a slight reduction in total number of follicles in AAmix450 and PM, but the differences were not statistically significant (Fig. 1C). When using percentage of follicles instead of absolute values, a significant reduction in primordial follicles was seen in groups exposed to AAmix450 ($p = 0.005$) and PM ($p = 0.01$) and there was a significant increase in secondary ($p = 0.05$) and tertiary ($p = 0.04$) follicles in the AAmix450 group (Fig. 1D). Furthermore, the number of recruited follicles (pool of primary, secondary and tertiary) was significantly increased in animals exposed to AAmix450 ($p = 0.01$) and PM ($p = 0.02$).

3.3 Hormone levels

There was a significant reduction ($p = 0.01$) in PRL levels after AAmix450 exposure, and visual evaluation of the data indicated a reduction also in the Totalmix450 and AAmix200 groups. However, these reductions were not statistically significant ($p = 0.15$ and $p = 0.27$, respectively) (Fig. 1E). No effects were seen on the levels of other pituitary hormones or inhibin A (Supplementary Figs. S2 and S3).

3.4 AGD, sexual maturation and estrous cyclicity

No significant differences between groups were observed for female AGD (Axelstad et al. 2014). Sexual maturation occurred significantly earlier in Totalmix200 ($p = 0.0002$), AAmix200 ($p = 0.02$) and Emix450 ($p = 0.04$) groups. Animals in Totalmix200 and AAmix200 groups had lower body weights at the day of VO ($p = 0.01$ and $p = 0.02$, respectively) (Fig. 2A and B). There were no effects on estrous cycle regularity at three months of age. At 12 months of age, overall analyses showed no effect on estrous cycle regularity, however pairwise comparison of control with each group showed significant effects in Totalmix100 ($p = 0.041$) and Totalmix200 ($p = 0.048$) (Fig. 2C). In a previous study on the same mixture (though only including Totalmix450) estrous cyclicity data was also collected at 12 months of age (Isling et al. 2014). As the power appeared low for estrous cyclicity, the control and Totalmix450 data from both studies were pooled, resulting in a significant difference between Totalmix450 and control ($p = 0.02$) (Fig. 2D). Irregular cycles were primarily characterized by longer than normal cycles with extended diestrous. Extended estrous and shorter than normal cycles were also observed, but to a lesser extent.

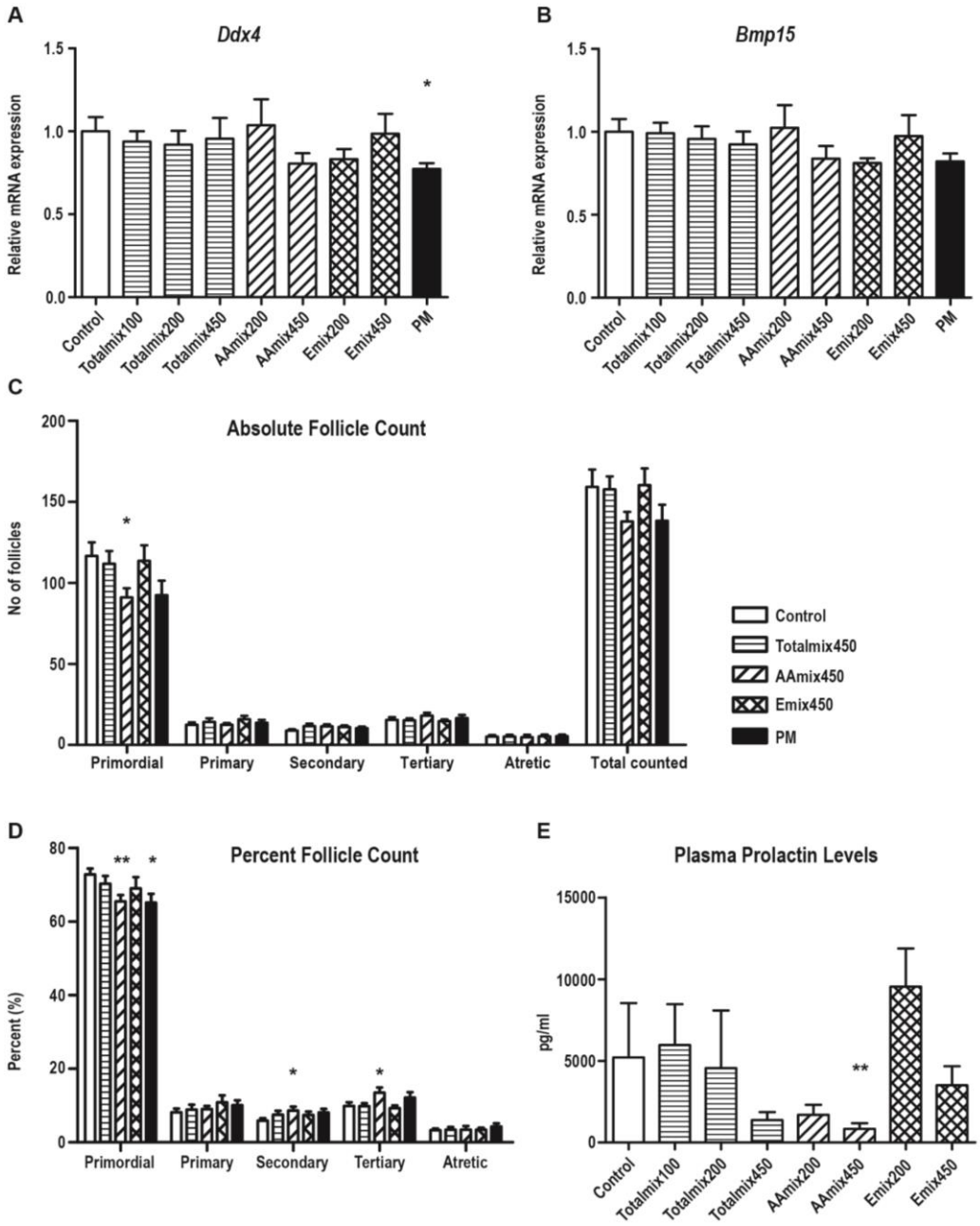


Figure 1 Results from PD 22. (A-B) A significant reduction in relative mRNA expression of *Ddx4* and a slight, but not statistically significant reduction, in *Bmp15* were seen in ovaries after PM treatment (n = 9-10 per group). C) Absolute follicle count showed significantly reduced numbers of primordial follicles in the AAmix450 group (n = 12-16 per group). D) Percentage of follicles (each animal's total number of follicles set to a 100%) showed a significant reduction in primordial follicles after AAmix450 and PM exposure. AAmix450 exposure also caused an increase in secondary and tertiary follicles. E) Prolactin level in plasma was reduced after AAmix450 exposure (n = 9-10 per group). Data presented as mean ± SEM, (*$p < 0.05$, **$p < 0.01$)

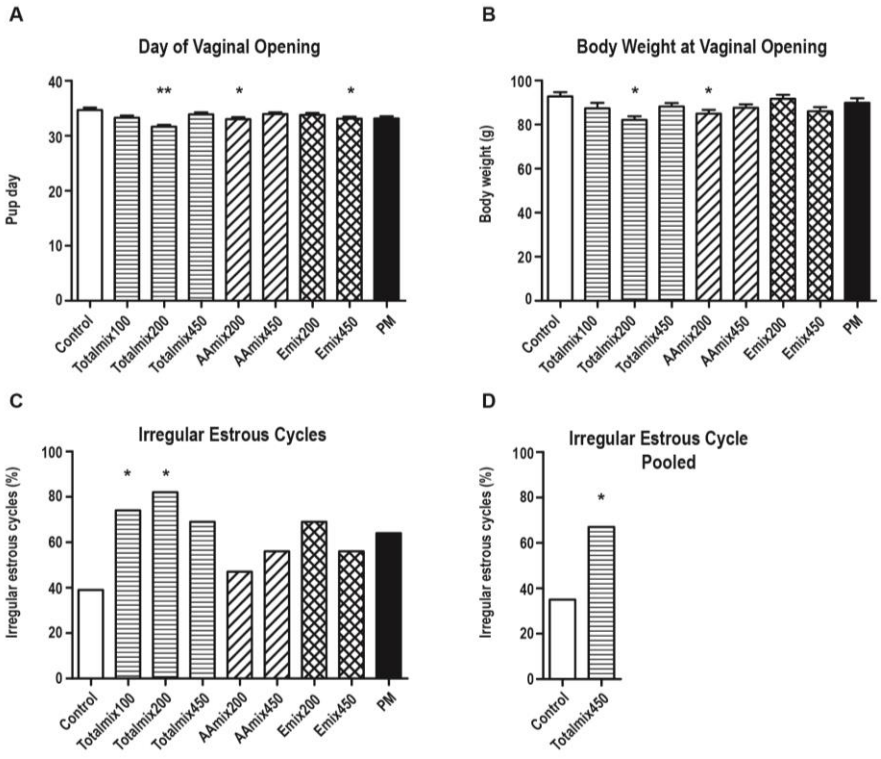


Figure 2 A) Sexual maturation, measured as day of vaginal opening (VO), occurred significantly earlier in animals exposed to Totalmix200, AAmix200 and Emix450 (n = 26-36 per group, corresponding to 13-19 litters per group). B) Body weight at VO was significantly lower in animals exposed to Totalmix200 and AAmix200 (n = 26-36 per group, corresponding to 13-19 litters per group). C) The incidence of animals with irregular estrous cycles was increased in the Totalmix100 and Totalmix200 (n = 11-14 animals per group from separate litters) in adult females (12 months of age). D) The incidence of animals with irregular estrous cycles was higher in the Totalmix450 (n = 31) compared to control (n = 30) when data was pooled with a previous study (Isling *et al*., 2014) in adult females (12 months of age). Data presented as mean ± SEM, (*$p < 0.05$, **$p < 0.01$)

3.5 Ovary weight and histology

At 13 months of age, ovary weight was significantly reduced in all Totalmix groups, both AAmix groups and the PM group (Fig. 3A). All females were killed whilst in proestrous or estrous, therefore observed effects were not considered to be due to variability in estrous cycle stage. No relevant changes in ovary weights were seen at PD22 (data not shown).

Histological examination at 13 months of age revealed a significant increase in incidences of rats presenting with complete absence of CL in AAmix200 ($p = 0.033$), AAmix450 ($p = 0.039$), and in PM ($p = 0.028$) groups (Fig. 3B). In addition, the mean number of CL was significantly reduced to 55% of control values in the Totalmix200 group ($p = 0.04$) and 54.7% of control values in the PM group ($p = 0.011$). The mean number of CL was also reduced in AAmix450, but did not reach statistical significance ($p = 0.056$) (Fig. 3C). The mean number of CL was positively correlated to ovary weight ($r^2 = 0.41$, $p < 0.0001$) (Fig. 3D).

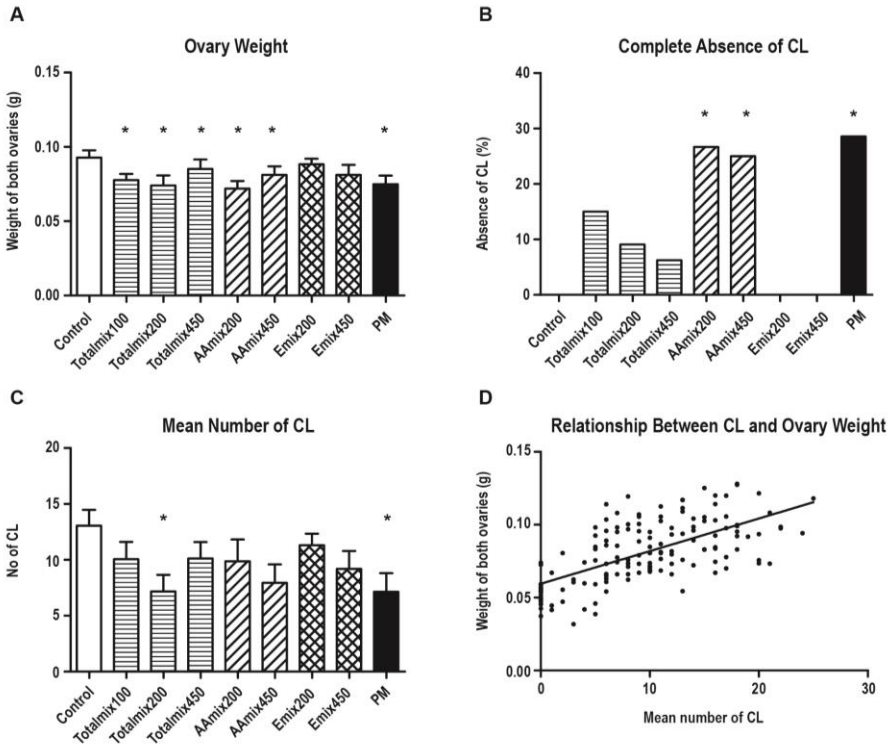


Figure 3 Effects on ovary weight and the presence of corpora lutea (CL) in 13 months-old animals. A) Mean ovary weight was reduced in animals exposed to Totalmix, AAmix and PM (n = 11-20). B) Complete absence of CL in animals exposed to AAmix and PM. C) Mean number of CL was significantly lower in Totalmix200 and PM (n = 11-20). D) Regression analysis showed that the mean ovary weight was associated with the mean number of CL, independent of exposure ($r^2 = 0.41$, slope significantly non-zero with $p < 0.0001$). Data presented as mean ± SEM, (*$p < 0.05$)

Follicular cysts and cyst-like structures were observed in all groups and are considered to be normal age-related changes. The number of animals with cyst-like structures in ovaries was significantly increased in the AAmix450 and the PM groups, and the same was seen when pooling data for cysts and cyst-like structures (Table 2).

3.6 Uterine and pituitary histology

Squamous metaplasia was observed in 1-3 rats per group in Totalmix450, AAmix450, Emix450 and PM groups, but not in controls (Table 2). Pituitary nodular hyperplasia or adenoma in *pars distalis* was not different between dose groups (Table 2). Six out of eight rats with squamous metaplasia in uterus also had absence of CL or 1-3 small degenerated CL suggesting a common endocrine effect. Only 6 out of 23 females with pituitary nodular hyperplasia, adenoma or macroscopic tumor also had few or absent CL, and only two had squamous metaplasia suggesting no clear relationships between pituitary findings and reproductive organ effects.

		Ovary						Pituitary			Uterus	
	N	Total number of CL	Ovaries with absence of CL	Ovaries with 1-3 degenerated CL	Ovaries with follicular cysts	Ovaries with cyst-like structures	Ovaries with follicular cysts and/or cyst-like structures	Nodular hyperplasia pars distalis	Adenoma pars distalis	Macroscopically observed tumor	Macroscopically observed tumor, nodular hyperplasia or adenoma pars distalis	Squamous metaplasia
Control	18	13.1 ± 6.0	0 (0%)	1 (6%)	1 (6%)	8 (44%)	8 (44%)	2 (11%)	1 (6%)	2 (12%)	4 (22%)	0 (0%)
Totalmix100	20	10.1 ± 6.9	3 (15%)	0 (0%)	3 (15%)	7 (35%)	8 (44%)	ND	ND	ND	ND	ND
Totalmix200	11	7.2 ± 4.9*	1 (9%)	1 (9%)	2 (18%)	8 (73%)	8 (73%)	ND	ND	ND	ND	ND
Totalmix450	16	10.1 ± 5.8	1 (6%)	1 (6%)	2 (13%)	11 (69%)	12 (75%)	1 (6%)	4 (25%)	4 (29%)	6 (38%)	3 (19%)
AAmix200	15	9.9 ± 7.6	4 (27%)*	0 (0%)	4 (27%)	9 (60%)	10 (67%)	ND	ND	ND	ND	ND
AAmix450	16	7.9 ± 6.6#	4 (25%)*	1 (6%)	5 (31%)	13 (81%)*	13 (81%)*	3 (19%)	3 (19%)	7 (64%)	7 (44%)	2 (13%)
Emix200	16	11.3 ± 4.1	0 (0%)	0 (0%)	4 (25%)	9 (56%)	10 (63%)	ND	ND	ND	ND	ND
Emix450	16	9.2 ± 6.4	0 (0%)	3 (19%)	1 (6%)	5 (31%)	5 (31%)	4 (25%)	0 (0%)	1 (7%)	4 (25%)	1 (6%)
PM	14	7.1 ± 6.2*	4 (29%)*	2 (14%)	2 (14%)	13 (93%)**	13 (93%)**	1 (7%)	1 (7%)	0 (0%)	2 (14%)	2 (14%)

Table 2 Summary of histopathological observations. Histological evaluation of ovaries from 13-month old rats exposed perinatally to mixtures of endocrine disrupting chemicals. Values are mean ± SD, or number of affected animals (% of affected rats). CL: corpora lutea. Asterisks indicate statistically significant difference from controls in a Fisher's exact test:
*p < 0.05, **p < 0.01, #p = 0.057.

4. DISCUSSION

This study showed effects of human relevant mixtures of EDCs on both pre-pubertal and adult female rats; reduction in primordial follicles, irregular cycling, and premature absence of CL. These symptoms resemble premature ovarian insufficiency syndrome in humans (Cox and Liu 2014), causing concern that perinatal exposure to EDCs can reduce the reproductive lifespan of women.

4.1 Early reproductive senescence

In rodents, onset of irregular estrous cycles, and eventually cycling arrest, can be a sign of reproductive senescence, usually initiating between 9 and 12 months of age (Maffucci and Gore 2006). We observed an increase in irregular cycles at 12 months of age following prenatal exposure to Totalmix. As no effect was seen on estrous cyclicity at three months of age, this could indicate that exposed rats entered reproductive senescence prematurely compared to the control group. We also observed a reduction in ovary weight in Totalmix, AAmix and PM groups. This was supported by a significantly reduced mean number of CL in Totalmix200 and PM groups, and an increased incidence of complete absence of CL and cyst-like structures in rats exposed to AAmix and PM. This accelerated rate of age-related changes – as compared to background levels - is considered adverse and a sign of early aging in exposed groups. It is possible that examination of slightly younger animals would have resulted in fewer background findings and therefore a clearer picture of the chemically induced histological changes. Nevertheless, our findings indicate that the AAmix and PM groups, as well as the Totalmix group, displayed signs of early reproductive senescence compared to the control group, despite only slight effects were seen on estrous cyclicity in those dose groups. Reproductive senescence may also present as uterine changes. Squamous metaplasia of the uterus occurs spontaneously among aged rats, and can be induced by continued administration of estrogenic compounds (Gopinath 1992). Due to low incidence, it is not clear whether the observed squamous metaplasia was related to early reproductive senescence. Reprogramming of the hypothalamic–pituitary–ovarian axis at central level may be related to early reproductive senescence (Gore et al. 2011) and in a study by Ref. (Isling et al. 2014), rats exposed to AAmix450 and Totalmix450 showed increased incidence of pituitary tumors at 19 months of age. In our study the changes in uteri and ovaries did not appear correlated with pituitary nodular hyperplasia or adenoma, thus we hypothesize that the late effects may be caused by direct effects on the ovaries.

4.2 Early versus delayed effects of anti-androgens in ovary

Effects on aging animals are rarely examined and are not a part of OECD test guidelines for reproductive toxicity studies (OECD 2001, 2011). Chemical effects on early reproductive senescence may thus be overlooked. We wanted to investigate if follicular development was affected at an earlier time-point as early changes could be useful biomarkers of late life effects. On PD22 we saw reduced expression of the germ cell markers *Ddx4* and *Bmp15* in ovaries from animals exposed to PM. This could be due to an overall loss of

oocytes, thus follicles were manually counted. The number of primordial follicles was reduced in rats exposed to AAmix and PM. This is worrying as females are considered to be born with a limited number of oocytes, such that any reduction in the number of primordial follicles can have permanent effects on fertility by reducing the reproductive life span (Hoyer and Keating 2014).

Interestingly, the reduction in primordial follicles was seen in animals exposed to anti-androgenic chemicals. It has become evident that androgens are important for ovarian function and follicular development (Lebbe and Woodruff 2013; Prizant et al. 2014). The androgen receptor (AR) knockout (KO) mouse (ARKO) has a phenotype resembling premature ovarian insufficiency with symptoms such as irregular estrous cycles, lack of CL and infertility (Sen and Hammes 2010; Shiina et al. 2006), effects similar to those observed in adult rats in our study. AR regulates downstream factors controlling folliculogenesis, and down-regulation in young individuals may cause impaired folliculogenesis at a later age (Shiina et al. 2006). It is therefore plausible that reduced AR signaling contributed to the observed late life effects on estrous cyclicity and number of CL.

Both AR antagonists and steroid synthesis inhibitors in the AAmix may have contributed to the observed effects on follicle numbers. Prenatal exposure to the AR antagonist vinclozolin reduced primordial follicle numbers in mice at 12 months of age, and a reduction in total number of oocytes was seen in explanted newborn rat ovaries, cultured and exposed to vinclozolin for ten days (Nilsson et al. 2012). Two studies on the steroidogenesis inhibitor DEHP and/or its metabolite MEHP have shown disruption of early folliculogenesis in explanted newborn mouse ovaries (Hannon et al. 2015; Zhang et al. 2014a). DEHP decreased the incidence of primordial follicles in ovaries of PND21 mice exposed during fetal life (Zhang et al. 2014b), and reduced the percentage of primordial follicles on PND15 and PND21 in mice following postnatal exposure (Zhang et al. 2013). Furthermore, an increase in secondary and antral follicles was registered by (Zhang et al. 2013, 2014b), a finding that agrees with the increased ratio of recruited versus non-recruited follicles in AAmix450 and PM groups. This indicates that in our study, increased folliculogenesis may have caused the reduction in primordial follicle numbers. However, slightly lower total follicle numbers were observed in AAmix450 and PM groups. Therefore it is unclear if the reduction was due to increased recruitment, if the follicle reserve initially was smaller, or a combination of both. Furthermore, both vinclozolin and DEHP have the potential to affect follicle numbers, but further studies are needed to evaluate whether the remainder of compounds in the AAmix also contributed to the observed effects.

