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Early-life exposure to potential endocrine disrupting chemicals and adverse developmental outcomes in Norwegian children.

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Oslo, December 2021

Anteneh A. Desalegn

Summary

Background: Humans are exposed to a large and ever-increasing number of complex chemical mixtures in the environment via multiple routes. Toxicity testing and regulations remain often based on a single chemical that may underestimate the potential risk. Endocrine Disrupting Chemicals (EDCs), unlike most chemicals, may produce toxicity at low dose, and pose a threat not only to the exposed organism but also to its future generations.

Aim: The overall aim of this thesis was to identify chemicals associated with adverse child health outcomes. The specific objectives for Paper I and IV were to investigate the association between early-life exposure to twenty-seven potential EDCs and cryptorchidism and autism spectrum disorders (ASD), respectively. For Paper II and III, the specific objectives were to determine whether androgen receptor and aryl hydrocarbon receptor activations were involved in the cryptorchidism development pathway.

Methods: Breast milk chemical exposure was used as a proxy for both gestational exposure as well as exposure during the breastfeeding periods covering a wide developmental window. Breast milk samples were collected from mothers enrolled in the Norwegian HUMIS birth cohort (2002-2009). The analysed chemicals fall into four main classes: polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polybrominated diphenyl ethers (PBDEs), and poly- and perfluoroalkyl substances (PFASs). Cryptorchidism was defined based on maternal report using repeated questionnaires at 1-, 6-, 12-, and 24- months after birth while ASD was ascertained based on a specialist-confirmed diagnosis when children were 13 years of old. We assessed the multiple exposures using elastic-net regression, a variable selection method to reduce multicollinearity in Paper I and IV, multivariate logistic regression to compare the level of receptor activities between cases and controls in Paper II and III. Multiple imputation was used to impute missing values for exposures and covariates. Paper IV also includes an experimental *in vivo* study using zebrafish larvae following a prospective cohort study in collaboration with Chemical Neuroscience Group, Centre for Molecular Medicine, University of Oslo.

Results: Early-life exposure to potential EDCs was associated with adverse child health outcomes among Norwegian children. PCBs (PCB-74, PCB-114, and PCB-194) and β-HCH were selected as important risk factors for congenital cryptorchidism (Paper I) while only β-HCH was selected as an important predictor for autism spectrum disorder (Paper IV). None of the 27 chemicals were selected as important predictors for recurrent or persistent cryptorchidism.

The *In vivo* experimental study on zebrafish embryo and larvae verified the neurodevelopmental toxicity of β-HCH at concentrations corresponding to lower quartile levels found in breast milk among Norwegian mothers with autistic children in the HUMIS study. The estimated daily intake of β-HCH among Norwegian children through breastfeeding in the HUMIS cohort was calculated to be 0.03 μg/kg of body weight (bw)/day slightly above the Dutch National Institute for Public Health and the Environment (RIVM) estimated Tolerable daily intake (TDI) for β-HCH (0.02 μg/kg of bw/day).

We found no evidence of an association between anti-androgenic receptor activity (Paper II) nor aryl hydrocarbon receptor activity (Paper III) and the development of cryptorchidism among Norwegian male babies. Estimated daily intake of both anti-AR activity (78 μg flutamide eq./kg of bw/day) not derived from natural hormones, and AhR activities (33.7 ± 17.9 pg TEQ/kg bw/day) among Norwegian children during the exclusive breastfeeding period were above the permitted daily exposure limits (50 μg/d for the androgen receptor antagonist flutamide, and WHO derived TDI range of 1-4 pg TEQs/kg body weight for total dioxin and dioxin-like compounds activity). Furthermore, the anti-androgenic activity when polar and non-polar breastmilk fractions were mixed together was smaller than the sum of the individual fraction. This may be due to an interaction and demonstrates the importance of basing risk assessments on real-life mixtures (Paper II). All dl-PCBs, and some of the ndl-PCBs (PCB-74, PCB-180, PCB-194), and two of the OCPs (HCB, β-HCH) were associated with AhR activity (pg CALUX-TEQ/g lipid) in breast milk whereas the association was null for PBDEs and PFASs (Paper III).

Conclusions: Early-life exposure to PCBs (PCB-74, PCB-114, and PCB-194) and β-HCH was associated with congenital cryptorchidism while only β-HCH was associated with autism among Norwegian children enrolled in the HUMIS study. β-HCH's neurotoxicity was further demonstrated in zebrafish larvae, with additional experiments suggesting perturbation of dopaminergic neurone network as a potential pathway for autism.