Unexpectedly, the reduction in primordial follicle numbers after AAmix and PM exposure was not seen in animals exposed to the Totalmix comprising AAmix, Emix and PM. Emix has been shown to have endocrine effects during prepuberty, as Emix exposure increased mammary outgrowth in PD22 females (Mandrup et al. 2015). For other endpoints such as male anogenital distance and pre-pubertal male reproductive organ

weights, the effects in Totalmix groups reflected the effects of the AAmix (Axelstad et al. 2014). We therefore propose that endocrine effects of Emix exposure can modulate the effects of AAmix and PM on the developing ovary.

Plasma levels of PRL were reduced in PD22 animals exposed to AAmix450. In young females, PRL is proposed to be involved in pubertal timing and reduced levels may cause delayed puberty (Picut et al. 2015). VO was not significantly affected in AAmix450, but visual evaluation indicated an earlier rather than delayed day of VO, which was also observed in AAmix200, Totalmix200 and Emix450, rendering PRL levels unlikely to be causative.

4.3 Human relevance of mixed chemical exposure

The EDCs included in this study were selected as to reflect a chemical exposure pattern relevant to humans (Axelstad et al. 2014; Christiansen et al. 2012). The doses were 100- to 450-times higher than estimated high-end human exposure levels and effects on estrous cyclicity and ovary weight were seen at all doses, suggesting that a standard regulatory safety margin of 100 is not present for highly exposed persons. PM was administered at a dose corresponding to the maximum recommended dose for humans (when taking into account the different kinetics of rats and humans). Such high exposure may seem unlikely to occur during the long time span modelled in the current study, but it is possible that PM exposure for a limited time period, during the most sensitive period of fetal reproductive development, can be sufficient to affect the reproductive function later in life. The observed reduction in primordial follicles, as well as irregular cycling and premature absence of CL resemble premature ovarian insufficiency syndrome in humans, a condition usually leading to premature menopause; before the age of 40 (Cox and Liu 2014). The cause for premature ovarian insufficiency is largely unknown (Cox and Liu 2014; Luisi et al. 2015), but EDCs have been suggested to be part of the etiology (Crain et al. 2008). This raises the concern that early life exposure to EDCs can compromise the reproductive lifespan of women. Such an effect, even if small, is problematic in today's society where the age at childbirth is delayed (Aitken 2013).

In summary, we investigated effects of perinatal exposure to human relevant mixtures of EDCs on female reproductive endpoints and found a reduced follicle pool in pre-pubertal animals after exposure to anti-androgenic chemicals or PM. In adults, signs of early reproductive senescence were seen: effects on estrous cycle regularity and reduced ovary weight after Totalmix exposure, and reduction in CL and ovary weight after anti-androgen and PM exposure. Together, the effects resemble what in humans is categorized as premature ovarian insufficiency, a condition where EDCs have been proposed as part of the etiology. As the mixture composition investigated resembles high-end everyday exposure for humans, the results raise concern for the reproductive lifespan of children of exposed women. The applied top-down approach,

starting from a human relevant exposure scenario, is considered highly relevant for human health assessment and leads the way for targeted mechanistic studies of sub-mixtures and individual compounds.

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Appendix. II Supplementary data

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Chapter 4: *Mixtures of endocrine disrupting chemicals alter the rat ovary proteome: a search for early biomarkers of late life adverse effects*

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ABSTRACT

In the female, the ovaries have a central role both in relation to reproductive and somatic health: they contain the germ cells delivering genetic material to the next generation, and are a critical component in homeostasis affecting the whole body. The size of the follicle pool is set during development and disruptions can have severe consequences on the reproductive lifespan in the adult female. The aim of this study was to investigate the effects of exposure to mixtures of human relevant EDCs on the ovarian proteome, with the objective of identifying candidate biomarker(s) of interrupted ovary development. Rats were exposed to a "real-life" mixture of phthalates, pesticides, UV-filters, bisphenol A, butyl-paraben, as well as paracetamol during pregnancy and lactation. The compounds were tested together (Totalmix) or in subgroups with either anti-androgenic (AAmix) or estrogenic (Emix) activity. Paracetamol was tested separately. Shotgun proteomics was conducted on ovaries from pup day 17 offspring. Three proteins were down-regulated in all four exposure groups: Trimethyllysine dioxygenase (TMLH), Keratin, type II cytoskeletal 8 (KRT8), and anti Müllerian hormone (AMH). Nine of the exposure-affected canonical pathways were common to all exposure groups. Among these were mTOR and HIPPO signaling pathways, which are known to be important for ovary function. In summary, this study showed that exposure to mixtures of EDCs affect the pre-pubertal rat ovary proteome to various degrees, and revealed potentially novel biomarkers that were down-regulated in all exposure groups. Further studies are warranted to better characterize the involvement and potential diagnostic utility of these marker proteins.

1. INTRODUCTION

In the female, the ovaries have a central role in reproductive and somatic health. They contain both germ cells and somatic cells required to fulfil the two main functions of the ovaries: production of oocytes and sex hormones, respectively. The generally accepted view is that the ovaries have a finite number of germ cells from birth, the oocyte pool, which is slowly depleted, inevitably leading to infertility and in some species, the menopause (Nelson et al. 2013).

The size of the oocyte pool is defined during fetal life in humans and neonatal life in mice (Grive and Freiman, 2015) and a reduction in the oocyte pool can lead to a shorter reproductive lifespan (McGee and Hsueh, 2000; Monniaux et al. 2014). Several stages during female development are sensitive to environmental chemical exposure (Chapter 2), and disruptions to these processes can potentially affect future ovary function, as described in the ovarian dysgenesis syndrome (ODS) hypothesis (Buck Louis et al. 2011; Crain et al. 2008).

ODS disorders such as polycystic ovarian syndrome (PCOS) and premature ovarian insufficiency (POI) are difficult to investigate because of the large time-gap between exposure during early development and phenotype manifestation much later in life. Such time gap may span over 30 years in humans. Likewise, the consequences for fertility following exposure to chemicals in aging animals are rarely investigated and not a part of the Organization for Economic Co-operation and Development (OECD) test guidelines for reproductive toxicity studies (OECD, 2011, 2001). Environmental chemicals contributing to ODS may therefore be overlooked when risk assessment is conducted. Early biomarkers of disrupted ovarian development after chemical exposure are, therefore, of great value to assess possible ovarian damage after chemical exposure. Recent advancements in proteomics approaches have enabled the identification and quantification of thousands of proteins, making the discovery of suitable protein biomarkers and/or proteomic fingerprints feasible (Huang et al. 2012; Lai and Chen, 2015).

Today, toxicity evaluation of chemicals is usually performed on one chemical at a time. However, a more realistic scenario is that humans are exposed to complex mixtures of chemicals. In a previous study, we found that mixtures of EDCs with mainly anti-androgenic potentials and the pharmaceutical paracetamol alone, reduced the ovarian follicle pool in pre-pubertal rats exposed during fetal life and lactation (Johansson et al. 2016). In the present study we used the same top-down approach and chemicals, starting from a human relevant mixture and subgroups of this mixture as previously described (Axelstad et al. 2014; Christiansen et al. 2012), with the aim to investigate the potential effects of exposure on the ovary proteome and identify candidate protein biomarkers and disrupted pathways linking chemical exposures to ovarian phenotypes.

2. MATERIALS AND METHODS

2.1 Chemicals

Chemicals used were: di-n-butyl phthalate (DBP) (purity >99.0 %, CAS no. 84-74-2), di-(2-ethylhexyl) phthalate (DEHP) (purity >99.5 %, CAS no. 117-81-7), vinclozolin (purity >99.5 %, CAS no. 50471-44-8), prochloraz (purity >98.5 %, CAS no. 67747-09-5), procymidone (purity >99.5 %, CAS no. 32809-16-8), linuron (purity >99.0 %, CAS no. 330-55-2), epoxiconazole (purity >99.0 %, CAS no. 106325-08-8), octyl methoxycinnamate (OMC) (purity >98.0 %, CAS no. 5466-77-3), dichlorodiphenyl-dichloroethylene (p,p'-DDE) (purity >98.5 %, CAS no.72-55-9); all purchased from VWR - Bie & Berntsen (Herlev, Denmark). 4-methyl-benzylidene camphor (4-MBC) (purity >98.0 %, CAS no. 36861-47-9), bisphenol A (BPA) (purity >99.5 %, CAS no. 80-05-7), butyl paraben (purity >99.0 %, CAS no. 94-26-8) and paracetamol (PM) (purity >99.0 %, CAS no. 103-90-2) were all purchased from Sigma-Aldrich (Brøndby, Denmark). Corn oil was used as a control compound and as vehicle; purchased from VWR - Bie & Berntsen (Herlev, Denmark).

Table 1 Mixture composition and dose for the tested mixtures in mg/kg per day. Design of the mixtures has previously been described (Axelstad et al. 2014, Christiansen et al. 2012).

	Mixture dose (mg/kg per day)			
Chemical	Totalmix-450	AAmix 450	Emix 450	PM
DBP	4.5	4.5	0	0
DEHP	9	9	0	0
Vinclozolin	4.05	4.05	0	0
Prochloraz	6.3	6.3	0	0
Procymidone	6.75	6.75	0	0
Linuron	0.27	0.27	0	0
Epoxiconazole	4.5	4.5	0	0
p,p'-DDE	0.45	0.45	0	0
4-MBC	27	0	27	0
OMC	54	0	54	0
Bisphenol A	0.675	0	0.675	0
Butyl paraben	27	0	27	0
Paracetamol	360	0	0	360

2.2 Chemical Mixtures

The composition of the mixtures was based on high-end human exposure levels as previously described (Axelstad et al. 2014; Christiansen et al. 2012). In short, the Totalmix contained all 13 compounds; the AAmix contained compounds considered to have predominantly anti-androgenic modes of action; the Emix

contained compounds considered to have predominantly estrogenic properties (Table 1). PM was included in the Totalmix, as well as tested on its own, but was not included in the AAmix or Emix. The mixtures were given at 450-times human high-end exposure and PM was given at 350 mg/kg, both in the Totalmix and in the single exposure (Table 1).

2.3 Animals and exposure

The animal study was as described in Axelstad et al. (2014). In brief, time-mated nulliparous Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were used and the day vaginal plug was detected was designated as GD 1 and the expected day of delivery (GD23) was designated as pup day (PD) 1. The dams were supplied at gestation day (GD) 3 of pregnancy. Animals were exposed to vehicle (controls), or one of the eight mixtures (Table 1). Each dose group comprised 16-20 dams, and 14-20 viable litters were obtained for each group. Rats were exposed by oral gavage from GD7-21, and again after birth from PD1-22. Exposure to PM was from GD13-19 and PD14-22, both in mixtures and single dosing. This was to avoid effects on embryo implantation (Gupta et al. 1981) and problems during parturition. The study was performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee.

2.4 Protein harvest and digestion

On PD17 one female offspring from each litter was killed and alternately the right or left ovary was collected and snap frozen in liquid nitrogen. The samples were stored at -80ºC. Protein was extracted and prepared from 10 ovaries from each group using the AllPrep kit ((#80004; QIAGEN, Manchester, UK) according to the manufacturer's instructions. Protein concentrations were quantified by a modified Lowry assay (Biorad Ltd., Hertfordshire, UK, cat.no. 500-0122) and from each sample 10 µg of protein extract was diluted to a total volume of 100 µl of 50 µM NH_4HCO_3. Proteins were digested in solution according to the PRIME-XS protocol (http://www.primexs.eu/protocols/Public-Documents/04---Protocols/PRIME-XS-Protocol-NPC-In-Solution-Digestion.pdf/). Briefly, proteins were reduced in 2 mM dithiothreitol for 25 min at 60°C and S-alkylated in 4 mM iodoacetamide for 30 min at 25°C in the dark, then digested by sequencing-grade modified trypsin (Promega) at a 1:50 ratio of trypsin:protein overnight at 37°C. The reaction was stopped by freezing at -80°C. Samples were dried by vacuum centrifugation (SpeedVac Plus SC110A, Savant) and dissolved in 50 µL 0.1% trifluoroacetic acid. Peptides were desalted using ZipTip C18 stage tips (Merck Millipore) according to the manufacturer's instructions. The eluate from the ZipTip was dried by SpeedVac and dissolved in 10 µL 2% acetonitrile/0.1% formic acid. One half (5 µL) of the peptide solution was analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), equivalent to 2.5 µg peptides assuming the ZipTip binding capacity was saturated. The LC-MS system comprised a Thermo Scientific Dionex UltiMate 3000 RSLC nano LC configured for pre-concentration onto a nano column, coupled to a Q

Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer fitted with an EASY-Spray nano-ESI source (Thermo Scientific). Peptide samples were injected onto a C18 PepMap 100 pre-column (300 µm i.d. x 5 mm) in loading pump solvent (2% acetonitrile, 0.1% formic acid) at a flow rate of 10 µL/min for 5 min. The pre-column was then reverse-flushed to the analytical column (PepMap RSLC C18; 50 µm i.d. x 15 cm)* at 0.3 µL/min using the nano pump. Peptides were separated using a gradient of acetonitrile* while MS/MS data were acquired by the Q Exactive in data-dependent mode (Top10 method)*.

* For 1 hour runs using the 15 cm column the LC and MS details were as follows:

Nano pump solvent A: 0.1% formic acid (in UHQ water)

Nano pump solvent B: 80% acetonitrile, 0.1% formic acid (in UHQ water)

LC gradient: 3-10% solvent B in 5 min, 10-40% solvent B in 30 min, 40-80% solvent B in 5 min, hold at 80% solvent B for 8 min, 80-3% solvent B in 1 min, hold at 3% solvent B for 15 min.

Parameters for the full scan/data-dependent MS2 (Top10) method were:

Full scan range 375-1750 m/z; resolution 70,000; AGC target 3e6; maximum IT 50 ms.

MS2 scan resolution 17,500; AGC target 5e4; maximum IT 100 ms; loop count 10; isolation window 1.6 m/z; NCE 26; underfill ratio 4%; charge states 2-5 included; peptide match preferred; exclude isotopes on; dynamic exclusion 40 s.

2.5 Analyses of LC-MS/MS output

All RAW files were simultaneously processed by MaxQuant (v 1.5.3.8) and searched against the UniProtKB FASTA database for *Rattus Norvegicus* (downloaded from www.UniProt.org 29-03-2016). MaxQuant parameters were set to default except label free quant: minimum ration count =1, and match between runs.

2.6 Statistical analyses

Data were handled in Microsoft Excel and analyzed in R (v3.31). Proteins were filtered to include only those with MaxLFQ-normalised intensity in at least 75% of the samples. After filtering, MaxLFQ-normalised protein intensities were log2 transformed. Missing values were imputed using the *missForest* package in R. Latent variation was removed using the *sva* package in R. Comparisons were performed using empirical moderated Bayes test statistics using the *limma* package in R. *P*-values were adjusted using the *limma*-adapted Benjamini-Hochbergh False Discovery Rate (FDR) approach. For each protein a z-score was calculated. The z-score is a measure of how many standard deviations (SD) below or above the population mean a raw score is, and ranges from -3 SD's to +3 SD's.

2.7 Pathway mapping

Ingenuity Pathway Analysis (IPA) V9.0 (Ingenuity Systems, http://www.ingenuity.com) was used to assign affected proteins to Canonical Pathways (Bellingham et al. 2013; Filis et al. 2015). Pathway mapping was performed using a liberal FDR threshold cut-off of 20%. To explore relationship between proteins and pathways in different exposure groups, Venn diagrams were constructed on the free online website: http://bioinformatics.psb.ugent.be/webtools/Venn/

3. RESULTS

3.1 Effects of exposure

Table 2 shows differentially expressed proteins in the four exposure groups, relative to control goup, at various cut-off FDR levels. In terms of number of proteins affected, Emix exposure was associated with the greatest number of differentially expressed proteins regardless of FDR cut-off, followed by Totalmix, PM and AAmix. The AAmix showed least differentially expressed proteins, and those with differential expression had FDR values above 10% (Table 2). A complete list of affected proteins is available upon request.

Table 2 Effects of exposure on the number of differentially regulated proteins at FDR adjusted p-values (5, 10, 20, and 30%). ↑ indicates upregulated proteins and ↓ down regulated proteins.

	5%	10%	20%	30%
C vs Totalmix	↑18 ↓4	↑38 ↓14	↑193 ↓117	↑331 ↓212
C vs AAmix	0	0	↑13 ↓8	↑63 ↓42
C vs Emix	↑211↓281	↑324↓395	↑460 ↓565	↑580 ↓703
C vs PM	↑2 ↓1	↑8 ↓11	↑49 ↓48	↑99 ↓92

Potential ovarian biomarkers of effect for each exposure regime were selected based on fold difference from control group expression levels, functional significance in the ovary, or if originating from blood (Table 3). Among these potential biomarkers three were down-regulated in all exposure groups; trimethyllysine dioxygenase (TMLH), Keratin, type II cytoskeletal 8 (KRT8), and anti Müllerian hormone (AMH). The protein calretinin (CALB2) was down regulated in Totalmix and AAmix, but upregulated in Emix. 3-oxo-5-alpha-steroid 4-dehydrogenase 1 (G8JLS2, also called SRD5A1) was upregulated in Totalmix and Emix, Estradiol 17-beta-dehydrogenase 1 (DHB1) was upregulated in AAmix and Emix, and Fragile X mental retardation protein 1 homolog (A0A0G2JZV8) and protein Hbb-b1 (A0A0G2JTW9) were upregulated in AAmix and PM.

Table 3 Potential biomarkers of effect for Totalmix, AAmix, Emix and PM groups. Proteins were chosen based on fold change, functional significance and blood provenance.

	Fold change	Protein Name	Gene names	Protein name (Full)
Totalmix	-3.93	CALB2	*Calb2*	Calretinin
	-2.53	KRT8	*Krt8*	Keratin, type II cytoskeletal 8
	-1.71	D3ZK97	*H3f3c*	Histone H3
	-1.71	AMH	*Amh*	Anti Müllerian hormone
	-1.71	ADA	*Ada*	Adenosine deaminase
	-1.66	D4A409	*Lama1*	Laminin, alpha 1
	-1.63	TMLH	*Tmlhe*	Trimethyllysine dioxygenase, mitochondrial
	-1.60	H31	*Hist1h31*	Histone H3.1
	-1.48	Q59IV9	*Esr2*	Estrogen receptor 2 beta, isoform CRA_c
	-1.42	D3ZJ08	*Hist2h3c2*	Histone H3
	4.45	CAH3	*Ca3*	Carbonic anhydrase 3
	2.18	A0A0G2JSR8	*Cyp17a1*	Cytochrome P450, family 17, subfamily a, polypeptide 1
	1.92	A0A0G2K8Q1	*Apoc3*	Apolipoprotein C-III
	1.75	A0A0G2JW12	*C4a*	Complement C4
	1.73	G8JLS2	*Srd5a1*	3-oxo-5-alpha-steroid 4-dehydrogenase 1
	1.63	M0RDM4	*LOC680322*	Histone H2A
	1.52	G3V679	*Tfrc*	Transferrin receptor protein 1
	1.51	DHB11	*Hsd17b11*	Estradiol 17-beta-dehydrogenase 11
	1.43	H10	*H1f0*	Histone H1.0
	1.38	A0A0G2K151	*Apoe*	Apolipoprotein E
	1.37	AL1A1	*Aldh1a1*	Retinal dehydrogenase 1
AAmix	-2.87	CALB2	*Calb2*	Calretinin
	-2.64	K2C8	*Krt8*	Keratin, type II cytoskeletal 8
	-1.90	TMLH	*Tmlhe*	Trimethyllysine dioxygenase, mitochondrial
	-1.88	MIS	*Amh*	Anti Müllerian hormone
	1.85	GRB2	*Grb2*	Growth factor receptor-bound protein 2
	1.63	A0A0G2JTW9	*Hbb-b1*	Protein Hbb-b1
	1.44	D3ZC01	*Rbbp5*	Protein Rbbp5
	1.33	A0A0G2JZV8	*Fmr1*	Fragile X mental retardation protein 1 homolog
	1.32	DHB1	*Hsd17b1*	Estradiol 17-beta-dehydrogenase 1
Emix	-2.08	MIS	*Amh*	Anti Müllerian hormone
	-2.08	NC2B	*Dr1*	Protein Dr1
	-1.78	TMLH	*Tmlhe*	Trimethyllysine dioxygenase, mitochondrial
	-1.76	HAT1	*Hat1*	Histone acetyltransferase type B catalytic subunit
	-1.76	K2C8	*Krt8*	Keratin, type II cytoskeletal 8
	-1.39	TRFE	*Tf*	Serotransferrin
	-1.35	B1WBQ7	*Msh2*	DNA mismatch repair protein Msh2
	-1.32	F7EY92	*Mbd3*	Methyl-CpG binding domain protein 3 (Predicted), isoform CRA_c
	-1.30	STAR	*Star*	Steroidogenic acute regulatory protein, mitochondrial
	-1.21	D4A0S1	*Foxl2*	Protein Foxl2
	1.72	F1MAN8	*Lama5*	Laminin, alpha 5, isoform CRA_a
	1.56	DHB1	*Hsd17b1*	Estradiol 17-beta-dehydrogenase 1
	1.48	A0A0G2JWD2	*Fxr1*	Fragile X mental retardation syndrome-related protein 1

Table 3 continued

	Fold change	Protein Name	Gene names	Protein name (Full)
Emix continued	1.46	G8JLS2	*Srd5a1*	3-oxo-5-alpha-steroid 4-dehydrogenase 1
	1.41	G3V8D4	*Apoc2*	Apolipoprotein C-II (Predicted)
	1.39	CALB2	*Calb2*	Calretinin
	1.20	A0A0G2JU18	*Zp2*	Zona pellucida glycoprotein 2, isoform CRA_a
PM	-2.01	K2C8	*Krt8*	Keratin, type II cytoskeletal 8
	-1.94	A0A0G2JSW3	*Hbb*	Hemoglobin subunit beta-1
	-1.80	TMLH	*Tmlhe*	Trimethyllysine dioxygenase, mitochondrial
	-1.94	A0A0G2JSW3	*Hbb*	Hemoglobin subunit beta-1
	-1.50	MIS	*Amh*	Anti Müllerian hormone
	1.75	Q1EG89	*Pxn*	Myocardial ischemic preconditioning associated protein 7
	1.68	H14	*Hist1h1e*	Histone H1.4
	1.58	A0A0G2JTW9	*Hbb-b1*	Protein Hbb-b1
	1.39	A0A0G2JZV8	*Fmr1*	Fragile X mental retardation protein 1 homolog

3.2 Ingenuity Pathway Analysis (IPA)

3.2.1 Canonical pathways

IPA analysis identified 53 canonical pathways for Totalmix, 38 for AAmix, 138 for Emix, and 33 for PM using a cut off $p < 0.05$. Among these, all four exposure groups had nine affected canonical pathways in common (mTOR signaling, G2/M DNA damage checkpoint regulation, EIF2 signaling, 14-3-3 mediated signaling, protein kinase A signaling, ERK/MAPK signaling, HIPPO signaling, IGF-1 signaling, and Regulation of eIF4 and p70S6K signaling) (Figure 1). The pathways with highest ratios (percent of the proteins that are present in the pathway) and the pathways with highest z-score (absolute figures) for all exposure groups are presented in Figure 2 A-D.

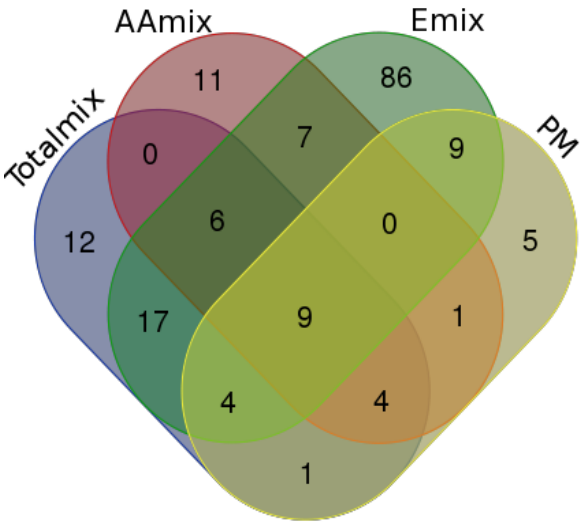

Figure 1 Venn diagram for canonical pathways. The exposed groups had 9 canonical pathways in common when all pathways with $p < 0.05$ were used for analysis.

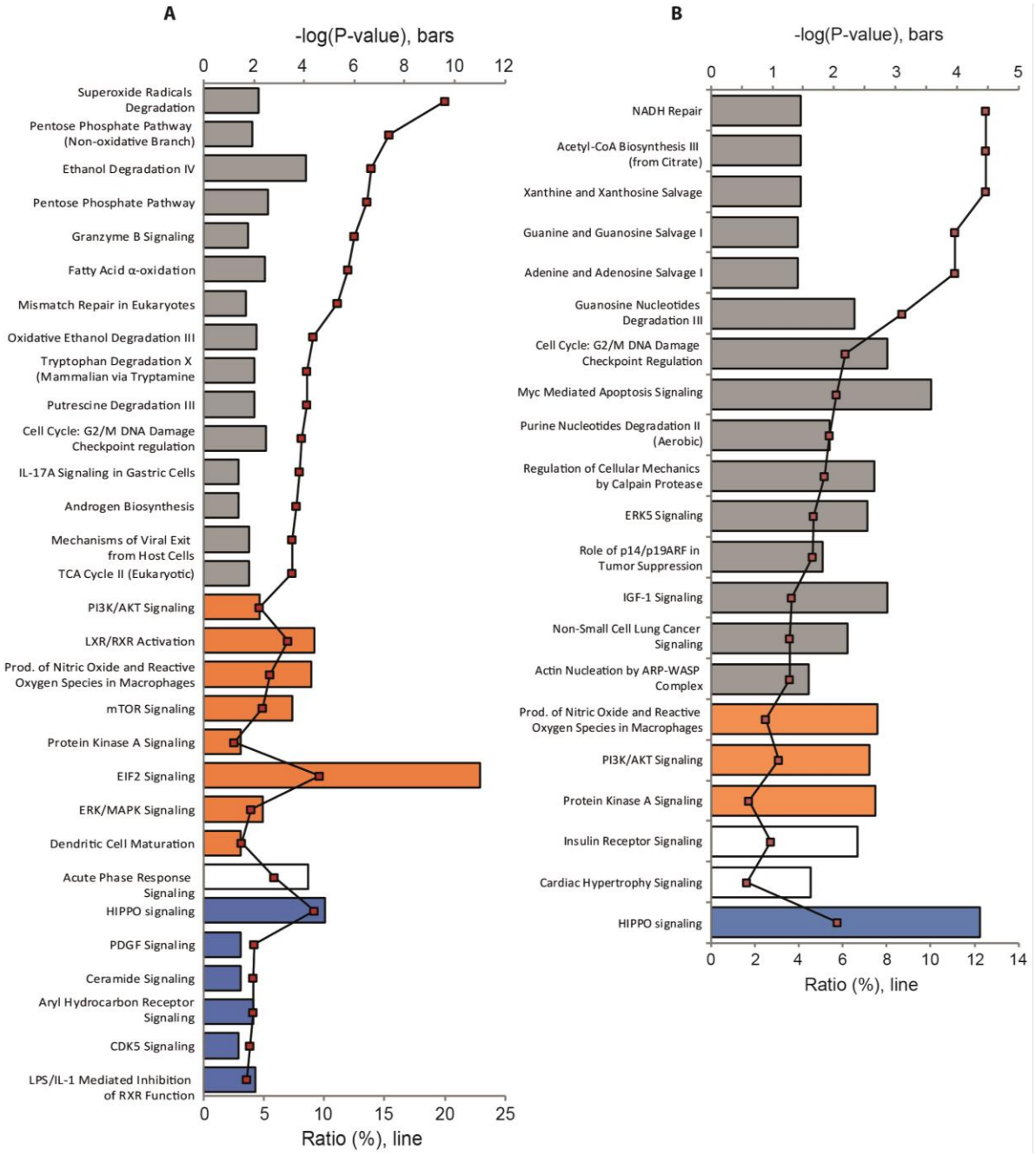

Figure 2 Top 15 pathways (or less if 15 pathways were not identified) with highest ratios (percent proteins of the pathway present among the proteins inserted in the IPA) and the 15 pathways with highest z-score (absolute figures). A) Totalmix, B) AAmix, C) Emix, and D) PM. Grey: no pattern, blue: down-regulated, orange: up-regulated. The line shows the ratio of altered proteins by total pathway proteins.

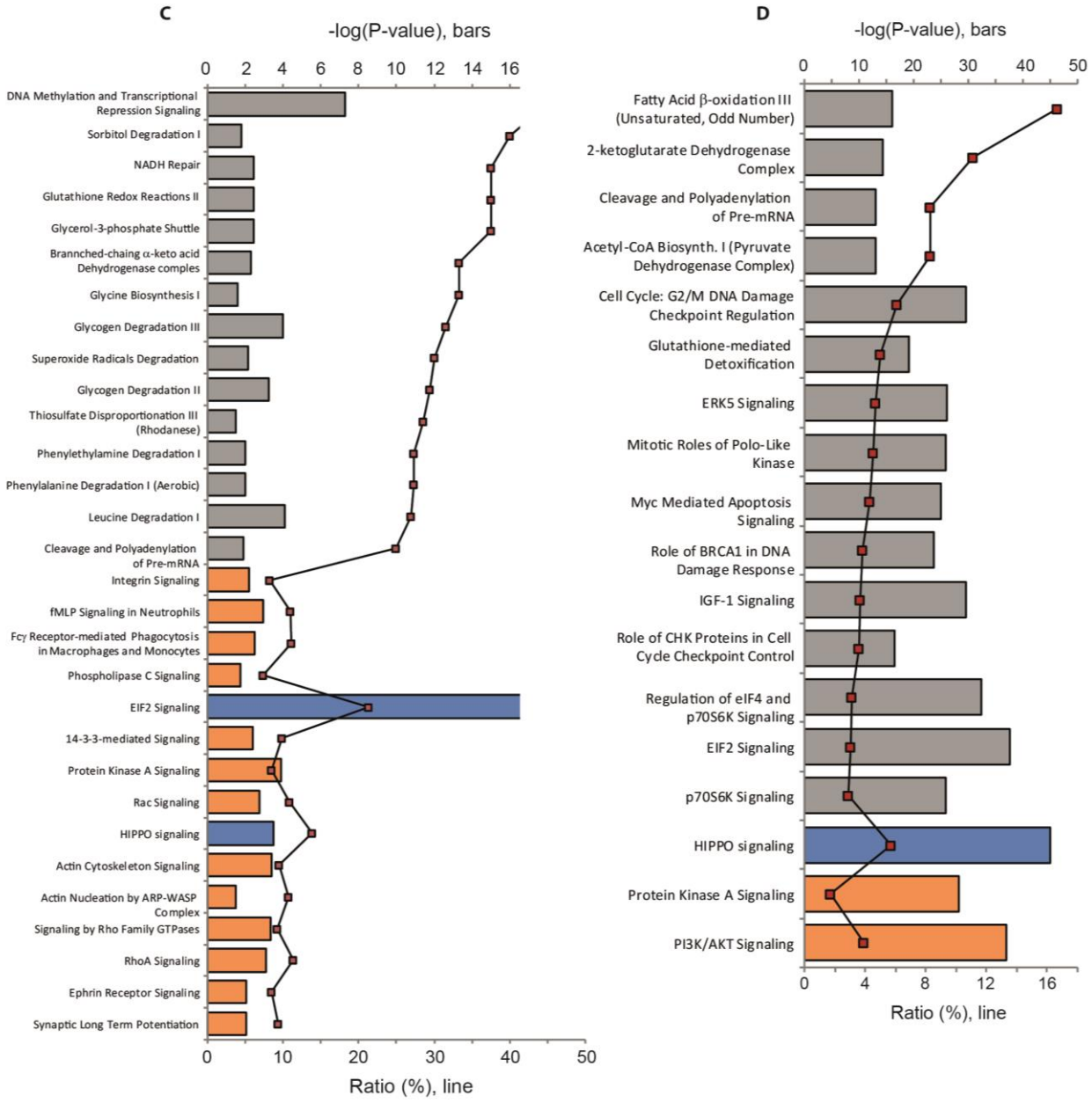

Figure 2 continued

3.2.2 Upstream regulation

For Totalmix, upstream regulation analysis showed 210 molecules with an activation z-score (suppl. material, available on request), and some interesting potential regulators emerged. The upstream regulator most strongly predicted to be activated was NFKBIA (z-score 2.7). Other activated proteins with a z-score above 1.5 were 17β-estradiol, FOXO1, PTEN, AR, dihydrotestosteone, and indomethacin. The most strongly upstream regulator predicted to be inhibited was sirolimus, also named rapamycin (z-score -2.8). Examples of other interesting upstream regulators predicted to be inhibited with a z-score less than -1.5 were tamoxifen, RICTOR, and ESR2.

For AAmix, fewer molecules were seen in the upstream regulation analysis: 42 molecules had an activation z-score (suppl. material, available on request). Among these, the molecule most strongly predicted to be activated was RICTOR (activation z-score 2.2) and the one most strongly predicted to be inhibited was ERBB2 (activation z-score -2.0).

Emix had 349 upstream regulators with an activation z-score (suppl. material, available on request). RICTOR was the molecule with largest activation z-score (5.0) and MYCN the regulator predicted to be most strongly inhibited (-4.3). Examples of interesting molecules with activation z-score above ± 1.5 are FSH and genistein.

There were in all 18 regulators with activation z-scores in PM-exposed ovaries (suppl. material, available on request). The highest absolute numerical activation z-scores were found for miR-124-3p (and other miRNAs) and LY294002.

4. DISCUSSION

The results showed three potential biomarkers of effect, TMLH, KRT8 and AMH, which were down-regulated in all exposure groups. Furthermore, the proteins CALB2, SRD5A1, DHB1, A0A0G2JZV8, and A0A0G2JTW9) were differentially expressed in more than one group. IPA analysis identified 9 pathways common to all exposure groups, among these the two ovary important pathways mTOR and Hippo signaling.

4.1 Differentially expressed proteins and potential biomarkers

In relation to identification of potential biomarker(s) of effect, three proteins were down regulated in all four exposure groups; TMLH, KRT8, and AMH. TMLH is the first catalytic enzyme in the carnitine biosynthesis pathway (Strijbis et al. 2010). Carnitine plays an essential role in the transfer of long-chain fatty acids into mitochondria where they are β-oxidized, and the plasma carnitine levels are age and sex dependent in both

rats and humans, suggesting that sex hormones may be involved in its regulation (Vaz and Wanders, 2002). Reduced levels of TMLH could indicate that the endogenous production of carnitine is reduced, which may then affect fatty acid oxidation. Significantly reduced carnitine levels have been found in women with PCOS, and may be associated with hyperandrogenism and/or insulin resistance (Fenkci et al. 2008). This is supported by a metabolomics study by Dong et al. (2015) who found reduced levels of carnitine in PCOS patients compared to controls. Interestingly, β-oxidation is important for oocyte developmental competence (Dunning and Robker, 2012), and addition of carnitine to cultured mouse follicles, from preantral to large antral stage, increase β-oxidation and improve maturity rates, fertilization rates and blastocyst development (Dunning et al. 2011). Carnitine supplementation *in vivo* has been shown to improve oocyte quality in a rodent model of ovarian aging (Miyamoto et al. 2010). Down-regulation of TMLH, therefore, has potential as a candidate biomarker of negative impact on ovary function, but further studies are needed, proposedly also involving measurement of plasma carnitine levels. Also, it should be kept in mind that *de novo* synthesis of carnitine only represents approximately 25% of total carnitine in mammals, whereas 75% comes from the diet (Rebouche, 1992) and that synthesis is primarily in the liver (Vaz and Wanders, 2002), implying that changes in ovarian production may be of less importance.

KRT8 has several functions in the body (Moll et al. 2008) and in relation to reproductive endpoints, KRT8 knockout mice show embryonic lethality between 12 and 13 dpc (Baribault et al. 1993), likely due to defective function of the placental barrier (Jaquemar et al. 2003). Interestingly, KRT8 is-upregulated in placentas from smokers (Huuskonen et al. 2016). KRT8 is expressed in the rat and mouse ovary during fetal life. Later, KRT8 is strongly expressed in primary follicles during the first two weeks after birth, whereas it is weak in growing follicles during the first week and disappears during the second week after birth (Appert et al. 1998; Fridmacher et al. 1992). A reduction in KRT8 on PD17 could therefore indicate a reduction in primary follicle numbers.

A third protein down regulated in all exposure groups was AMH. AMH expression is initiated in the granulosa cells when primordial follicles are recruited to grow, with highest expression found in pre-antral and small antral follicles (Dewailly et al. 2014). AMH participates in regulation of the follicle reserve by inhibiting recruitment of primordial follicles (Durlinger et al. 2002), and in AMH knockout mice, more pre-antral and antral follicles are seen at the ages of 25 days and 4 months, and at 13 months the ovarian follicle pool is almost completely depleted (Durlinger et al. 1999). A corresponding situation is found in women, where AMH is reduced with advancing age reflecting the declining size of the primordial follicle pool. To date AMH is one of the most promising human biomarkers of ovarian reserve (Broer et al. 2014). In a previous study, littermates of the animals used herein were found to have reduced numbers of primordial follicles at PD22 after AAmix and PM exposure and at approximately 1 year of age, signs of early reproductive senescence were seen, especially in the Totalmix group (Johansson et al. 2016). This may point

to an early depletion of the ovarian follicular reserves and is in line with the observed reduction in AMH at PD 17. The utility of AMH as a biomarker for ovarian effects of EDC exposure is therefore highly relevant. Calretinin (CALB2) was down regulated in Totalmix and AAmix, and up regulated in Emix. In the adult human ovary, CALB2 is expressed in the germinal epithelium and in androgen excreting cells (Bertschy et al. 1998). CALB2 is a useful marker for diagnosing several types of tumours in the female reproductive organs (Portugal and Oliva, 2009). Thus the fact that CALB2 was down-regulated after both Totalmix and AAmix exposure, suggests that it may be a useful biomarker of anti-androgenic disruption. Furthermore, as the animals exposed to Totalmix and AAmix showed signs of reproductive senescence (Johansson et al. 2016), CALB2 may be useful as an early marker of later life adverse ovarian effects.

Two other proteins having potential as biomarkers for interrupted ovarian development after exposure to anti-androgens were A0A0G2JZV8 and A0A0G2JTW9, both upregulated in AAmix and PM. Furthermore, the protein SRD5A1, which converts testosterone to dihydrotestosterone, was upregulated in Totalmix and Emix. This indicates that the chemicals present in Emix may affect androgen synthesis, and increased level of SRD5A1 has been associated with PCOS (Vassiliadi et al. 2009). It would be of value to further investigate these proteins as potential biomarkers of effect.

4.2 Subtle effects on the ovarian proteome following AAmix and PM exposure

Based on our previous study where we saw effects of exposure on primordial follicle numbers in AAmix and PM groups (Johansson et al. 2016), we expected to see more marked changes to protein expression in these two groups compared to the other mixture groups. Surprisingly, dysregulation of proteins was less pronounced in the AAmix group than the other exposure groups, with no significantly affected proteins at FDR correction levels of 5 and 10%. In the PM group, dysregulation of proteins was seen at a FDR correction level of 5%. However, much fewer proteins were affected than in the Totalmix or Emix groups. Notably, one of the drawbacks with shotgun proteomics is that only the most abundant proteins are quantified (Cayer et al. 2016). However, the most abundant proteins are not necessarily the most important with respect to the biological effects caused by chemical exposure. The lower numbers of affected proteins found in AAmix and PM compared to Totalmix and Emix merely implies that the most abundant proteins were less affected. It is therefore possible to miss potential biomarkers of effect. In this study we chose to use a non-conservative approach, emphasizing fold change and previously known physiological significance in female reproduction more than the FDR cut-off levels. If a potential biomarker is found, further studies have to include confirmation of the findings and thereby identification of false positives. Strategically, it is better to include more potential biomarkers in the screen, as the irrelevant ones are likely to be sorted from the relevant in the confirmation process.

The ovary is a plastic organ undergoing dramatic changes during the menstrual/estrous cycle in adult individuals and also during pre-pubertal development (Picut et al. 2015). In Johansson et al. (2016) the exposure-associated reductions in primordial follicle numbers were observed at PD22, whereas in the present study the ovaries from PD17 animals were examined. Due to the rapid progression of ovarian development around this age, five days is a relatively long time allowing for significant changes in the pre-pubertal ovary. During the time interval from approximately PD17 to PD22 the estradiol negative feedback system is initiated, contributing to decline in FSH/LH levels, and circulating prolactin and inhibin B concentrations increase. On a histological level apoptosis of granulosa cells and atresia of follicles is initiated after PD21 (Picut et al. 2015). This could be part of the explanation for the relatively small effects seen in the PD17 AAmix and PM exposed proteomes. Another more likely possibility is that the reduction in ovarian oocyte pool identified at PD22 occurred much earlier in development, and at the time of proteomics investigation the proteomes did not express any changes which could explain the reduction. Exposure covered several sensitive developmental windows, such as primordial germ cell migration and gonadal sex determination, meiosis, and primordial follicle assembly, which are all sensitive to chemical insult (Chapter 2).

4.3 Affected pathways and upstream regulators

Within the ovary, the spatial environment may be just as important for follicle activation as hormonal signaling (Woodruff and Shea, 2007). The Hippo signaling pathway is involved in regulation of follicle development and defects in Hippo signaling genes are associated with POI, PCOS, ovarian follicle reserve, and ovarian tumorigenesis (Hsueh et al. 2015). In Totalmix, the Hippo signaling pathway was inhibited (negative z-score) indicating that regulation of follicle development might be disrupted.

Another pathway in common for all groups was mTOR, which involves the two complexes mTORC1 and mTORC2 (Weber and Gutmann, 2012). Activation of mTORC1 leads to primordial follicle recruitment, but this effect can be stopped by rapamycin which affects mTORC1 (Adhikari et al. 2010). Interestingly, in the upstream analysis for Totalmix, rapamycin was predicted to be inhibited, meaning that the pathway acted as if rapamycin was not present, thus activation of the primordial follicle pool would be possible. Also, the Totalmix was composed of chemicals with both estrogenic and anti-androgenic potentials and examples of other upstream regulators predicted in Totalmix were β-estradiol, androgen receptor, dihydrotestosterone, and indomethacin. The chemicals in Totalmix can be expected to act in ways similar to several of these molecules.