Neither anti-androgenic receptor activity (μg flutamide eq./g of milk) nor aryl hydrocarbon receptor activity (pg CALUX-TEQ/g lipid) were implicated as having roles in cryptorchidism development pathways. Our study was limited to investigating interaction with receptors while EDCs may also affect other processes (synthesis, storage, release, and metabolism) that require further investigation. However, the anti-androgenic receptor activity was above permitted daily exposure calculated based on European Medicines Agency directive for the androgen receptor antagonist flutamide. Aryl hydrocarbon receptor activity (pg TEQ/g lipid) were also above tolerable daily intake for dioxins and dioxin-like substances set by WHO among Norwegian

children during the exclusive breastfeeding period. This should be taken into consideration in the future risk-benefit assessment of breastmilk. The current levels of dioxin and dl-PCBs expressed as AhR activity (pg TEQ/g lipid) in breast milk falls in the declining trend in Norway from 1986 to 2005 although the level remains above permitted daily exposure limit.

Multipollutant analysis, using elastic net, demonstrated the importance of using appropriate statistical models to handle highly correlated chemicals simultaneously to reduce false-positive associations, as we observed markedly different results from the single pollutant analyses. This is important for risk assessment as single-pollutant analysis may be confounded by other chemicals co-existing in the mixture if they are also highly correlated as indicated in our analysis.

Sammendrag (Norwegian summary)

Bakgrunn: Mennesker eksponeres for et stadig økende omfang av kjemikalier fra flere kilder. Likevel gjennomføres risikovurdering for hvert enkelt stoff separat. Da eksponering for en miks av ulike stoffer kan ha både additiv og synergistisk effekt betyr dette at den potensielle risikoen undervurderes. Kjemikalier med hormon-hermende effekter (EDC) kan, i motsetning til de fleste andre kjemikalier, ha toksiske effekter i svært lave doser og utgjøre en trussel både for den eksponerte organismen, og også for dets avkom.

Det overordnede målet med denne oppgaven var å identifisere kjemikalier som økte risikoen for sykdom hos barn. De spesifikke målene med Paper I og IV var å undersøke forholdet mellom tidlig eksponering for tjuesju potensielle EDC-er og henholdsvis kryptorkisme og autismespekterforstyrrelser (ASD). I Paper II og III var de spesifikke målene å undersøke om aktivering av androgen- og arylhydrokarbonreseptor var involvert i utvikling av kryptorkisme.

Metode: Miljøgifter målt i morsmelk ble brukt som proxy for barnets eksponering både i fosterlivet og under ammeperioden, og dekket derfor ett bredt vindu i utviklingen. Morsmelk ble samlet inn fra mødre som deltok i den norske morsmelkestudien (HUMIS, 2002-2009). Fire klasser av kjemikalier ble analysert: polyklorerte bifenyler (PCB), organiske klorpesticider (OCP), polybromerte difenyletere (PBDE) og poly- og perfluoralkylstoffer (PFAS). Kryptorkisme var basert på mødrenes rapporting i spørreskjemaer fylt ut ved 1-, 6-, 12- og 24-måneder etter fødselen, mens ASD diagnosen var basert på spesialistopplysinger frem til barnet var 13 år. Vi analyserte mange høyt korrelerte miljøgifteksponeringer med elastisk net regresjon, en seleksjonsmetode som reduserer multikollinearitet, i Paper I og IV. Multivariat logistisk regresjon ble brukt for å sammenligne nivået av reseptoraktivitet i kasus-kontroll-studier i Paper II og III. Multippel imputering ble brukt for å imputere manglende verdier for eksponeringer og kovariater. Paper IV inkluderer også en eksperimentell in vivo studie med sebrafisklarver, for å validere funnene i den prospektive kohortstudien, utført i samarbeid med Chemical Neuroscience Group, Senter for molekylær medisin, Universitet i Oslo.