In the Emix-exposed ovaries genistein emerged as an activated upstream regulator. Genistein is a known estrogen (Vitale et al. 2013) and this indicates that the Emix affected downstream pathways in an expected, estrogenic way.

In summary, this study showed that exposure to representative mixtures of EDCs affect the pre-pubertal rat ovarian proteome to various degrees. Three proteins with potential as biomarkers of effect on ovary development were affected in all four exposure groups: TMLH, KRT8 and AMH. The latter demonstrates biological consistency of the approach taken since it is already employed as a biomarker for ovarian reserve. Further work investigating their potential role as biomarkers is warranted.

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Chapter 5: *Endocrine disrupting chemicals with anti-androgenic potential reduce the number of primordial follicles and disturb follicle assembly in neonatal rat ovary explants: A pilot study*

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Study report

ABSTRACT

Androgens play an important role in females as well as males, thus dysregulation of androgen-sensitive signaling pathways can have negative consequences for fertility in both sexes. In a previous study we found that perinatal exposure to a mixture of anti-androgenic pesticides and phthalates (AAmix) can cause a reduced follicle pool in pre-pubertal female rats. In the present study, we aimed to further investigate the etiology of this phenotype, as well as discerning whether the pesticides or the phthalates are the main drivers of the effect. Explanted fetal rat ovaries collected around the day of birth were cultured in the presence of AAmix, or sub-mixtures of pesticides (PEmix) or phthalates (PHmix), as well as the di-(2-ethylhexyl) phthalate (DEHP)-metabolite mono(2-ethylhexyl)phthalate (MEHP) tested separately. No significant effects were observed in AAmix-exposed and PHmix-exposed ovaries as assessed by gene expression analysis and morphological assessments of the oocytes. However, ovaries exposed to PEmix displayed a significant reduction in primordial follicles. Although preliminary, these data suggests that some effects caused by selected pesticides are alleviated when they are present in mixtures with the phthalates, but the mechanisms behind these interactions remain unknown. Further investigations with appropriate group size are warranted in relation to effects on the primordial follicle pool.

1. INTRODUCTION

Androgens are typically referred to as male sex hormones, but it has become evident that they also play an important role in females, including maintenance of normal ovary function (Lebbe and Woodruff 2013; Prizant et al. 2014). Therefore, dysregulated androgen levels can have negative consequences for fertility, which has been observed in women with adrenal insufficiency or PCOS, which causes reduced or increased androgen levels, respectively (Lebbe and Woodruff 2013). Also, mice lacking the androgen receptor (AR) show irregular estrous cycling, lack of corpora lutea, and are infertile (Sen and Hammes 2010; Shiina et al. 2006). Thus, androgens play a crucial role in female reproduction and are not simply present as precursor molecules for estrogen synthesis.

It is generally accepted that females have a set number of eggs around the time of birth, the so called ovary reserve, which is established during fetal life in humans (Grive and Freiman 2015), and neonatally in mice and rats (Pepling 2012). The establishment of the follicle pool is a delicate process occurring over a prolonged period and involves breakdown of germ cell cysts, programmed cell death, and assembly of primordial follicles (Pepling 2012). Disruptions to any steps of this process can reduce the total number of primordial follicles and consequently affect reproductive function and physiology later in life. It has become increasingly clear that some EDCs can negatively impact follicle assembly in rodents, including di-(2-ethylhexyl) phthalate (DEHP) (or its metabolite mono(2-ethylhexyl)phthalate (MEHP)) (Hannon et al. 2015; Zhang et al. 2014) and vinclozolin (Nilsson et al. 2012). Prenatal exposure to either DEHP or vinclozolin also causes reduced primordial follicle numbers in both pre-pubertal and adult mice, respectively (Nilsson et al. 2012; Zhang et al. 2015), attesting to the fact that more efforts should be spent on how female reproductive function is affected by EDCs.

We recently showed that *in utero* exposure to a mixture of anti-androgenic EDCs can reduce the follicle reserve in pre-pubertal rats (Johansson et al. 2016). However, as that study focused primarily on effect endpoints, our present study was carried out to better understand the potential mechanisms that underpin the late life phenotypes, and to better understand what components of the chemical mixtures are most likely to drive the effects. To achieve this, explanted neonatal ovaries were exposed to the original mixture (AAmix), as well as sub-mixtures of pesticides (PEmix), phthalates (PHmix) and the sole phthalate metabolite MEHP. Expression of genes known to be important for ovary development and function was investigated as well as histological evaluation of ovary sections. As this was a pilot study, only one exposure dose per mixture was included, and the group size was small (n = 2-4).

2. MATERIALS AND METHODS

2.1 Chemicals

Test compounds were: di-n-butyl phthalate (DBP) (purity >99.0 %, CAS no. 84-74-2), di-(2-ethylhexyl) phthalate (DEHP) (purity >99.5 %, CAS no. 117-81-7), mono(2-ethylhexyl)phthalate (MEHP) (purity 97%, CAS no. 4376-20-9), vinclozolin (purity >99.5 %, CAS no. 50471-44-8), prochloraz (purity >98.5 %, CAS no. 67747-09-5), procymidone (purity >99.5 %, CAS no. 32809-16-8), linuron (purity >99.0 %, CAS no. 330-55-2), epoxiconazole (purity >99.0 %, CAS no. 106325-08-8), p,p'-dichlorodiphenyl-dichloroethylene (p,p'-DDE) (purity >98.5 %, CAS no.72-55-9); all purchased from VWR - Bie & Berntsen (Herlev, Denmark).

2.2 Mixtures

DMEM/F-12 (1:1) (1x) F-12 nutrient mixture (HAM) media (Thermo Fisher Scientific), Gentamicin (1:1000, Millipore, Thermo Fisher Scientific), Fungizone (1:100, Millipore, Thermo Fisher Scientific), and 10% fetal bovine serum (Catalog no. F6765, Sigma Aldrich) was used for culture.

Stock solutions were prepared in DMSO and added to the media to reach a combined concentration of 100 µM in the AAmix. The phthalate mix (PHmix) contained DBP and DEHP, and the pesticide mix (PEmix) vinclozolin, procholoraz, procymidone, linuron, epoxiconazole, and p,p'-DDE at concentrations listed in Table 1. Since MEHP is an active metabolite of DEHP it was tested separately at concentration corresponding to DBP and DEHP (Table 1). The ratio between the chemicals corresponded to the ratios used in previous *in vivo* studies (Axelstad et al. 2014; Christiansen et al. 2012). DMSO was added at equal concentrations to all exposure and control groups.

Table 1 Chemical composition of the different mixtures. Ratios between the chemicals correspond to the ratios in (Axelstad et al. 2014; Christiansen et al. 2012).

	Mixtures in molar (µM)			
Compound	Aamix	Phthalate mix	Pesticide mix	MEHP
DBP	14.7	14.7		35.6*
DEHP	20.9	20.9		
Vinclozolin	12.9		12.9	
Prochloraz	15.2		15.2	
Procymidone	21.6		21.6	
Linuron	1.0		1.0	
Epoxiconazole	12.4		12.4	
p,p'-DDE	1.3		1.3	
Sum	100	35.6	64.4	35.6

*Corresponding to the sum of DBP and DEHP concentrations

2.3 Animals and *ex vivo* fetal ovary culture

Six time-mated Sprague Dawley rats (Taconic Europe, Ejby, Denmark) were delivered two weeks into pregnancy. Fetal age was designated as noon of the day following overnight mating corresponding to 0.5 days post coitum (dpc). Fetuses were collected at 21 or 22 dpc and dissected directly into growth medium. The ovaries were collected with the top part of the fallopian tubes (*infundibulum* and *fimbrae*) attached. For culturing, the ovaries were placed on top of a filter (MF™ Membrane Filters, 0.45um HA, HAWP02500, Merck Millipore Ltd.) and floated on top of 400µl of growth medium containing chemicals in 24-well plates (Costar, Corning Incorporated). The ovaries were cultured at 37 °C, 5 % CO_2 for 72 h, with medium changed after 24 and 48 h. After 72 h, ovaries were snap-frozen and stored at -80 ºC.

Animal experiments were performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee.

2.4 Gene expression

Relative gene expression was analyzed by RT-qPCR as previously described (Svingen et al. 2015). In short, ovaries were pooled together in groups of three, total RNA isolated using a RNeasy Micro Kit (Qiagen, Hilden, Germany) and quantified on a NanoDrop-1000 Spectrophotometer, then cDNA was synthesized from 500 ng RNA in the presence of Random Primer mix (New England Biolabs, Ipswich, MA, USA) using an Omniscript kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. RT-qPCR reactions were performed in duplicates on a 7900 HT qPCR system (Applied Biosystems, Foster City, CA, USA) in 20 µl reactions containing TaqMan Fast Universal Master mix (Life Technologies, Carlsbad, CA, USA), 3 µl diluted (1:20) cDNA and gene-specific TaqMan assays (Life Technologies Europe BV, Naerum, Denmark): *Ar* (Rn00560747), *Trpm2* (Rn00562081), *Ddx4* (Rn01489814), , *Fst* (Rn00561225), *Inhba* (Rn01538592), *Inhbb* (Rn01753772), *Inha* (Rn00561423), and *Pcna* (Rn01514538), *Sycp3* (RN 01648688). *Foxl2* was designed in house (forward primer: ACG AGT GCT TCA TCA AGG TG, reverse primer: GGT AGT TGC CCT TCT CGA AC, probe: TAG TTG CCC TTG CGC TCG CC) and amplification efficiency of the *FoxL2* assay was tested to be 98% using a 6 serial 10-fold dilution in triplicates. Data were analyzed by the comparative Ct-method normalized with the geometric mean of verified reference genes *Rps18* (Rn01428913) and *Sdha* (Rn00590475). Intra-assay variability of technical replicates was < 0.5 cycles.

2.5 Histological evaluation of germ cell and follicle numbers

Ovaries cultured for 72h were fixed in 10% formalin for 1h and processed for paraffin embedding. The ovaries were sectioned at 5 µm and stained with hematoxylin and eosin (H&E) following standard procedures. The slides were evaluated for the presence or not (yes = 1 / no = 0) of germ cell cysts, single germ cells, and primordial follicles on every third section by a person blinded to exposure group. For each

germ cell category, the total numbers of sections denoted with a 'yes' were added and the number divided with the total number of sections, giving the percentage of sections with the specified germ cell developmental stage. After this the blinding key was revealed.

2.6 Statistical analysis

Unpaired, two-tailed Student's t-test was used to compare control group with exposure group in the statistical software GraphPad Prism 5 (GraphPad Software, San Diego California, USA). As this was a pilot study, a less conservative statistical approach with no correction for multiple comparisons was applied.

3. RESULTS

In this study, neonatal ovaries were investigated for effects after exposure to EDCs with anti-androgenic potentials for 72 h in culture. Due to logistical restraints concerning experimental setup, conduction of two separate experiments was required to get a sample size fit for this pilot study. Unfortunately the age of the rat pups therefore differed with approximately 24 h between the two experiments; in Ex1 pups were killed a few hours after birth, and in Ex2 they were extracted with cesarean section the day before birth.

3.1 Effects of exposure

The pooled data from Ex1 and Ex2 did not show any effects on gene expression (Figure 1). To investigate if there were differences between the two groups, data from control and AAmix groups in the two experiments (n = 2 per group) was compared. A significant effect was seen on expression of *Pcna* when comparing the AAmix groups of Ex1 (AAmix 1) and Ex2 (AAmix 2) ($p = 0.02$), and a tendency was seen for *Ar* ($p = 0.06$) and *Sycp3* ($p = 0.06$) for the same groups (Figure 2). No significant effects were seen in the controls or on the expression of the reference genes *Rps18* and *Sdha*.

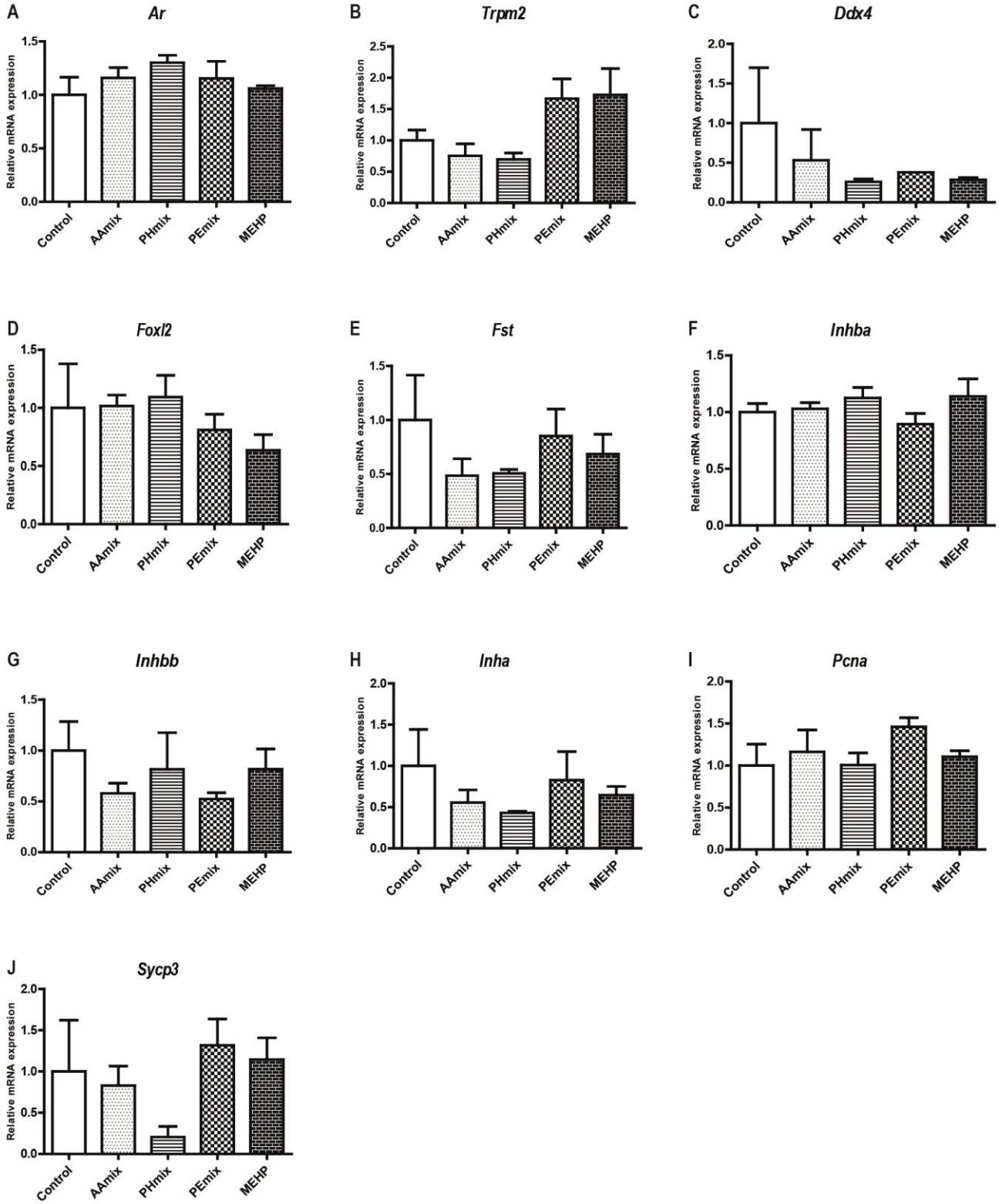

Figure 1 (A-J) Gene expression results from the pooled analysis of Ex1 and Ex2. No statistically significant effects were seen on gene expression after exposure to AAmix, PHmix, PEmix and MEHP. Mean ±SEM.

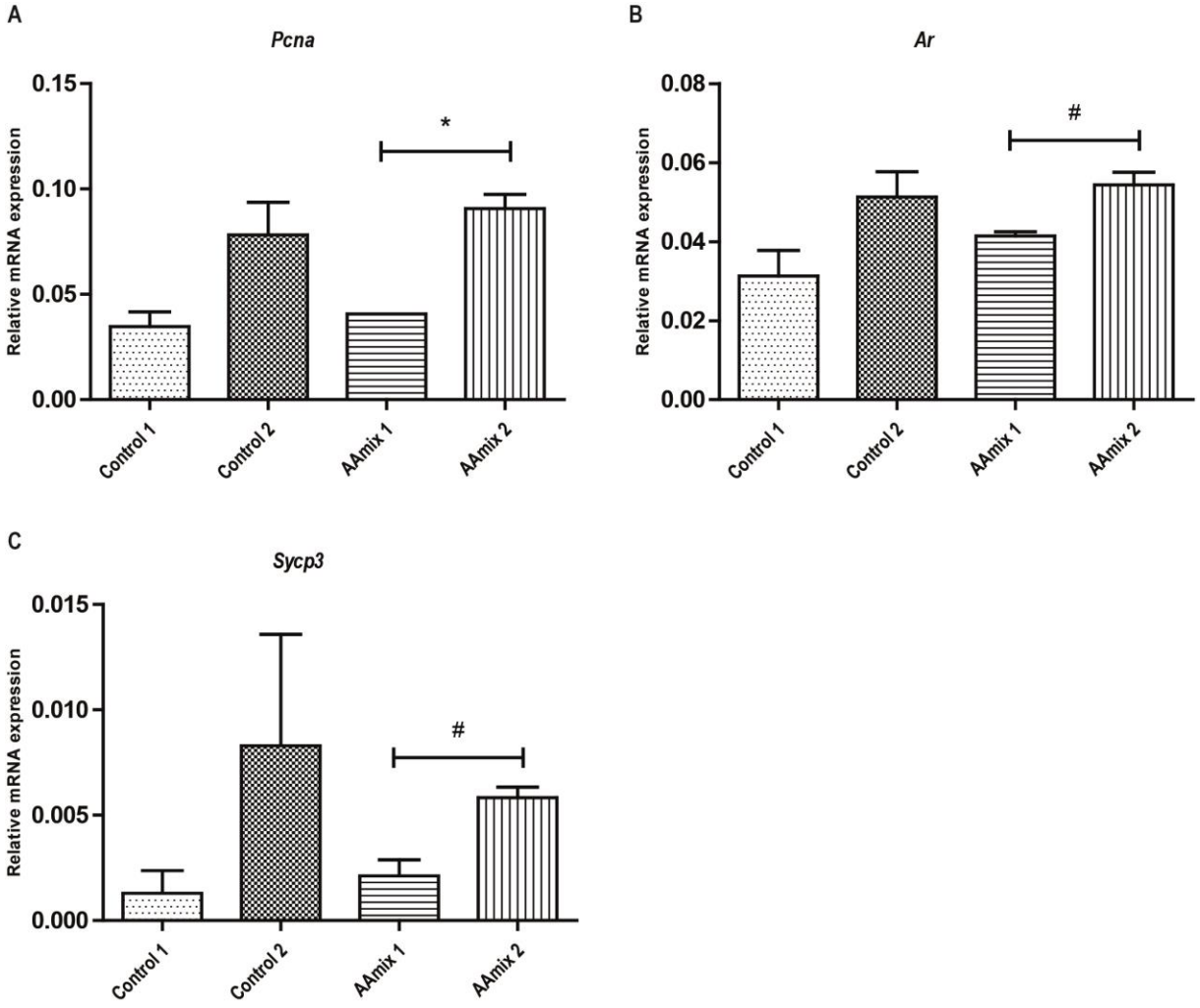

Figure 2 Comparison of gene expression results between Ex1 and Ex2. A) A significant effect was seen on expression of *Pcna* when comparing the AAmix groups ($p = 0.02$). B-C) A tendency towards effect on expression of *Ar* and *Sycp3* was seen between the AAmix groups ($p = 0.06$), ($n = 2$ per experiment, $^{*}p < 0.05$, $^{\#}p < 0.1$). Mean ±SEM.

Histological evaluation of ovary sections showed a significant reduction of primordial follicles after exposure to PEmix ($p = 0.01$) and a slight, but not statistically significant, reduction after MEHP exposure ($p = 0.07$). No effects were seen after exposure to AAmix or PHmix (Figure 3A). No effect was seen on single germ cells or germ cell cysts (Table 2). To ensure that the effect seen on primordial follicles after PEmix exposure was not due to difference in age of the ovaries, data from Ex2 only was evaluated. This showed that a reduction in primordial follicles in PEmix compared to controls was still present ($p = 0.008$) (Figure 3B). Comparison of controls and PEmix exposed ovaries in Ex1 was not possible since group size was too small when Ex2 animals were removed. Example of a germ cell cyst, single germ cell and primordial follicle can be seen in Figure 4A-B.