Resultater: Tidlig eksponering for potensielle hormonhermere var assosiert med sykdomstilstander hos norske barn. PCB-er (PCB-74, PCB-114 og PCB-194) og β-HCH ble identifisert som risikofaktorer for medfødt kryptorkisme (Paper I) mens kun β-HCH ble valgt som en viktig prediktor for autismespekterforstyrrelse (Paper IV). Den eksperimentelle in vivo-studien på sebrafiskembryoer og -larver bekreftet at β-HCH har nevropsykologisk effekt ved konsentrasjoner tilsvarende det laveste kvartilnivået i morsmelk blant norske mødre med autistiske barn i HUMIS-studien. Det estimerte daglige inntaket av β-HCH blant norske barn

gjennom ammeperioden ble estimert til å være 0,03/g/kg kroppsvekt/daglig, noe som er litt over det det Nederlandske nasjonale institutt for folkehelse og miljø (RIVM) anslår som tolerabelt daglig inntak (TDI) for β-HCH (0,02 μg/kg kroppsvekt/daglig). Vi fant ingen bevis for en sammenheng mellom antiandrogen reseptoraktivitet (Paper II), eller arylhydrokarbon reseptoraktivitet (Paper III), og utvikling av kryptorkisme blant norske gutter. I perioden barnet ble eksklusivt ammet var det estimert daglig inntak av anti-AR-aktivitet, utover den fra naturlige hormoner, (78 flg flutamidekv./kg kroppsvekt/dag), og AhR-aktivitet (33,7 ± 17,9 pg TEQ/kg kroppsvekt/dag) over de tillatte eksponeringsgrensene (50 μg/d for androgenreseptorantagonisten flutamid, og WHO-avledet TDI-område på 1-4 pg TEQs/kg kroppsvekt for total dioksin og dioksinlignende forbindelsesaktivitet). Videre var den anti-androgene aktiviteten når polare og ikke-polare morsmelkfraksjoner ble blandet sammen, mindre enn summen av de individuelle fraksjonene. Dette kan skyldes en interaksjon og viser viktigheten av å basere risikovurderinger på reelle blanding av kjemikalier (Paper II). Alle dl-PCB-ene, og noen av ndl-PCB-ene (PCB-74, PCB-180, PCB-194), og to av OCP-ene (HCB, β-HCH) var assosiert med AhR-aktivitet (pg CALUX-TEQ / g lipid) i morsmelk mens det ikke var noen sammenheng med PBDE og PFAS (Paper III).

Konklusjon: Prenatal eksponering for PCB-er (PCB-74, PCB-114 og PCB-194) og β-HCH var assosiert med medfødt kryptorkisme, mens kun β-HCH var assosiert med autisme blant barn som var med i den norske HUMIS-studien. Nevrotoksisiteten til β-HCH ble bekreftet i eksperimentelle forsøk på sebrafisklarver, hvor det også ble vist påvirkning av det dopaminerge nevrale nettverket, som en mulig mekanisme for utvikling av autisme. Multipollutant analysis, using elastic net, demonstrated the importance of using appropriate statistical models to handle highly correlated chemicals simultaneously to reduce false-positive associations, as we observed markedly different results from the single pollutant analyses. This is important for risk assessment as single-pollutant analysis may be confounded by other chemicals co-existing in the mixture if they are also highly correlated as indicated in our analysis. Multipollutantanalyse ved bruk av elastisk net regresjon viste viktigheten av å bruke adekvate statistiske modeller som kan håndtere høyt korrelerte kjemikalier, for å redusere falskt positive assosiasjoner, da vi observerte svært forskjellige resultater i forhold til de individuelle analysene. Dette er viktig for risikovurderinger. Verken antiandrogen reseptoraktivitet (pg flutamidekv./g melk) eller arylhydrokarbon reseptoraktivitet (pg CALUX-TEQ/g lipid) var av betydning for utviklingen av kryptorkisme i henhold til funnene våre. Imidlertid var studien vår begrenset til å undersøke interaksjon med disse reseptorene, mens hormonhermende miljøgifter også kan påvirke andre prosesser (syntese, lagring, frigjøring og metabolisme), noe som krever videre studier. Men den

antiandrogene reseptoraktiviteten var over tillatt eksponering ifølge European Medicines Agency-direktivet for androgenreseptorantagonisten flutamid. Arylhydrokarbonreseptoraktivitet (pg TEQ/g lipid) var også over tolerabelt daglig inntak for dioksiner og dioksinlignende stoffer satt av WHO, hos de norske barna mens de ble eksklusivt ammet. Dette bør tas i betraktning i den fremtidige nytte-risikovurderingen av morsmelk. Dagens nivåer av dioksin og dl-PCB uttrykt som AhR-aktivitet (pg TEQ /g lipid) i morsmelk viser en fallende trend i Norge fra 1986 til 2005, selv om nivået fortsatt holder seg over tillatt eksponeringsgrense.

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2. ***Anti-androgenic compounds in breast milk and cryptorchidism among Norwegian boys in the HUMIS birth cohort.*** *(Science of The Total Environment. 2022. Volume 803, 106815)*
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4. ***Early-life exposure to endocrine disrupting chemicals and autism spectrum disorder: a multi-pollutant analysis of Norwegian birth cohort and evaluation of developmental neurotoxicity in zebrafish embryos and larvae.*** *(Submitted)*
   Anteneh A. Desalegn, Wietske van der Ent, Virissa Lenters, Nina Iszatt, Hein Stigum, Jan Ludvig Lyche, Karolina J. Kirstein-Smardzewska, Gezime Seferi, Camila Vicencio, & Merete Eggesbø

Abbreviations

ADHD	Attention deficit hyperactivity disorder
AhR	Aryl hydrocarbon receptor
Anti-AR	Anti-androgenic receptor
ARNT	AhR nuclear translocator
ASD	Autism spectrum disorder
CALUX	Chemical Activated LUciferase gene eXpression
CPP	The U.S. Collaborative Perinatal Project
DAGs	Directed acyclic graphs
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
Dl-	Dioxin-like compounds
EDCs	Endocrine disrupting chemicals
EDI	Estimated daily intake
EMA	The Early Markers for Autism study
EPA	Environmental Protection Agency
ERs	Oestrogen receptors
GR	Glucocorticoid receptor
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HOME	Health Outcomes and Measures of the Environment study
HUMIS	The Norwegian Human Milk Study
INSL3	Insulin-like hormone 3
IQR	Interquartile range
LOD/Q	Limit of detection/quantification
MBRN	Medical Birth Registry of Norway
MI	Multiple imputation
MoBA	The Norwegian Mother, Father and Child Cohort Study
(N)dl	(Non)-dioxin-like

OCPs	Organochlorine pesticides
(P)BDE	(poly)brominated diphenyl ether
PCB	Polychlorinated biphenyl
PCDDs	2,3,7,8-substituted polychlorinated dibenzo-p-dioxins
PCDFs	2,3,7,8-substituted polychlorinated dibenzofurans
PDE	Permitted daily exposure
PFASs	Poly- and perfluoroalkyl substances
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
POPs	Persistent Organic Pollutants
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDI	Tolerable Daily Intake
TDS	Testicular dysgenesis syndrome
TEQ	TCDD toxic equivalent
WHO	World Health Organization
β-HCH	Beta- hexachlorocyclohexane

1. Introduction

1.1. Endocrine disrupting chemicals

Exposure to the ever-increasing number of chemicals in the environment has become unavoidable, and we are exposed to thousands of chemicals every day. According to the World Health Organisation (WHO) about 800 chemicals have known or suspected potential to interfere with the endocrine system, however, most have not been studied for their endocrine disrupting properties in animals or humans (WHO/UNEP, 2013).The endocrine system consists of several endocrine glands located throughout the body (Figure 1). Endocrine glands release hormones mainly into the bloodstream where specific receptors in different organs and tissues bind the hormones and respond. The hormones regulate various physiologic processes in the body such as homeostatic functions, reproduction, and development (Gore et al., 2015).