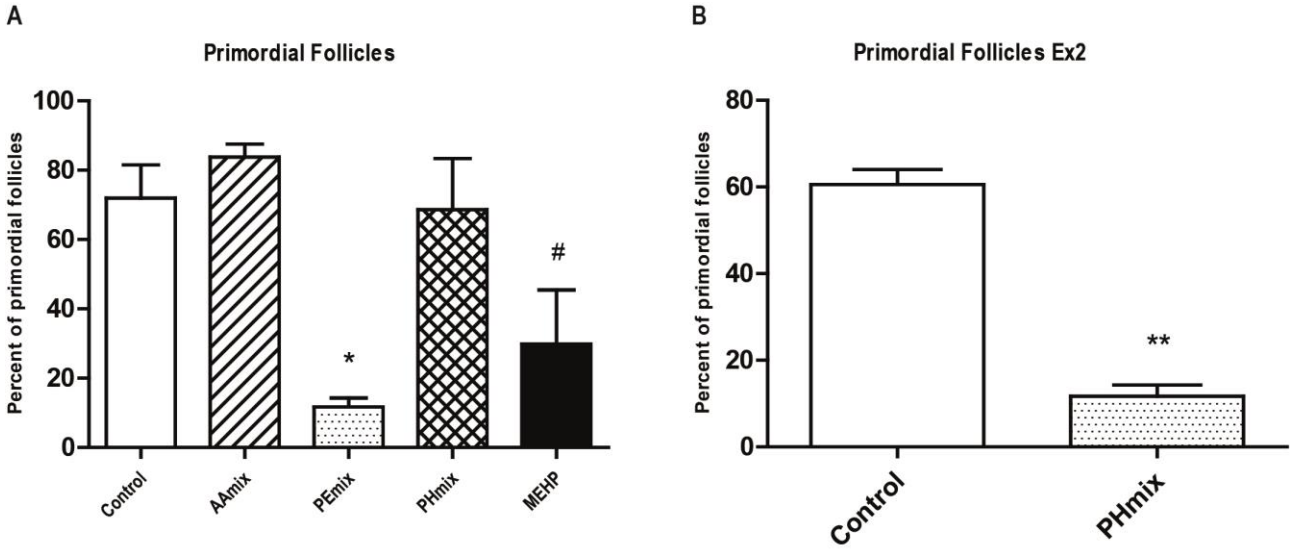

Figure 3 Sections/total number of sections per animal (%) is shown. Mean ±SEM. A) Presence of primordial follicles was significantly reduced after PEmix exposure ($p = 0.01$) and there was a tendency towards reduction after MEHP exposure ($p = 0.07$) ($n = 4$ (control), $n = 2$ (exposed)). B) Data on primordial follicles from control and PEmix from Ex2 only were also tested separately to ensure that the effect seen was not due to age differences. The results showed that the effect was still present ($p = 0.008$) ($n = 2$). Control was compared to exposed groups by use of students t-test, ($^*p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.1$).

	N	Naked germ cells	Germ cell cysts	Primordial Follicles
Control	4	99	67	72
Aamix	2	100	51	84
PEmix	2	100	81	12*
PHmix	2	100	58	69
MEHP	2	100	86	30

Table 2: Histological analysis of ovaries after EDC exposure**.** The percentage of sections per animal with presence of the three different oocyte classes was evaluated for each animal. A significant reduction in percentage of section with primordial follicles was seen in PEmix (t-test, ($^* = p < 0.01$)

During the scoring process, recruited follicles (primary and secondary) were found in some samples. These ovaries also had few germ cell cysts compared to other ovaries. When the blinding key was removed, all the ovaries with presence of recruited follicles were from Ex1. In Figure 4C a section from an Ex1 ovary with presence of recruited follicles is shown.

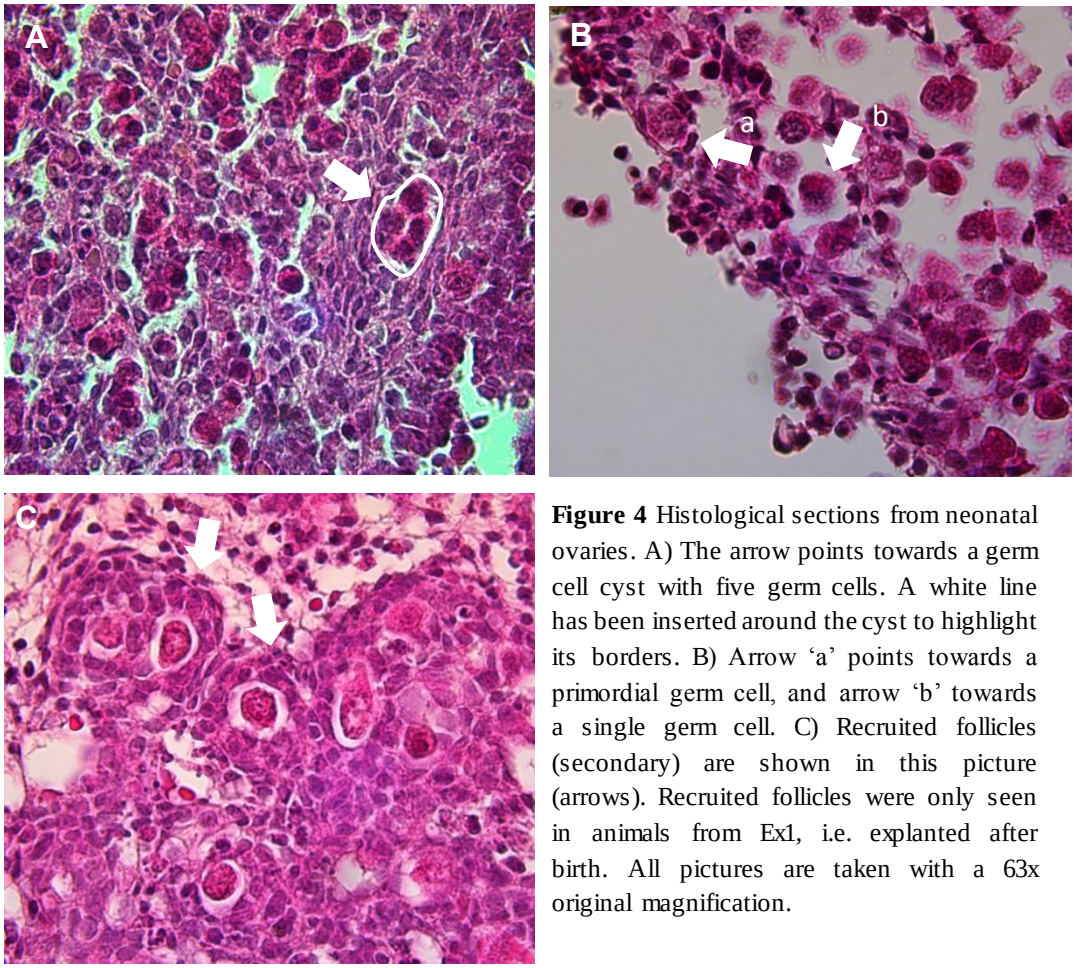

Figure 4 Histological sections from neonatal ovaries. A) The arrow points towards a germ cell cyst with five germ cells. A white line has been inserted around the cyst to highlight its borders. B) Arrow 'a' points towards a primordial germ cell, and arrow 'b' towards a single germ cell. C) Recruited follicles (secondary) are shown in this picture (arrows). Recruited follicles were only seen in animals from Ex1, i.e. explanted after birth. All pictures are taken with a 63x original magnification.

4. DISCUSSION

4.1 AR antagonism and effects on follicle assembly

In utero exposure to the AR antagonist flutamide has been shown to increase the number of germ cell cysts and reduce the number of follicles in fetal pig ovaries (Knapczyk-Stwora et al. 2013). Since the majority of pesticides in the PEmix are thought to act mainly as AR antagonists (Orton et al. 2011; Vinggaard et al. 1999), except prochloraz and epoxiconazole acting primarily via testosterone inhibition (Kjærstad et al. 2010), it can be speculated that AR antagonism underpins the reduction in primordial follicles that we observed in the cultured rat ovaries. Interestingly, vinclozolin has been shown to reduce oocyte numbers in rat ovaries cultured from 4-14 days *post partum* (dpp), albeit only at a high concentration of 500 µM; at lower concentrations (50-200 µM) no effects were seen (Nilsson et al. 2012). Here we used 12.9 µM vinclozolin which is almost four times lower than the lowest no effect dose of 50 µM used by Nilsson et al. (2012). It is therefore unlikely that vinclozolin alone drives the reduction in primordial follicle numbers.

Only a few studies have investigated the effect of prochloraz on females, but in fish it has been shown to cause several adverse effects. For instance, prochloraz exposure can reduce the number of eggs laid by the fathead minnow (Ankley et al. 2005), it triggers oocyte maturation in the rainbow trout (Monod et al. 2004; Rime et al. 2010), and can reduce estradiol levels without affecting testosterone levels in cultured brown trout ovaries (a Marca Pereira et al. 2011). Although making direct comparisons between the reproductive systems of fish and mammals can be problematic, these results nevertheless indicate that prochloraz can adversely affect oocytes across animal phyla.

Although there are no studies showing that procymidone, epoxiconazole, nor p,p'-DDE can adversely affect oocyte development in mammals, procymidone has been shown to have estrogenic effects by activating mitogen-activated protein kinase (MAPK) in both MCF-7 breast carcinoma cells (Radice et al. 2006) and in rainbow trout hepatocytes (Radice et al. 2004). MAPK has important oocyte-specific functions, such as resumption of meiosis and meiotic spindle formation (Pomerantz and Dekel 2013). Epoxiconazole also seem able to interfere with female development causing masculinization of female rats after fetal exposure (Taxvig et al. 2007), as well as triggering oocyte maturation in rainbow trout (Monod et al. 2004).

Herein, the concentrations of the individual compounds were relatively low and the effect seen in PEmix-exposed ovaries may likely have been caused by the combined effect of two or more pesticides with similar effects. Mixture effects showing dose addition have previously been shown *in vivo* in male rats for several of the compounds present in the PEmix (Hass et al. 2007; Hotchkiss et al. 2010; Jacobsen et al. 2010). However, to determine if mixture effects - possibly by way of dose addition - did occur in the explanted ovaries, individual testing of the compounds would be necessary.

It is surprising that no significant effects in AAmix-exposed ovaries were observed, which comprised both PEmix and PHmix. It is possible that there were interactions between the pesticides and phthalates in the AAmix, but without further experimentation, this remains an unsupported conjecture. Nevertheless, there are examples where similar phenomena have been seen in *in vivo* mixture studies, where one mixture on its own causes effect, but when combined with a second mixture, the effect disappears (Johansson et al. 2016).

4.2 The PHmix and lack of effect

No significant effects were seen after exposure to the PHmix containing DBP and DEHP. Previous studies using mouse *in vitro* ovary cultures have shown that DEHP can affect follicle assembly (Mu et al. 2015; Zhang et al. 2014). The concentrations used in the different studies are in the same dose-range as used herein, and both Zhang et al. (2014) and Mu et al. (2015) observed effects of DEHP at concentrations lower than the PHmix concentration (35.6 µM). Thus, differences in concentrations cannot explain the lack of effect in our *ex vivo* culture.

In intact animals, effects caused by DEHP exposure are thought to be due to the activity of its metabolite MEHP. Biotransformation from DEHP to MEHP is likely to occur at a very low rate, or not at all, in transplanted ovaries, but this remains uncertain until more knowledge is obtained about ovarian metabolic capacity (Bhattacharya and Keating 2012). Nevertheless, we exposed ovaries to MEHP alone and found a tendency towards reduction in primordial follicles ($p = 0.07$). The lack of statistical significance may be due to the small group size (control $n = 4$, exposed $n = 2$) and a relatively crude method for counting oocytes and will be repeated in follow-up studies. These preliminary results are in agreement with Hannon et al. (2015), who also saw no effects on early folliculogenesis following DEHP exposure, but significant effects following MEHP exposure.

4.3 The importance of developmental age of the ovary

During development, transcriptional regulation is very dynamic and can change significantly during short windows of time, including in the developing ovary (Lawson et al. 2011; Lea et al. 2016). This fact must be considered when comparing data from differently staged tissues. In this study two separate experiments were conducted using rat ovaries collected immediately before and after birth. Although only representing one day difference in developmental age, it is possible that parturition itself can affect the ovarian transcriptome. When comparing the transcript levels of selected genes between control samples from the two groups, no changes to gene expression levels were evident. At the histological level, however, the postpartum ovaries had recruited follicles present and very few germ cell cysts. This was not observed in the ovaries collected from the 21 dpc fetuses. These observations are in line with the fact that ovaries from newborn mice contain almost zero single oocytes/primordial follicles; a number that changes to almost 100% of the oocytes being single/primordial five to six days after birth (Pepling and Spradling 2001).

Minor differences to ovarian gene expression levels were seen between the differently staged ovaries that had been exposed to AAmix for 72 h. There were no differences in the control ovaries, but further studies are needed to investigate any potential effects.

4.4 Histological evaluation – a crude approach

For this study we chose not to count oocytes and follicles, but rather evaluate presence or absence of three different stages. This was due to the time limitations and difficulties in identification of the oocytes in H&E sections. However, both H&E staining (Hannon et al. 2015; Kezele and Skinner 2003; Zhou et al. 2015) and other staining methods such as immunohistochemical staining for oocyte markers (Pepling and Spradling 2001) have previously been used successfully for oocyte and follicle counting at this age.

5. CONCLUDING REMARKS

The aim of this preliminary study was to investigate if AAmix could affect follicle assembly in neonatal rat ovaries and if a potential effect was caused by phthalates or pesticides, or both. Surprisingly, AAmix did not affect follicle assembly, whereas the PEmix, a submix of AAmix, reduced the number of primordial follicles. Also, no effects were seen after PHmix exposure, but exposure to MEHP showed a tendency towards reduction in primordial follicles. This indicates that AAmix may not interfere with primordial follicle assembly, but should be interpreted with care as the study involved few biological replicates. It would be most valuable to perform a larger study on MEHP to better understand the molecular mechanisms driving potential effects on the germ cell population.

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Chapter 6: In Closing

6.1 Overview

A bullet-point summary of the main findings from chapter 2-5 is given below.

Chapter 2:
Chemical exposure and ovarian dysgenesis: Sensitive developmental windows

- The aim was to synthesize current knowledge and to define knowledge gaps within the defined research area.
- Four potentially EDC-sensitive female developmental stages and effects were identified:
  1) Primordial germ cell proliferation and gonadal sex determination: PM can affect primordial germ cell proliferation, and tamoxifen and BPA seem able to disturb the pathways governing the balance between male and female cell fates.
  2) Meiosis: BPA, DEHP, PM and indomethacin all seem able to disturb meiosis. BPA probably exerted its effect via ERβ antagonism and the other chemicals via not yet identified mechanisms.
  3) Follicle assembly: BPA and DEHP (or its metabolite MEHP), genistein and DES are all able to affect follicle assembly, but the precise mechanism of action is not known.
  4) Early folliculogenesis: BPA and DEHP seem able to increase recruitment of first wave follicles, potentially causing earlier reproductive senescence.

Chapter 3:
Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging

- The aim was to investigate how perinatal exposure to mixtures of EDCs affects the ovary in young and senescent rats and apply a targeted approach to identify potential biomarkers.
- In the pre-pubertal rats, reduction in primordial follicles was found after AAmix and PM exposure.
- In the 1 year old rats, Totalmix caused a higher incidence of irregular estrous cycles. Reduced ovary weights were seen in Totalmix, AAmix, and PM exposed groups. Also, the incidence of rats with complete absence of CL was increased after AAmix and PM exposure. The mean number of CL, which was positively correlated to ovary weight, was reduced in Totalmix200 and PM groups.

- Together the results resemble premature ovarian insufficiency in humans and point to a possible role of EDCs in the pathogenesis of this disorder.

Chapter 4:

Mixtures of endocrine disrupting chemicals alter the rat ovary proteome: a search for early biomarkers of late life adverse effects

- The aim was to apply a non-targeted approach to investigate how the rat ovary proteome was affected by exposure to mixtures of EDCs and if one or more differentially regulated proteins could be potential biomarkers of effect.
- Regardless of what FDR cut off level was used, Emix showed the largest number of dysregulated proteins, whereas proteins in AAmix ovaries were least affected.
- Among proteins chosen as potential biomarkers of effect, three were in common for all exposure groups: Trimethyllysine dioxygenase (TMLH), Keratin, type II cytoskeletal 8 (KRT8), and anti müllerian hormone (AMH). Also, proteins involved in androgen signaling were affected.
- The three proteins common for all groups, and proteins differentially regulated after exposure to the AAmix or Emix should be further studied to elucidate if they are potential biomarkers of adverse effects on the developing ovary causing late life effects.

Chapter 5:

Endocrine disrupting chemicals with anti-androgenic potential reduce the number of primordial follicles and disturb follicle assembly in neonatal rat ovary explants: A pilot study

- The aim was to follow up on the results found in Chapter 3 by investigating, by a targeted approach, if the AAmix perturbed follicle assembly in the neonatal rat ovary in explanted ovaries, and if it was possible to deduce if phthalates or pesticides were driving a potential effect.
- Histological evaluation showed significant reduction of primordial follicles after PEmix exposure and a reduction, however not significant, after MEHP exposure.
- This indicates that the pesticides in AAmix, and likely also MEHP, affects follicle assembly and further studies are needed to investigate the mechanisms driving the effects.

6.2 Discussion

6.2.1 Morphological biomarkers of effect

In Chapter 3 follicular counts showed a reduced ovary reserve in histological sections from ovaries of pre-pubertal (PD22) rats after PM and AAmix exposure. PM has been shown to affect the ovary reserve in two other rodent *in utero* exposure studies. Dean et al. (2016) found a reduced number of primordial follicles in rats after PM exposure from 13.5-21.5 dpc, whereas Holm et al. (2016) found a reduced number in mice after exposure from 7 dpc to birth. Based on these recent studies, the effects seen in Chapter 3 may be caused by delayed meiosis (Dean et al. 2016), or inhibited proliferation of primordial germ cells (Holm et al. 2016), or both. However, since PM exposure was initiated at 7 dpc and continued until weaning, several sensitive developmental windows were included (Lea et al. 2016; (Chapter 2)), such that disruption to postnatal processes such as follicle assembly and early folliculogenesis cannot be excluded.

The effect seen on the ovary reserve was most pronounced in animals exposed to AAmix. Two of the compounds in the AAmix have previously been shown to affect the follicle pool. Vinclozolin reduced primordial follicle numbers in adult mice after prenatal exposure (Nilsson et al. 2012), and DEHP decreased the incidence of primordial follicles in ovaries of pre-pubertal mice exposed during fetal life (Zhang et al. 2015) or postnatally (Zhang et al. 2013). Both vinclozolin and DEHP, as well as its metabolite MEHP, have been shown to interfere with germ cell numbers in explanted ovaries from newborn rodents (Hannon et al. 2015; Mu et al. 2015; Nilsson et al. 2012; Zhang et al. 2014; Zhou et al. 2015). We therefore wanted to investigate if the reduced number of primordial follicles after AAmix exposure could be explained by disruption of follicle assembly. This was addressed in Chapter 5 where neonatal ovaries were cultured *in vitro* and exposed to AAmix or subgroups of pesticides (PEmix), phthalates (PHmix), or MEHP alone.

In Chapter 5 we found a significant reduction in primordial follicles in the PEmix group and a tendency towards effect in the MEHP group. Unexpectedly, no effects were seen in the AAmix group, which could be due to the effect on ovary reserve seen in Chapter 3 occurring earlier, or due to the phthalates and pesticides affecting each other and thereby the response (see 6.2.5 Mixture exposure). It should be carried in mind that this was a preliminary study with low power, having a high risk of false negatives.

In humans, reduced ovary reserves can cause premature ovarian insufficiency, where reproductive function is lost before the age of 40 (Cox and Liu 2014). Based on the findings in Chapter 3, a reduced follicle pool in pre-pubertal animals may be a potential marker for shortened reproductive lifespan after EDC exposure. However, the link between ovary reserves and late life effects is not clear, as no effects were seen on the follicle reserve after Totalmix exposure, despite showing signs of earlier reproductive senescence at 1 year of age. Furthermore, using follicle counts as a biomarker is not optimal, with the method having some

drawbacks, the most important being that follicle count is time consuming and labor intensive using the currently available methods. Also comparisons of values between studies are not recommended (Tilly 2003). A molecular marker would therefore be of great value.

6.2.2 Molecular biomarkers of effects

In Chapter 3, 1 year old animals showed signs of early reproductive senescence, corresponding to early menopause in women. There were no signs of pituitary dysregulation, and plasma levels of the pituitary hormones FSH and LH were unaffected, which led us to hypothesize that the ovaries were directly affected and additional studies focused on the ovary were conducted.

Plasma levels of the ovarian hormones AMH and inhibin A were measured. The concentration of AMH was below the limit of detection, and could therefore not be assessed (results not shown), and no effects were seen on inhibin A levels. The expression level of *Amh, Bmp15, Ddx4, Fshr, Fst,* and *Lhr* were also investigated. No effects were seen on *Amh, Fshr, Fst or Lhr*, indicating that at least the ovarian cellularity was relatively unaffected by exposure. The germ cell-specific gene *Ddx4* was significantly reduced in the PM group though, and there was a tendency towards downregulation of *Bmp15*, indicating that the overall number of germ cells was reduced. PM has previously been found to reduce *Ddx4* expression in female ovaries after early fetal exposure, and in the same study the number of primordial follicles was reduced at 7 weeks of age (Holm et al. 2016) which supports the findings in Chapter 3. As no effect was seen on expression of *Ddx4* or *Bmp15* in the AAmix exposed group, where the most significant effect on follicle reserve was seen, it is likely that the effect on *Ddx4* was specifically due to PM, as also seen by Holm et al. (2016). Interestingly, sheep exposed to androgen (testosterone propionate) during fetal life show increase in DDX4 protein and increased numbers of germ cells enclosed in follicles (Comim et al. 2015), and in human fetal ovaries exposed to smoking, increase in DDX4 positive oocytes and increase in primordial follicle numbers are seen in the second trimester (Fowler et al. 2014). Based on this, and on the fact that the effect seen after PM exposure was relatively weak, *Ddx4* and *Bmp15* would not be considered sensitive biomarkers of effect on the follicular reserve after a general EDC exposure.