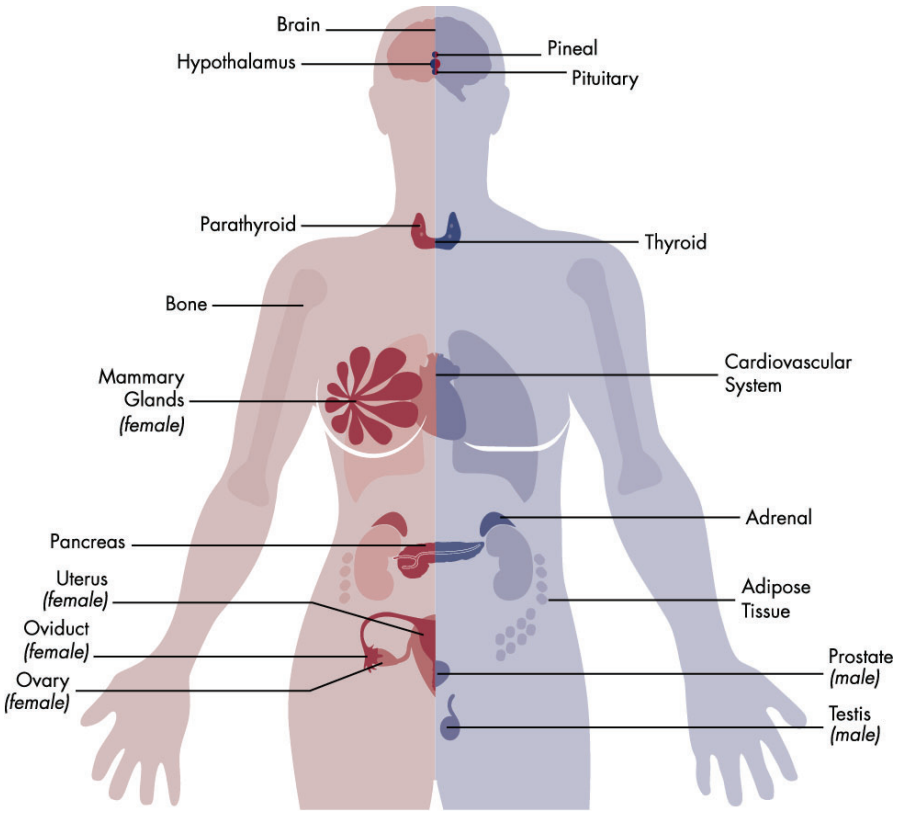

Figure 1. Diagram of the body's endocrine glands in females (left) and males (right). Reproduced with permission from Gore et al., (2015) Copyright © 2015 by the Endocrine Society.

The physiological actions of hormones are mediated by the receptors that they bind to. Hormone receptors exhibit non-linear dose-response relationships. The response increases in a logarithmic

manner as the concentration of hormone increases until a saturation point is reached. This means that a small change in the concentration of hormones will have a larger effect at the lower end of the curve than a similar change near the saturation point of the curve (Figure 2A). The potency of a hormone depends on the number of receptors it binds to. The more receptors a hormone binds to, the more potent it is, i.e., a lower concentration of the hormone will produce the same response (Figure 2B). Hormone receptors may also exhibit nonmonotonic dose-response relationships, specifically an "inverted U" shape dose-response, where low doses increase the response, but overstimulation at high doses down-regulate the receptor and decrease the response (Figure 2C) (Zoeller et al., 2012).