In Chapter 4, investigation of protein expression was conducted by proteomics and three proteins, TMLH, KRT8, and AMH were found to be significantly dysregulated in all exposure groups. It would be interesting and relevant to further investigate the effects on these proteins and the pathways they are part of, to see if they are indeed affected after EDC exposure and if there is a direct relationship with effects on reproductive senescence. If so, one or several proteins could prove to be good effect biomarkers for early disruption of ovary development causing later life adverse effects. CALB2 could also be worth studying further, since it is used as a tumor marker in female reproductive organs (Portugal and Oliva 2009) and could be a potential

biomarker of adverse effects after exposure to EDCs with primarily anti-androgenic potential, as it was down-regulated in both Totalmix and AAmix.

In Chapter 5, neonatal ovaries were exposed to mixtures of EDCs with anti-androgenic potentials for 72 h in culture. Due to experimental constraints, two separate experiments (Ex1 and Ex2) were conducted and pooled. For the second experiment the rats proved to be mislabeled so that we were forced to conduct caesarian sections rather than collect ovaries from partitioned animals. Hence, the ovaries from these pups were one day younger than Ex1. At the gene expression level, a significant difference between Ex1 and Ex2 was only seen for *Pcna* expression when comparing the AAmix groups. As statistically significant effects were not seen on other genes, we pooled the data and ran analyses, but again, no effects were observed. For many of the genes tested, the variability in the control group was high, which makes comparison between groups difficult and increases the uncertainty of the results (Crawley 2005). The high variability could be due to various factors, not least the low transcript abundance of some of the target genes. But also, when grown in culture there is expected to be a more significant variability in tissue cellularity between biological replicates, with certain cell types being more susceptible to insult, such as germ cells, as well as the mesenchyme expanding more than during *in vivo* conditions. Finally, the differences in age likely contributed some to the variability even though no statistically significant differences were seen between the two experiments.

The two approaches used have both advantages and shortcomings. An advantage of the targeted approach is that the role of the chosen endpoints in ovary function is largely known, and there are established methods for identification. A shortcoming is that many potentially important molecular components can be missed by the simple fact that they remain to be characterized. This problem could be solved by use of methods such as shotgun proteomics where thousands of proteins are identified. However, a limitation to this approach is that only the most abundant proteins are identified, which means that less abundant proteins can go undetected even if they are significantly affected by chemical exposure (Cayer et al. 2016).

6.2.3 Females and EDCs

Within the area of endocrine disruption, male reproduction has been the main focus for many years, centralized around the TDS hypothesis (Skakkebæk et al. 2001). Female reproductive health has gained much less attention, one of the reasons likely being that female fetal development is considered to occur largely independent of sex hormones, and hence not vulnerable to EDC exposure. However, disturbance of endocrine functions or tissues are seen in female test animals exposed to EDCs during fetal life and early development (Chao et al. 2012; Gámez et al. 2015; Mandrup et al. 2015; Rodríguez et al. 2010; Santamaría et al. 2016; Zhang et al. 2013). This indicates that hormones might be more involved in female fetal

development than previously anticipated or that functions programming later life endocrine functions are affected by exposure.

In medical research there are examples of illnesses that were viewed as typically male diseases, for example cardiovascular disease, which later proved to affect women as well, but with different symptoms (Wenger 2012). In relation to EDCs, a possible relationship between reproductive effects and exposure appears to be more difficult to identify in females than in males due to difficulties in examination and choice of appropriate endpoints. However, the consequences of exposure may be harsh in females, such as effects on reproductive capacity (Holm et al. 2016; Zhang et al. 2015, Fernández et al. 2010).

A great challenge working with female reproductive tissues is the constant hormonal and physiological changes during the menstrual or estrous cycle (Farage et al. 2009; Goldman et al. 2007). When conducting experiments, it is therefore important that endpoints are evaluated at the same stage of menstrual or estrous cycling. Changes occurring due to exposure can be similar to one of the natural phases in the cycle and be missed if thought of as a normal (Gopinath 2013). Furthermore, evaluation of endpoints during different stages of cycling can cause great intra-group variability, which can lead to failure of establishing an effect of exposure when it actually exists (Goldman et al. 2007). However, evaluation of endpoints at the same estrous stage can be a great logistical challenge as it requires time and flexibility. Females are rarely completely synchronized and must therefore be examined on several different days.

Characterization of estrous cycling itself after chemical exposure can also give important information, but can be challenging in relation to determination of cycling stage, as a transition between stages is often present, and statistical analysis of data needs to be carefully conducted not to mask effects (Goldman et al. 2007).

6.2.4 Mixture exposure

Mixtures of chemicals can cause a greater effect than each chemical on its own, and humans are exposed to a mixture of different man-made chemicals with EDC properties on a daily basis (Svingen and Vinggaard 2016). In this thesis a top-down approach was implemented to have the exposure anchored in a real-life situation using a human relevant mixture pattern. Top-down approaches have been used in several other studies using different exposure periods and endpoints, and in common for these experiments is the use of a human or environmentally relevant mixture of chemicals (Berger et al. 2014; Hadrup et al. 2016; Lefevre et al. 2016).

A problem with exposing to mixtures of chemicals is that it is not possible to deduce if the increased effects seen are due to synergism or addition of the effect of each compound, or if some chemicals have opposing

effects. For this, testing the chemicals individually and in smaller mixtures is needed, as done by Hass et al. (2007).The subgrouping used in Chapter 3 was an attempt to look at groups of compounds having similar main modes of action instead of looking at single compounds. This was to see which ones contributed to potential effects in the mixture comprised of all compounds. Doing this, an interesting phenomenon was seen in Chapter 3, and also in the explanted ovaries in Chapter 5, which might be due to opposing modes of action. It appeared that effects seen for AAmix and PM on the follicular reserve in Chapter 3 was opposed by the presence of Emix in the Totalmix, as no effects were seen after Totalmix exposure. We proposed that endocrine effects of Emix exposure can modulate the effects of AAmix and PM on the developing ovary. Similarly, in Chapter 5 the PEmix affected primordial follicle numbers, whereas the AAmix, which contained both PEmix and PHmix, did not. Pesticides belonging to both different and the same chemical classes have previously been reported to sometimes reduce each other's potency when mixed (Rizzati et al. 2016). Opposing actions of different chemicals or chemical mixtures may not only be due to antagonistic actions on a specific receptor, but may very well reflect several endocrine modes of action appearing at the same time. One compound, or group of compounds, could increase the metabolism or excretion of another or the compounds could interact directly with each other when mixed. It is possible that combining the different compounds in Totalmix, as in Chapter 3, and AAmix, as in Chapter 5, caused antagonistic interactions leading to a no-effect result. However, given the limited power of these studies, further investigations are necessary to determine whether the findings reflect actual differences between the mixture groups.

6.2.5 Extrapolation between species

In the work conducted for this PhD thesis, the rat was used as a model for potential effects in the human after EDC exposure. To enable comparison between species such as rat and human, it is important to choose similar stages of development (Habert et al. 2014). This is especially critical to keep in mind when comparing processes occurring at different ages in the different species such as follicle assembly, which occurs during fetal life in humans and neonatally in the rat (Grive and Freiman 2015). However, this still does not ensure a total comparability as there can be great differences in molecular signaling pathways, gene expression, protein folding, endocrine processes and so forth, between species. One example of this is estrogen signaling in the testis. The estrogen hypothesis was presented by Sharpe and Skakkebaek (1993) stating that estrogen exposure from various sources may underlie male reproductive disorders. However, in 2013, Mitchell et al. published a paper showing that the estrogen hypothesis was not valid because ESR1, which mediates the effects of estrogens on the fetal rat testis, was not expressed in human fetal testis (Mitchell et al. 2013). Another example is the difference in susceptibility to phthalate exposure between the rat and human fetal testis. Phthalates affect expression of steroidogenic genes and testosterone levels in the

rat, but not in the human fetal testis (Heger et al. 2012; Mitchell et al. 2012). This illustrates the importance of mechanistic knowledge when extrapolating data from one species to another.

6.3 Perspectives

The proteomics study (Chapter 4) revealed that several proteins were dysregulated following exposure to EDCs. The factors that were dysregulated typically varied between the different exposure groups, but among the chosen potential biomarkers three proteins were affected in all groups: TMLH, KRT8 and AMH. It would be of great interest to confirm the differential regulation of these proteins by other methods. Also in relation to TMLH to investigate if other proteins involved in L-carnitine biosynthesis or β-oxidation of long-chain fatty acids are dysregulated in the exposed ovaries. It could be tempting to speculate that TMLH or a related factor, could be a general biomarker for ODS resulting from EDC exposure. On the other hand, the idea that a single biomarker should function as a marker for all potential ovarian pathologies caused by EDC exposure is perhaps unrealistic. Different etiologies can vary greatly both with respect to mechanisms of action and resulting pathology. Therefore, one would more likely expect specific biomarkers for specific effect-endpoints, such as AMH for reduced ovarian reserve, and perhaps a panel of biomarkers, a fingerprint of sorts, functioning as a general 'biomarker' for ODS more broadly. The continued search for effect biomarkers could involve experiments using chemicals with known mechanisms/modes of action to pinpoint common denominators that are affected at either the histological or molecular levels.

The concept of mixture effects is an issue that is attracting increasing attention. Today, the general requirement when conducting risk assessment is to evaluate each chemical on its own, which could underestimate the effects as they may add up. However, as shown in Chapter 3 and Chapter 5, the chemical makeup of mixtures can greatly affect the effect outcome; in this case a sub-mixture gave greater effects than a total mixture containing a larger number of chemicals. Further studies on why this phenomenon appears would be of great value for understanding mixture interactions.

The work performed during this PhD study not only revealed novel effects of EDCs on the ovary, but also highlighted knowledge gaps with regard to basic female reproductive biology. In females, the potential role of hormones during fetal and neonatal development is not well established, and the consequences of antagonism or activation of receptors by EDCs during early development remains to be clarified with respect to adverse health outcomes later in life. There may be a 'feminization window', corresponding to the 'masculinization window' (Welsh et al. 2008), even though female development is considered to be largely independent of sex hormones. However, this remains to be empirically verified, albeit some time periods and processes during development seem more sensitive to insult than others (Lea et al. 2016; (Chapter 2)). This raises several questions concerning the mechanisms behind the effects exerted by EDCs on the developing

female. Are the observed effects due to chemicals mimicking endogenous hormones and their activity? Or are they due to the chemicals affecting pathways that modulates or participate in programming of reproductive tissues and the endocrine system later in life, or both? To answer these questions, further targeted approaches for the purpose of specifically looking into effects in females are needed.

6.4 Conclusion

An early life effect biomarker for late life disease would be of great value when evaluating effect of EDCs on female reproductive health. Several periods during female development were identified as sensitive to chemical insult, and experimental data showed that exposure to mixtures of EDCs during the entire perinatal period cause reduction in follicle count in pre-pubertal rats and earlier reproductive senescence at 1 year of age. In addition, using a proteomics approach showed that exposure to mixtures of EDCs caused reduced levels of the proteins TMLH, KRT8 and AMH. These proteins could function as biomarkers of effects on the developing ovary and holds potential as early biomarkers of late life effects.

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Appendices

Appendix I:

Johansson HK, Jacobsen PR, Hass U, Svingen T, Vinggaard AM, Isling LK, Axelstad M, Christiansen S, Boberg J. *Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging*. Reprod Toxicol 2016, Jun;61: 186-94

Appendix II:

Supplementary material to:

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Appendix I

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Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging

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ABSTRACT

Exposure to endocrine disrupting chemicals (EDCs) during development can have negative consequences later in life. In this study we investigated the effect of perinatal exposure to mixtures of human relevant EDCs on the female reproductive system. Rat dams were exposed to a mixture of phthalates, pesticides, UV-filters, bisphenol A, butylparaben, as well as paracetamol. The compounds were tested together (Totalmix) or in subgroups with anti-androgenic (AAmix) or estrogenic (Emix) potentials. Paracetamol was tested separately. In pre-pubertal rats, a significant reduction in primordial follicle numbers was seen in AAmix and PM groups, and reduced plasma levels of prolactin was seen in AAmix. In one-year-old animals, the incidence of irregular estrous cycles was higher after Totalmix-exposure and reduced ovary weights were seen in Totalmix, AAmix, and PM groups. These findings resemble premature ovarian insufficiency in humans, and raises concern regarding potential effects of mixtures of EDCs on female reproductive function.

1. Introduction

Exposure to endocrine disrupting chemicals (EDCs) during development can have long-lasting consequences extending into adulthood, for instance compromised reproductive health [1,2]. Numerous studies have investigated the link between early exposure to EDCs and late-life effects on the male reproductive system, often referred to as the testicular dysgenesis syndrome (TDS) hypothesis, comprising a range of male disorders presumed to have a common developmental origin [3,4]. Studies addressing comparative questions in females, however, are far fewer. An ovarian dysgenesis syndrome (ODS) hypothesis has been proposed though. And as with males, pathologies presenting in adulthood; impaired placental function, early pregnancy loss, breast cancer, pubertal timing, and polycystic ovarian syndrome (PCOS), are suspected to share a common developmental origin [5,6].

The generally accepted view, although challenged by a few studies (reviewed in Ref. [7]), is that females are born with a set number of follicles that depletes throughout their reproductive lifespan, inevitably leading to infertility. Disturbed establishment of the follicle pool during development may therefore be detrimental to fertility in the adult female [8]. Rodent studies have indicated that oocyte meiosis, ovarian folliculogenesis, fertility, and the onset of reproductive senescence can be altered by environmental contaminants, including EDCs [9–15]. This has led to an increased level of concern regarding female reproductive health, particularly since reduced fecundity has been associated with EDCs in humans [16].

When evaluating toxicity of a chemical, the state-of-the-art has been to assess one chemical at a time. A more realistic scenario, however, is that humans are exposed to a mixture of different chemicals simultaneously. Thus, there is a need for evaluation of toxicity of mixtures rather than single compounds only [17–19]. The aim of this study was to address effects of perinatal exposure to mixtures of EDCs on female reproductive endpoints in a top-down approach starting from a human relevant mixture and subgroups of this mixture. Thirteen EDCs, including phthalates, pesticides, UV-filters, Bisphenol A, butylparaben, and the pharmaceutical drug paracetamol (PM) were tested in a mixture ratio based on high-end human exposure levels as previously described [20,21]. The tested phthalates are known to influence steroid synthesis (reviewed in Ref. [22]) and indications of altered ovarian development have been seen in rodent studies [15,23]. The tested pesticides are known to act mainly as androgen receptor antagonists [24,25] and the UV-filters, Bisphenol A and butylparaben are known estrogen receptor agonists [26–28], but the compounds may also act via other modes of action [24,26]. PM is a prostaglandin synthesis inhibitor with

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Table 1
Mixture composition and dose for the tested mixtures in mg/kg per day. Design of the mixtures has previously been described [20,21].

Chemical	Mixture dose (mg/kg per day)							
	Totalmix 100	Totalmix 200	Totalmix 450	AAmix200	AAmix450	Emix 200	Emix 450	PM
DBP	1	2	4.5	2	4.5	0	0	0
DEHP	2	4	9	4	9	0	0	0
Vinclozolin	0.9	1.8	4.05	1.8	4.05	0	0	0
Prochloraz	1.4	2.8	6.3	2.8	6.3	0	0	0
Procymidone	1.5	3	6.75	3	6.75	0	0	0
Linuron	0.06	0.12	0.27	0.12	0.27	0	0	0
Epoxiconazole	1	2	4.5	2	4.5	0	0	0
p,p'-DDE	0.1	0.2	0.45	0.2	0.45	0	0	0
4-MBC	6	12	27	0	0	12	27	0
OMC	12	24	54	0	0	24	54	0
Bisphenol A	0.15	0.30	0.675	0	0	0.30	0.675	0
Butyl paraben	6	12	27	0	0	12	27	0
Paracetamol	80	160	360	0	0	0	0	360
Sum (mg/kg per day)	112	224	504	16	36	48	109	360

possible anti-androgenic modes of action [29]. To evaluate whether effects of the Totalmix could be attributed to one or more components of the mixture, we also tested subgroups of chemicals with anti-androgenic or estrogenic potentials, and PM alone (Table 1). The mixtures were administered in doses 100–450 times high-end human exposure levels, as these doses were predicted to affect anti-androgenic endpoints in male offspring, whereas the PM dose was corresponding to human exposure levels, see also [21].

Effects of these EDC mixtures on early male reproductive development [21], and mammary gland development [30] have previously been published. In the current study we hypothesized that perinatal exposure to mixtures of EDCs adversely affects the ovary in young and senescent animals, and investigated the impact of perinatal EDC exposure on ovarian gene expression, number of follicles, pituitary hormone levels in plasma, sexual maturation, estrous cyclicity and ovarian histology in rat offspring. Together, the results from young and senescent animals indicated premature ovarian insufficiency after exposure to this human-relevant EDC mixture, and these effects were likely caused by the anti-androgen mixture components and PM.

2. Materials and methods

2.1. Test compounds

Test compounds were: di-*n*-butyl phthalate (DBP) (purity >99.0%, CAS no. 84-74-2), di-(2-ethylhexyl) phthalate (DEHP) (purity >99.5%, CAS no. 117-81-7), vinclozolin (purity >99.5%, CAS no. 50471-44-8), prochloraz (purity >98.5%, CAS no. 67747-09-5), procymidone (purity >99.5%, CAS no. 32809-16-8), linuron (purity >99.0%, CAS no. 330-55-2), epoxiconazole (purity >99.0%, CAS no. 106325-08-8), octyl methoxycinnamate (OMC) (purity >98.0%, CAS no. 5466-77-3), dichlorodiphenyl-dichloroethylene (p,p'-DDE) (purity >98.5%, CAS no.72-55-9); all purchased from VWR—Bie & Berntsen (Herlev, Denmark). And: 4-methyl-benzylidene camphor (4-MBC) (purity >98.0%, CAS no. 36861-47-9), bisphenol A (BPA) (purity >99.5%, CAS no. 80-05-7), butyl paraben (purity >99.0%, CAS no. 94-26-8) and paracetamol (PM) (purity >99.0%, CAS no. 103-90-2); all purchased from Sigma-Aldrich (Brøndby, Denmark). Corn oil was used as a control compound and as vehicle; purchased from VWR—Bie & Berntsen (Herlev, Denmark).

2.2. Mixtures

The mixture compositions were based on high-end human exposure levels of 13 well-characterized endocrine disrupters, as previously described [20,21]. Totalmix contained all 13 compounds; AAmix contained compounds with predominantly anti-androgenic modes of action; Emix contained compounds with predominantly estrogenic properties (Table 1). PM was included in the Totalmix, as well as tested on its own. Totalmix was given at 100-, 200- or 450-times human high-end exposure, the AAmix and Emix at 200- and 450-times human high-end exposure. PM was given at 350 mg/kg, which corresponds to the dose given in the Totalmix450 (Table 1).

2.3. Animals and exposure

A detailed design of the animal study can be found in Ref. [21]. In short, time-mated nulliparous Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) 3 of pregnancy. The day vaginal plug was detected was designated as GD 1 and the expected day of delivery (GD23) was designated as pup day (PD) 1. The dams received vehicle (controls), or one of the eight mixtures (Table 1). Each dose group comprised 16–20 dams, with 14–20 viable litters obtained for each group. Rats were exposed by oral gavage from GD7-21, and again after birth from PD1-22. PM exposure was from GD13-19 and PD14-22, both in mixtures and single dosing, to avoid possible effects on embryo implantation [31] and problems during parturition. At PD22, 1–2 females per litter were weaned and kept until adulthood. One female pup per litter was killed at weaning (PD22) and one or two female pups per litter were killed at 13 months of age whilst in estrous or proestrous, evidenced by vaginal smears in the morning. Blood was collected for hormone analysis, and ovaries were weighed and prepared for histological examination and/or gene expression analysis. The study was performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee.

2.4. In vivo measurements

2.4.1. Anogenital distance and sexual maturation

Anogenital distance (AGD) was measured, by the same technician, in all offspring. The technician was blinded with regard to exposure group. Measurements were performed using an ocular stereomicroscope with unit markings on the ocular. Onset of puberty was defined as day of vaginal opening (VO) and assessed daily from PD28 until VO was detected in all female offspring. Age and body weights were recorded on the day when VO was first observed.

2.4.2. Estrous cyclicity

Vaginal smears were taken daily between 8 and 10 a.m., for 21 consecutive days at 3 and 12 months of age. A swab moist-

ened in saline was inserted into the vaginal lumen and cells were transferred to a microscope glass slide to air dry. The smears were fixed in 96% ethanol and stained with Gill's hematoxylin, Orange G6 and eosin-azure 50 (VWR—Bie & Berntsen, Herlev, Denmark) according to the adapted Papanicolaou (PAP stain) procedure [32]. The smears were mounted in Eukit (VWR—Bie & Berntsen, Herlev, Denmark) and examined by light microscopy under blinded conditions. Classification was done according to stages; estrous, metestrous, diestrous or proestrous, or transitions between stages [33,34].

The animals were categorized as either being regularly cycling (cycles lasting four to five days) or irregularly cycling (cycles lasting less than four days or more than five days) [35]. Episodes of three to four consecutive days of vaginal estrous and/or four to five days of diestrous were considered extended [33].

2.5. Histological examination

Ovaries from one female per litter, alternately left and right, were examined at PD22 as follows: control, Totalmix450, AAmix450, Emix450, and PM (n = 12–16) ovaries were fixed in formalin, processed for paraffin embedding, sectioned (5 μm sections at 90 μm intervals, all sections were counted) and stained with hematoxylin and eosin (H&E). In all sections, primordial (oocyte surrounded by flat pre-granulosa cells), primary (oocyte surrounded by one layer of cuboidal granulosa cells), secondary (oocyte surrounded by two or more layers of granulosa cells and theca cells), tertiary (presence of antrum), and atretic follicles (presence of condensed, dark cells) were counted, provided a nucleolus was visible.

At 13 months of age, histological evaluation was performed on one section per ovary (all groups), uterus and pituitary (control, Totalmix450, AAmix450, Emix450 and PM groups). The number of corpora lutea (CL) and the presence of follicular cysts (follicles devoid of oocytes, displaying a large antrum surrounded by 1–2 layers of flattened granulosa cells and a thecal cell layer) and cyst-like structures (follicles devoid of oocytes, displaying a large antrum surrounded by a few layers of granulosa cells) were investigated. In uterus the number and appearance of endometrial glands were evaluated, and the presence of squamous metaplasia, endometrial cysts and endometrial stromal polyps was registered. Pituitary glands were examined with emphasis on the presence of nodular hyperplasia and adenoma in pars distalis [36].

2.6. Plasma hormone levels

On PD22, blood was collected in heparin-coated vials, centrifuged and plasma withdrawn. Plasma levels of adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF), follicle stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH), prolactin (PRL) and thyroid stimulating hormone (TSH) was measured using a Milliplex map Rat Pituitary Magnetic Bead Panel (Cat. No. RPTMAG-86K; Merck Life Science A/S, Hellerup, Denmark). The PM group was not included. Plasma levels of Inhibin A were measured by ELISA (Cat. No. CSB-E08239r, CUSABIO Biotech Co.) Measurements were conducted according to the manufacturer's instructions.

2.7. Gene expression

For all nine dose groups (n = 9–10 per group), the alternate left and right ovary was excised from one female per litter at PD22 and stored in RNAlater (Qiagen, Hilden, Germany) at −80 °C. Relative gene expression was analyzed by RT-qPCR as previously described [37]. In short, total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified on a NanoDrop-1000 Spectrophotometer. RT-qPCR reactions were run in duplicates on a 7900HT qPCR system (Applied Biosystems, Foster City, CA) in 20 μl reactions including 3 μl diluted (1:20) cDNA and gene-specific TaqMan assays (Life Technologies Europe BV, Naerum, Denmark: *Amh* (Rn01535314_g1), *Bmp15* (Rn00572320_m1), *Ddx4* (Rn01489814_m1), *Fshr* (Rn01648507_m1), *Fst* (Rn00561225_m1), *Lhr* (Rn00564309_m1)). Intra-assay variability of technical replicates was <0.5 cycles. Data were analyzed by the comparative Ct-method normalized with the geometric mean of verified reference genes *Rps18* (Rn01428913_gH) and *Sdha* (Rn00590475_m1) [37].

2.8. Statistical analysis

For all analyses, the alpha level was set at 0.05. Data from continuous endpoints were examined for normal distribution and homogeneity of variance and if required, logarithmic transformation was performed. For endpoints where ANOVA and Dunnett's post-test were used, data were compared as follows: Control versus Totalmix, Control versus AAmix, Control versus Emix. PM was compared to control by use of Student's t-test. For non-normally distributed data Kruskal-Wallis and Dunn's post-test or Mann-Whitney was used. Data from follicle count were investigated by t-test as only highest doses were used. Litter was used as an independent, random and nested factor when more than one pup from each litter was examined.

AGD data were analyzed using pups' birth weights as covariate and by the AGD-index, i.e. AGD divided by the cube root of body weight. Statistical analyses were adjusted using litter as an independent, random, and nested factor. Age and weight at sexual maturation was analyzed by ANOVA using body weight at PD22 as a covariate to compensate for size differences. Estrous cyclicity data were tested using logistic regression and tested for over-dispersion with Deviance and Pearson Goodness-of-Fit tests. Correction for over-dispersion due to litter effects was used when appropriate.

Organ weights were analyzed by ANOVA using body weight as a covariate. Histological data were evaluated using Fisher's Exact Test. Regression was used to investigate relationship between ovary weight and number of CL.

The statistical software SAS (SAS Enterprise Guide 4.3), R [38], and GraphPad Prism 5 (GraphPad Software, San Diego California USA) were used for analysis.

3. Results

3.1. Gene expression

No significant changes in overall expression were observed for any of the somatic markers (Suppl. Fig. S1), suggesting that the overall ratio of cell-specific populations were relatively unchanged. A significant reduction in *Ddx4* (p = 0.03) and trend to a reduction in *Bmp15* (p = 0.07) transcript levels were observed in the PM-exposed group (Fig. 1A and B). As this suggested a smaller number of oocytes, manual counting of follicles was performed.

3.2. Follicle count

Primordial follicles were significantly reduced (78.2% of control values, p = 0.02) in rats exposed to AAmix450, and a tendency towards reduction was seen in the PM-exposed group (p = 0.06) (Fig. 1C). There were no statistically significant differences in number of recruited follicles (pool of primary, secondary and tertiary) between groups. Visual evaluation of the total number of follicles (total number in all sections counted), indicated a slight reduction in total number of follicles in AAmix450 and PM, but the differences

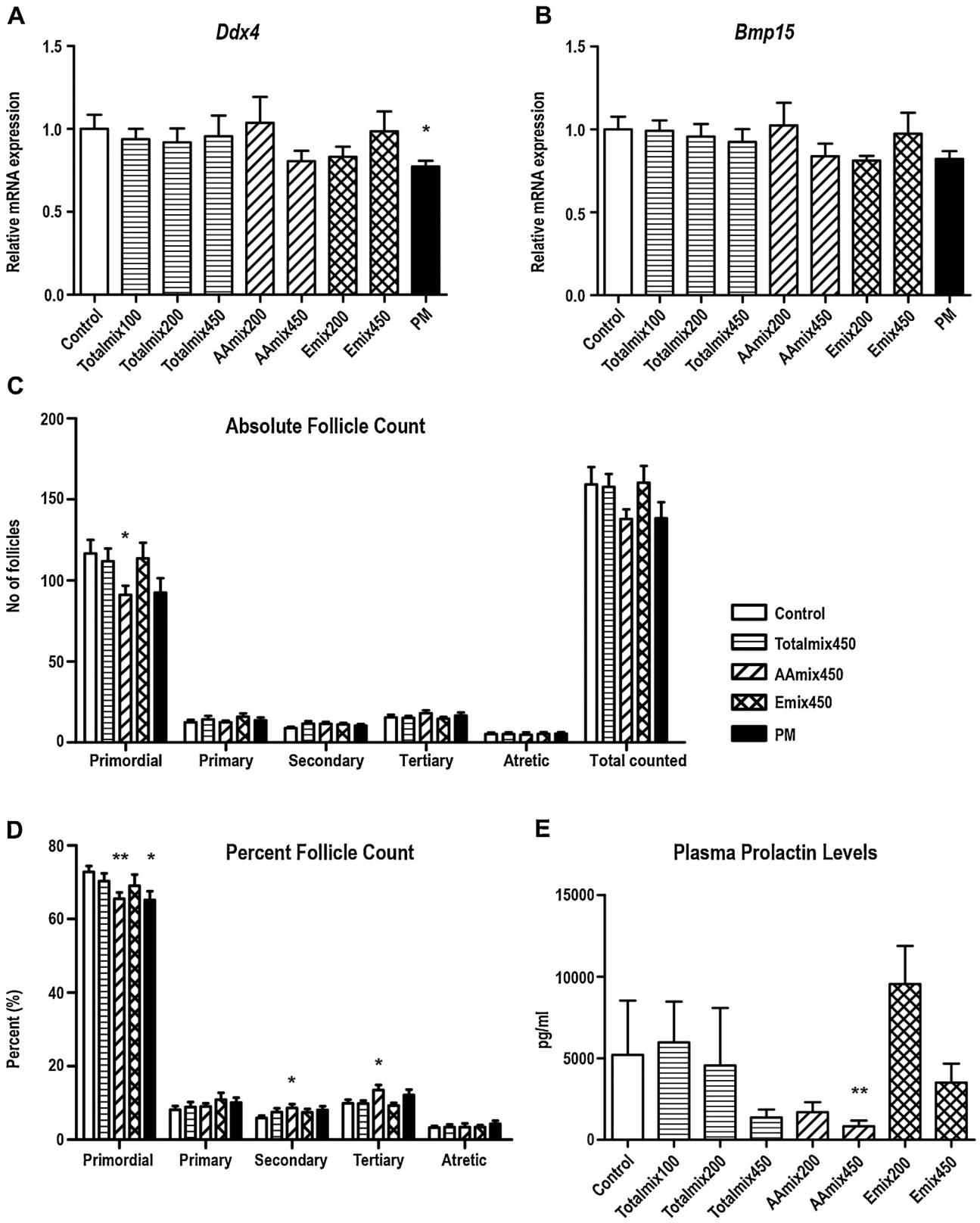

Fig. 1. Results from PD 22. (A and B) A significant reduction in relative mRNA expression of *Ddx4* and a slight, but not statistically significant reduction, in *Bmp15* were seen in ovaries after PM treatment (n = 9–10 per group). (C) Absolute follicle count showed significantly reduced numbers of primordial follicles in the AAmix450 group (n = 12–16 per group). (D) Percentage of follicles (each animal's total number of follicles set to a 100%) showed a significant reduction in primordial follicles after AAmix450 and PM exposure. AAmix450 exposure also caused an increase in secondary and tertiary follicles. (E) Prolactin level in plasma was reduced after AAmix450 exposure (n = 9–10 per group). Data presented as mean ± SEM, (*$p < 0.05$, **$p < 0.01$).

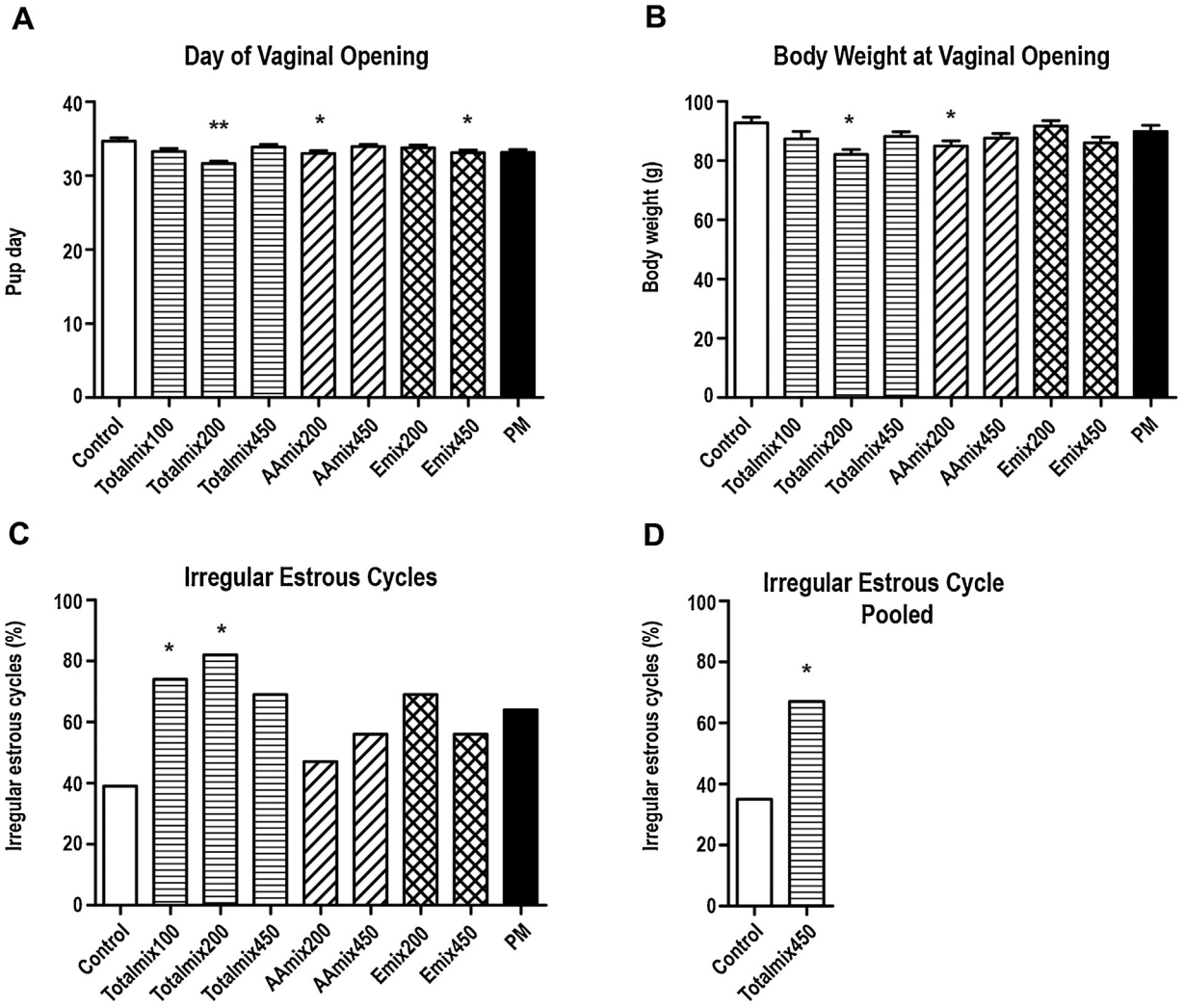

Fig. 2. (A) Sexual maturation, measured as day of vaginal opening (VO), occurred significantly earlier in animals exposed to Totalmix200, AAmix200 and Emix450 (n = 26–36 per group, corresponding to 13–19 litters per group). (B) Body weight at VO was significantly lower in animals exposed to Totalmix200 and AAmix200 (n = 26–36 per group, corresponding to 13–19 litters per group). (C) The incidence of animals with irregular estrous cycles was increased in the Totalmix100 and Totalmix200 (n = 11–14 animals per group from separate litters) in adult females (12 months of age). (D) The incidence of animals with irregular estrous cycles was higher in the Totalmix450 (n = 31) compared to control (n = 30) when data was pooled with a previous study (Isling et al., 2014) in adult females (12 months of age). Data presented as mean ± SEM, (*p < 0.05, **p < 0.01).

were not statistically significant (Fig. 1C). When using percentage of follicles instead of absolute values, a significant reduction in primordial follicles was seen in groups exposed to AAmix450 (p = 0.005) and PM (p = 0.01) and there was a significant increase in secondary (p = 0.05) and tertiary (p = 0.04) follicles in the AAmix450 group (Fig. 1D). Furthermore, the number of recruited follicles (pool of primary, secondary and tertiary) was significantly increased in animals exposed to AAmix450 (p = 0.01) and PM (p = 0.02).

3.3. Hormone levels

There was a significant reduction (p = 0.01) in PRL levels after AAmix450 exposure, and visual evaluation of the data indicated a reduction also in the Totalmix450 and AAmix200 groups. However, these reductions were not statistically significant (p = 0.15 and p = 0.27, respectively) (Fig. 1E). No effects were seen on the levels of other pituitary hormones or inhibin A (Supplementary Figs. S2 and S3).

3.4. AGD, sexual maturation and estrous cyclicity

No significant differences between groups were observed for female AGD [21]. Sexual maturation occurred significantly earlier in Totalmix200 (p = 0.0002), AAmix200 (p = 0.02) and Emix450 (p = 0.04) groups. Animals in Totalmix200 and AAmix200 groups had lower body weights at the day of VO (p = 0.01 and p = 0.02, respectively) (Fig. 2A and B). There were no effects on estrous cycle regularity at three months of age. At 12 months of age, overall analyses showed no effect on estrous cycle regularity, however pairwise comparison of control with each group showed significant effects in Totalmix100 (p = 0.041) and Totalmix200 (p = 0.048) (Fig. 2C). In a previous study on the same mixture (though only including Totalmix450) estrous cyclicity data was also collected at 12 months of age [1]. As the power appeared low for estrous cyclicity, the control and Totalmix450 data from both studies were pooled, resulting in a significant difference between Totalmix450 and control (p = 0.02) (Fig. 2D). Irregular cycles were primarily characterized by longer than normal cycles with extended diestrous. Extended estrous and shorter than normal cycles were also observed, but to a lesser extent.

3.5. Ovary weight and histology

At 13 months of age, ovary weight was significantly reduced in all Totalmix groups, both AAmix groups and the PM group (Fig. 3A).

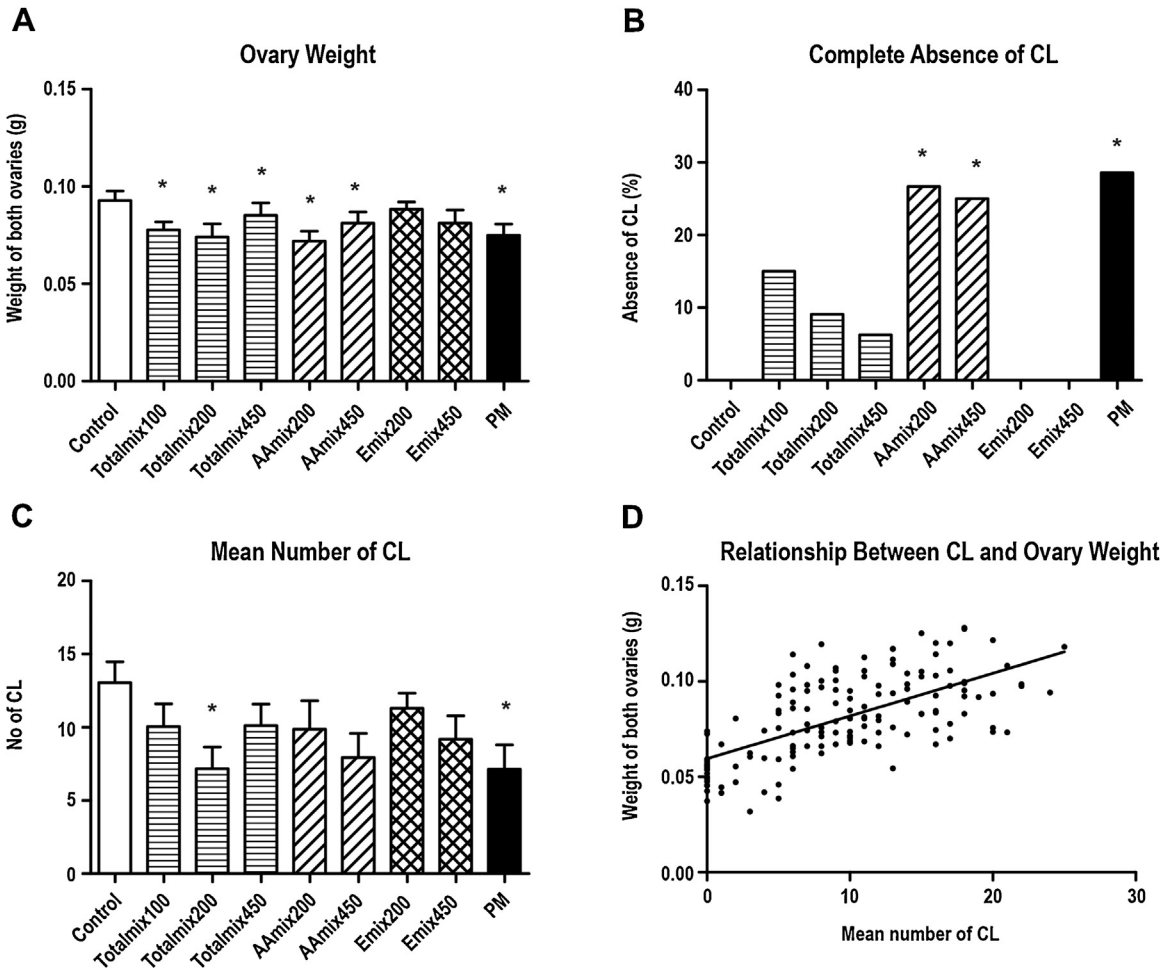

Fig. 3. Effects on ovary weight and the presence of corpora lutea (CL) in 13 months-old animals. (A) Mean ovary weight was reduced in animals exposed to Totalmix, AAmix and PM (n = 11–20). (B) Complete absence of CL in animals exposed to AAmix and PM. (C) Mean number of CL was significantly lower in Totalmix200 and PM (n = 11–20). (D) Regression analysis showed that the mean ovary weight was associated with the mean number of CL, independent of exposure ($r^2 = 0.41$, slope significantly non-zero with $p < 0.0001$). Data presented as mean ± SEM, (*$p < 0.05$).

All females were killed whilst in proestrous or estrous, therefore observed effects were not considered to be due to variability in estrous cycle stage. No relevant changes in ovary weights were seen at PD22 (data not shown).

Histological examination at 13 months of age revealed a significant increase in incidences of rats presenting with complete absence of CL in AAmix200 ($p = 0.033$), AAmix450 ($p = 0.039$), and in PM ($p = 0.028$) groups (Fig. 3B). In addition, the mean number of CL was significantly reduced to 55% of control values in the Totalmix200 group ($p = 0.04$) and 54.7% of control values in the PM group ($p = 0.011$). The mean number of CL was also reduced in AAmix450, but did not reach statistical significance ($p = 0.056$) (Fig. 3C). The mean number of CL was positively correlated to ovary weight ($r^2 = 0.41$, $p < 0.0001$) (Fig. 3D).