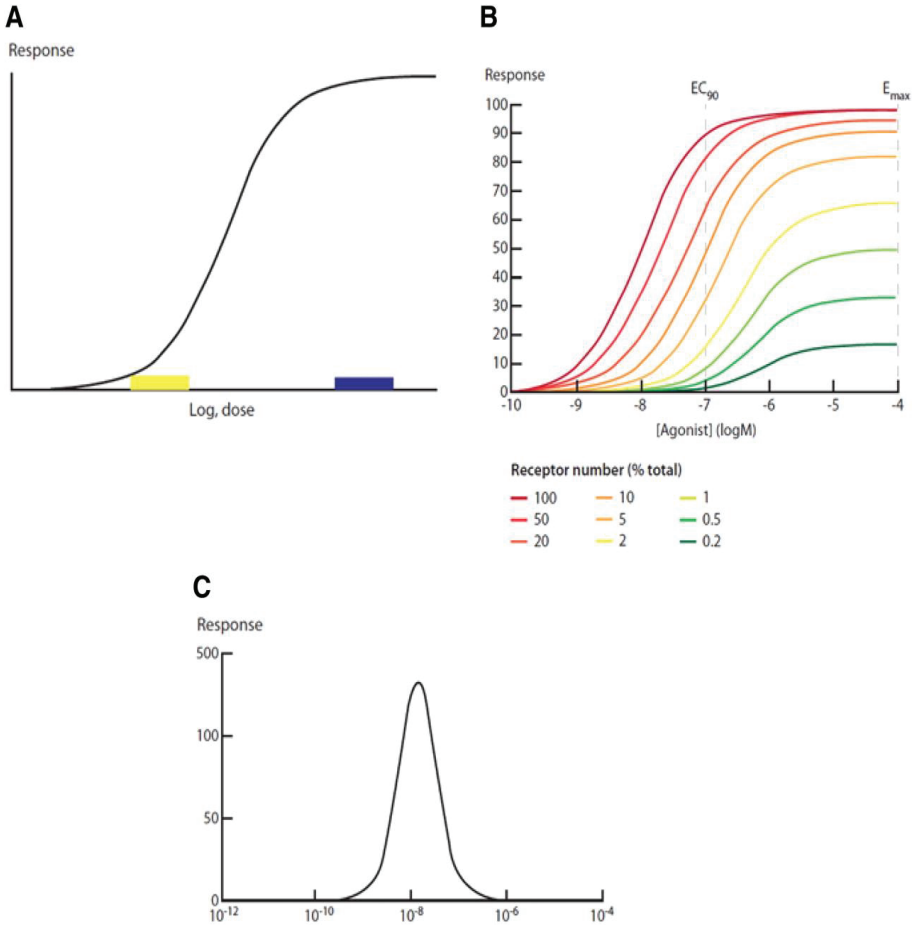

Figure 2. Dose-response curve for hormones. ***(A)*** *sigmoidal dose-response curve for hormones* ***(B)*** *The dose response to the hormone depends on receptor concentration.* ***(C)*** *Nonmonotonic dose response curve. Reproduced with permission from* Zoeller et al.*, (2012) Copyright © 2012 by The Endocrine Society.*

Endocrine Disrupting Chemicals (EDCs) are defined slightly differently across various organizations or agencies worldwide. The definitions from EU and WHO mainly focus on the outcome (adverse health effects) while the U.S. Environmental Protection Agency (EPA) and the U.S. Endocrine Society's definition are based on the mode of action (ability to interfere with hormone action).

1. European Union defines EDCs as "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. A potential endocrine disruptor is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism." (European Commission, 1996).
2. World Health Organization**,** the International Programme on Chemical Safety (IPCS), defines EDC as: " exogenous substance or mixture that alters the normal function(s) of endocrine systems and consequently cause adverse health effects in an intact organism, or its progeny, or (sub) populations." (IPCS, 2002).
3. United States Environmental Protection Agency (EPA) defines EDCs as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process."(Kavlock et al., 1996).
4. The Endocrine Society (U.S.) defines EDC as "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action." (Zoeller et al., 2012).

The actions of EDCs are mainly mediated by nuclear hormone receptors, particularly estrogen receptors (ERs), androgen receptors (AR), thyroid hormone receptors (ThR), progesterone receptor (PR), glucocorticoid receptor (GR), peroxisome proliferator-activated receptors (PPARs), the orphan receptor aryl hydrocarbon receptor (AhR), and membrane steroid hormone receptors. EDCs can either directly stimulate or inhibit these receptors or indirectly act by interfering with intracellular signalling by the receptors and their coregulatory elements. Furthermore, EDCs can also act beyond receptors to impact hormone synthesis (steroidogenesis), release, transport, distribution, metabolism, and clearance of hormones (Gore et al., 2015, La Merrill et al., 2020).

Figure 3 below shows 10 potential sites of actions for EDCs: 1) interaction with or activating hormone receptors, 2) antagonizing hormone receptors, 3) alteration of hormone receptor

expression, 4) alteration of signal transduction (including changes in protein or RNA expression, post-translational modifications and/or ion flux) in hormone-responsive cells, 5) induction of epigenetic modifications in hormone-producing or hormone-responsive cells, 6) alteration of hormone synthesis, 7) alteration of hormone transport across cell membranes, 8) alteration of hormone distribution or circulating hormone levels, 9) alteration of hormone metabolism or clearance, 10) alteration of the fate of hormone-producing or hormone-responsive cells (La Merrill et al., 2020).