Follicular cysts and cyst-like structures were observed in all groups and are considered to be normal age-related changes. The number of animals with cyst-like structures in ovaries was significantly increased in the AAmix450 and the PM groups, and the same was seen when pooling data for cysts and cyst-like structures (Table 2).

3.6. Uterine and pituitary histology

Squamous metaplasia was observed in 1–3 rats per group in Totalmix450, AAmix450, Emix450 and PM groups, but not in controls (Table 2). Pituitary nodular hyperplasia or adenoma in *pars distalis* was not different between dose groups (Table 2).

Six out of eight rats with squamous metaplasia in uterus also had absence of CL or 1–3 small degenerated CL suggesting a common endocrine effect. Only 6 out of 23 females with pituitary nodular hyperplasia, adenoma or macroscopic tumor also had few or absent CL, and only two had squamous metaplasia suggesting no clear relationships between pituitary findings and reproductive organ effects.

4. Discussion

This study showed effects of human relevant mixtures of EDCs on both prepubertal and adult female rats; reduction in primordial follicles, irregular cycling, and premature absence of CL. These symptoms resemble premature ovarian insufficiency syndrome in humans [39], causing concern that perinatal exposure to EDCs can reduce the reproductive lifespan of women.

4.1. Early reproductive senescence

In rodents, onset of irregular estrous cycles, and eventually cycling arrest, can be a sign of reproductive senescence, usually initiating between 9 and 12 months of age [40]. We observed an increase in irregular cycles at 12 months of age following prenatal exposure to Totalmix. As no effect was seen on estrous cyclic-

Table 2
Summary of histopathological observations. Histological evaluation of ovaries, pituitary and uterus from 13-month old rats exposed perinatally to mixtures of endocrine disrupting chemicals. Values are mean ± SD, or number of affected animals (% of affected rats). CL: corpora lutea. Asterisks indicate statistically significant difference from controls in a Fisher's exact test: $^{*}p < 0.05$, $^{**}p < 0.01$, #p = 0.057.

		Ovary					Pituitary			Uterus
	N	Total number of CL	Ovaries with absence of CL	Ovaries with 1–3 degenerated CL	Ovaries with follicular cysts	Ovaries with cyst-like structures	Ovaries with follicular cysts and/or cyst-like structures	Nodular hyperplasia pars distalis	Adenoma pars distalis	Macroscopically observed tumor	Macroscopically observed tumor, nodular hyperplasia or adenoma pars distalis	Squamous metaplasia
Control	18	13.1 ± 6.0	0 (0%)	1 (6%)	1 (6%)	8 (44%)	8 (44%)	2 (11%)	1 (6%)	2 (12%)	4 (22%)	0 (0%)
Totalmix100	20	10.1 ± 6.9	3 (15%)	0 (0%)	3 (15%)	7 (35%)	8 (44%)	ND	ND	ND	ND	ND
Totalmix200	11	7.2 ± 4.9*	1 (9%)	1 (9%)	2 (18%)	8 (73%)	8 (73%)	ND	ND	ND	ND	ND
Totalmix450	16	10.1 ± 5.8	1 (6%)	1 (6%)	2 (13%)	11 (69%)	12 (75%)	1 (6%)	4 (25%)	4 (29%)	6 (38%)	3 (19%)
AAmix200	15	9.9 ± 7.6	4 (27%)*	0 (0%)	4 (27%)	9 (60%)	10 (67%)	ND	ND	ND	ND	ND
AAmix450	16	7.9 ± 6.6#	4 (25%)*	1 (6%)	5 (31%)	13 (81%)*	13 (81%)*	3 (19%)	3 (19%)	7 (64%)	7 (44%)	2 (13%)
Emix200	16	11.3 ± 4.1	0 (0%)	0 (0%)	4 (25%)	9 (56%)	10 (63%)	ND	ND	ND	ND	ND
Emix450	16	9.2 ± 6.4	0 (0%)	3 (19%)	1 (6%)	5 (31%)	5 (31%)	4 (25%)	0 (0%)	1 (7%)	4 (25%)	1 (6%)
PM	14	7.1 ± 6.2*	4 (29%)*	2 (14%)	2 (14%)	13 (93%)**	13 (93%)**	1 (7%)	1 (7%)	0 (0%)	2 (14%)	2 (14%)

ity at three months of age, this could indicate that exposed rats entered reproductive senescence prematurely compared to the control group. We also observed a reduction in ovary weight in Totalmix, AAmix and PM groups. This was supported by a significantly reduced mean number of CL in Totalmix200 and PM groups, and an increased incidence of complete absence of CL and cyst-like structures in rats exposed to AAmix and PM. This accelerated rate of age-related changes—as compared to background levels—is considered adverse and a sign of early aging in exposed groups. It is possible that examination of slightly younger animals would have resulted in fewer background findings and hence a clearer picture of the chemically induced histological changes. Nevertheless, our findings indicate that the AAmix and PM groups, as well as the Totalmix group, displayed signs of early reproductive senescence compared to the control group, despite only slight effects were seen on estrous cyclicity in those dose groups. Reproductive senescence may also present as uterine changes. Squamous metaplasia of the uterus occurs spontaneously among aged rats, and can be induced by continued administration of estrogenic compounds [41]. Due to low incidence, it is not clear whether the observed squamous metaplasia was related to early reproductive senescence. Reprogramming of the hypothalamic–pituitary–ovarian axis at central level may be related to early reproductive senescence [42] and in a study by Ref. [1], rats exposed to AAmix450 and Totalmix450 showed increased incidence of pituitary tumors at 19 months of age. In our study the changes in uteri and ovaries did not appear correlated with pituitary nodular hyperplasia or adenoma, thus we hypothesize that the late effects may be caused by direct effects on the ovaries.

4.2. Early versus delayed effects of anti-androgens in ovary

Effects on aging animals are rarely examined and are not a part of OECD test guidelines for reproductive toxicity studies [43,44]. Chemical effects on early reproductive senescence may thus be overlooked. We wanted to investigate if follicular development was affected at an earlier time-point as early changes could be useful markers of late-life effects. On PD22 we saw reduced expression of the germ cell markers *Ddx4* and *Bmp15* in ovaries from animals exposed to PM. This could be due to an overall loss of oocytes, thus follicles were manually counted. The number of primordial follicles was reduced in rats exposed to AAmix and PM. This is worrying as females are considered to be born with a limited number of oocytes, such that any reduction in the number of primordial follicles can have permanent effects on fertility by reducing the reproductive life span [45].

Interestingly, the reduction in primordial follicles was seen in animals exposed to anti-androgenic chemicals. It has become evident that androgens are important for ovarian function and follicular development [46,47]. The androgen receptor (AR) knockout (KO) mouse (ARKO) has a phenotype resembling premature ovarian insufficiency with symptoms such as irregular estrous cycles, lack of CL and infertility [48,49], effects similar to those observed in adult rats in our study. AR regulates downstream factors controlling folliculogenesis, and down-regulation in young individuals may cause impaired folliculogenesis at a later age [49]. It is therefore plausible that reduced AR signaling contributed to the observed late-life effects on estrous cyclicity and number of CL.

Both AR antagonists and steroid synthesis inhibitors in the AAmix may have contributed to the observed effects on follicle numbers. Prenatal exposure to the AR antagonist vinclozolin reduced primordial follicle numbers in mice at 12 months of age, and a reduction in total number of oocytes was seen in explanted newborn rat ovaries, cultured and exposed to vinclozolin for ten days [50]. Two studies on the steroidogenesis inhibitor DEHP and/or its metabolite MEHP have shown disruption of early fol-

liculogenesis in explanted newborn mouse ovaries [51,52]. DEHP decreased the incidence of primordial follicles in ovaries of PND21 mice exposed during fetal life [23], and reduced the percentage of primordial follicles on PND15 and PND21 in mice following postnatal exposure [15]. Furthermore, an increase in secondary and antral follicles was registered by [15,23], a finding that agrees with the increased ratio of recruited versus non-recruited follicles in AAmix450 and PM groups. This indicates that in our study, increased folliculogenesis may have caused the reduction in primordial follicle numbers. However, slightly lower total follicle numbers were observed in AAmix450 and PM groups. Therefore it is unclear if the reduction was due to increased recruitment, if the follicle reserve initially was smaller, or a combination of both. Furthermore, both vinclozolin and DEHP have the potential to affect follicle numbers, but further studies are needed to evaluate whether the remainder of compounds in the AAmix also contributed to the observed effects.

Unexpectedly, the reduction in primordial follicle numbers after AAmix and PM exposure was not seen in animals exposed to the Totalmix comprising AAmix, Emix and PM. Emix has been shown to have endocrine effects during prepuberty, as Emix exposure increased mammary outgrowth in PD22 females [30]. For other endpoints such as male anogenital distance and prepubertal male reproductive organ weights, the effects in Totalmix groups reflected the effects of the AAmix [21]. We therefore propose that endocrine effects of Emix exposure can modulate the effects of AAmix and PM on the developing ovary.

Plasma levels of PRL were reduced in PD22 animals exposed to AAmix450. In young females, PRL is proposed to be involved in pubertal timing and reduced levels may cause delayed puberty [53]. VO was not significantly affected in AAmix450, but visual evaluation indicated an earlier rather than delayed day of VO, which was also observed in AAmix200, Totalmix200 and Emix450, rendering PRL levels unlikely to be causative.

4.3. Human relevance of mixed chemical exposure

The EDCs included in this study were selected to reflect a chemical exposure pattern relevant to humans [20,21]. The doses were 100- to 450-times higher than estimated high-end human exposure levels and effects on estrous cyclicity and ovary weight were seen at all doses, suggesting that a standard regulatory safety margin of 100 is not present for highly exposed persons. PM was administered at a dose corresponding to the maximum recommended dose for humans (when taking into account the different kinetics of rats and humans). Such high exposure may seem unlikely to occur during the long time span modelled in the current study, but it is possible that PM exposure for a limited time period during the most sensitive period of fetal reproductive development can be sufficient to affect the reproductive function later in life. The observed reduction in primordial follicles, as well as irregular cycling and premature absence of CL resemble premature ovarian insufficiency syndrome in humans, a condition usually leading to premature menopause; before the age of 40 [39]. The cause for premature ovarian insufficiency is largely unknown [39,54], but EDCs have been suggested to be part of the etiology [55]. This raises the concern that early-life exposure to EDCs can compromise the reproductive lifespan of women. Such an effect, even if small, is problematic in today's society where the age at childbirth is delayed [56].

In summary, we investigated effects of perinatal exposure to human relevant mixtures of EDCs on female reproductive endpoints and found a reduced follicle pool in pre-pubertal animals after exposure to anti-androgenic chemicals or PM. In adults, signs of early reproductive senescence were seen: effects on estrous cycle regularity and reduced ovary weight after Totalmix exposure, and reduction in CL and ovary weight after anti-androgen and PM exposure. Together, the effects resemble what in humans is categorized as premature ovarian insufficiency, a condition where EDCs have been proposed as part of the etiology. As the mixture composition investigated resembles high-end everyday exposure for humans, the results raise concern for the reproductive lifespan of children of exposed women. The applied top-down approach, starting from a human relevant exposure scenario, is considered highly relevant for human health assessment and leads the way for targeted mechanistic studies of sub-mixtures and individual compounds.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The http://dx.doi.org/10.1016/j.reprotox.2016.03.045 associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2016.03.045.

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Appendix II

Supplementary Material

Johansson HK, Jacobsen PR, Hass U, Svingen T, Vinggaard AM, Isling LK, Axelstad M, Christiansen S, Boberg J. *Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging*. Reprod Toxicol 2016, Jun;61: 186-94

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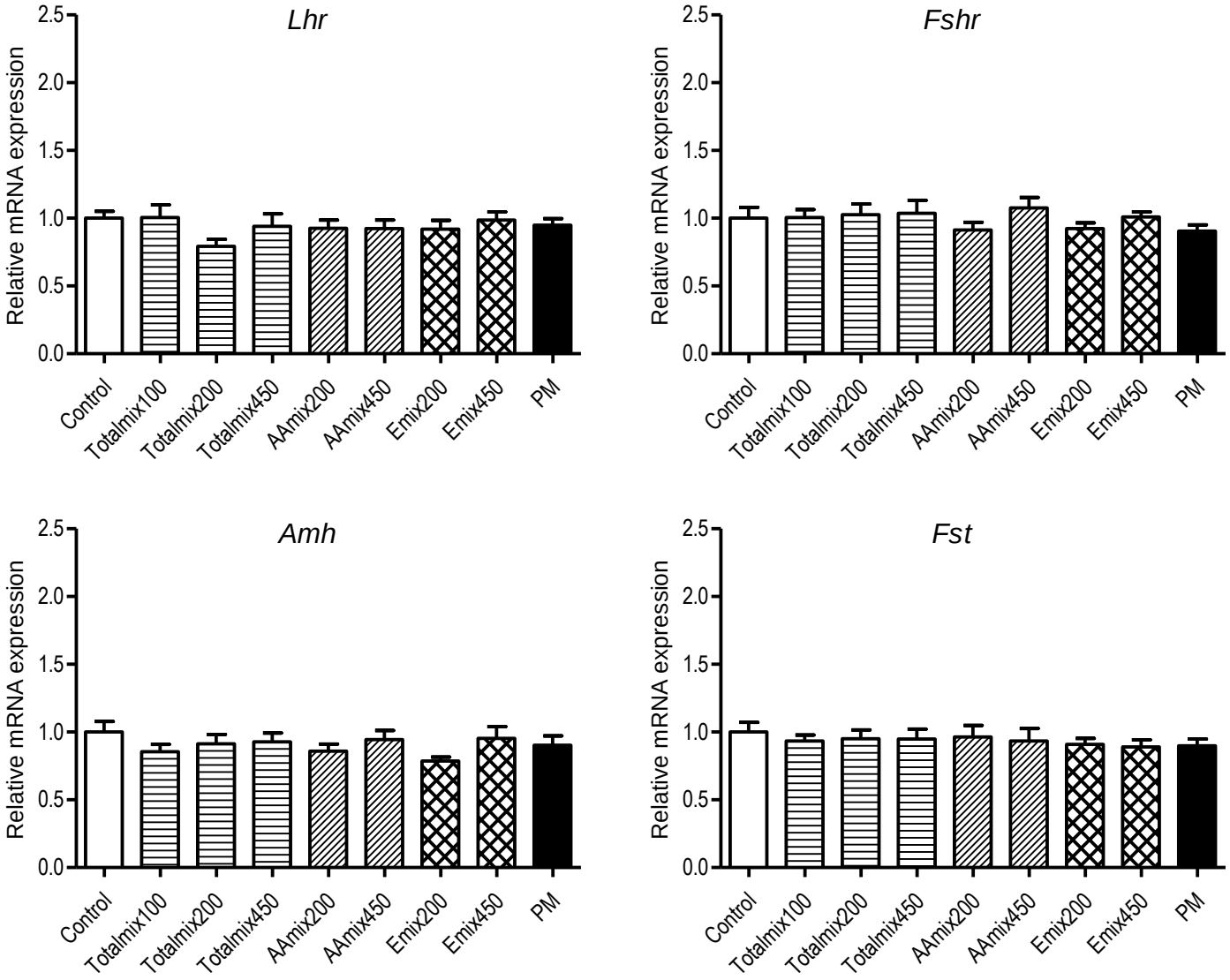
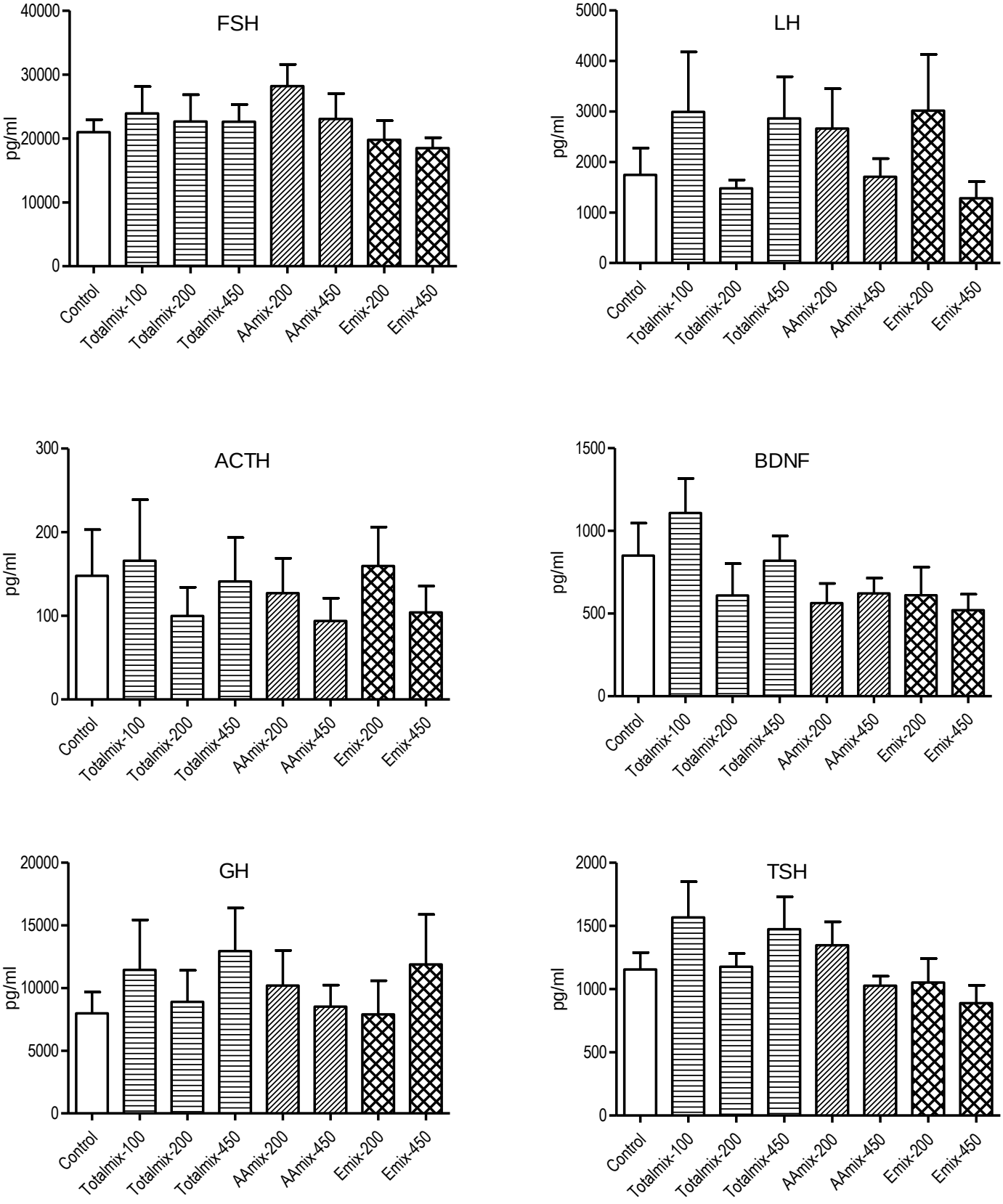
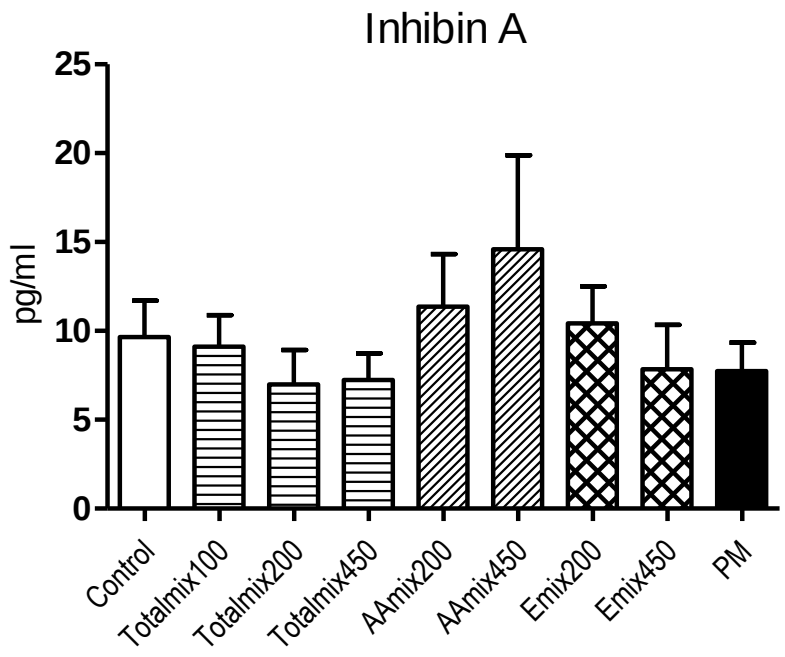


Supplementary Fig S1: Relative mRNA expression of somatic markers in ovaries on PD22. No statistically significant effects were seen ($p>0.05$, n=9-10).



Supplementary Fig S2: Plasma levels (pg/ml) of pituitary hormones on PD22. No statistically significant effects were seen ($p>0.05$, n=8-11, FSH= Follicle stimulating hormone, LH= Luteinizing hormone, ACTH= Adrenocorticotropic hormone, BDNF= Brain-derived neurotrophic factor, GH= Growth hormone, TSH= thyroid stimulating hormone.



Supplementary Fig S3: Plasma levels (pg/ml) of Inhibin A on PD22. No statistically significant effects were seen ($p>0.05$, n=7-10).