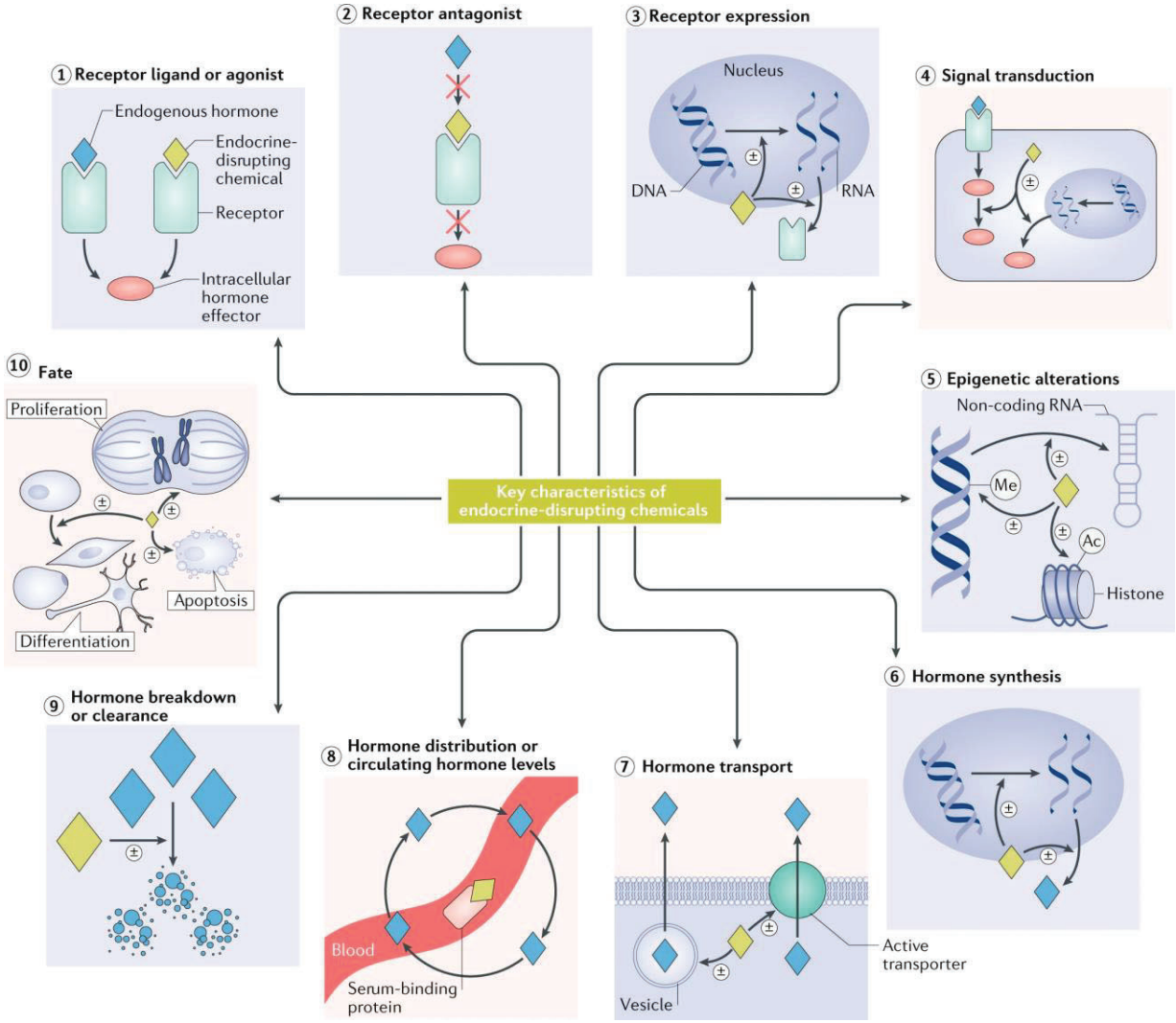

Figure 3. Potential key sites actions for endocrine disrupting chemicals. The ± symbol indicates that an EDC can increase or decrease processes and effects. Ac, acetyl group; Me, methyl group. Reproduced from La Merrill et al., (2020) CC BY 4.0

Over the past decades, there has been an increasing global trend in endocrine-related disorders in humans and wildlife worldwide attributed to exposure to EDCs such as low birth weight, preterm birth, genital malformation in baby boys (cryptorchidism, hypospadias), attention deficit hyperactivity disorder, lower intelligence quotient (IQ), pulmonary disorders (asthma, COPD), early puberty, infertility, obesity, type 2 diabetes, cardiovascular disease, leukemia, breast cancer, testicular cancer, and thyroid disorders (Bergman et al., 2013, Gore et al., 2014).

Humans are exposed to a cocktail of EDCs from multiple sources with life-long exposure (Fig 4). Exposure can occur via a variety of routes, including oral (consumption of food and water), inhalation of contaminated air or dust, dermal (skin contact), placental (mother to foetus), or via lactation (breastfeeding) (Figure 4) (Longnecker et al., 1999, Sharpe and Irvine, 2004, van den Berg et al., 2017b).

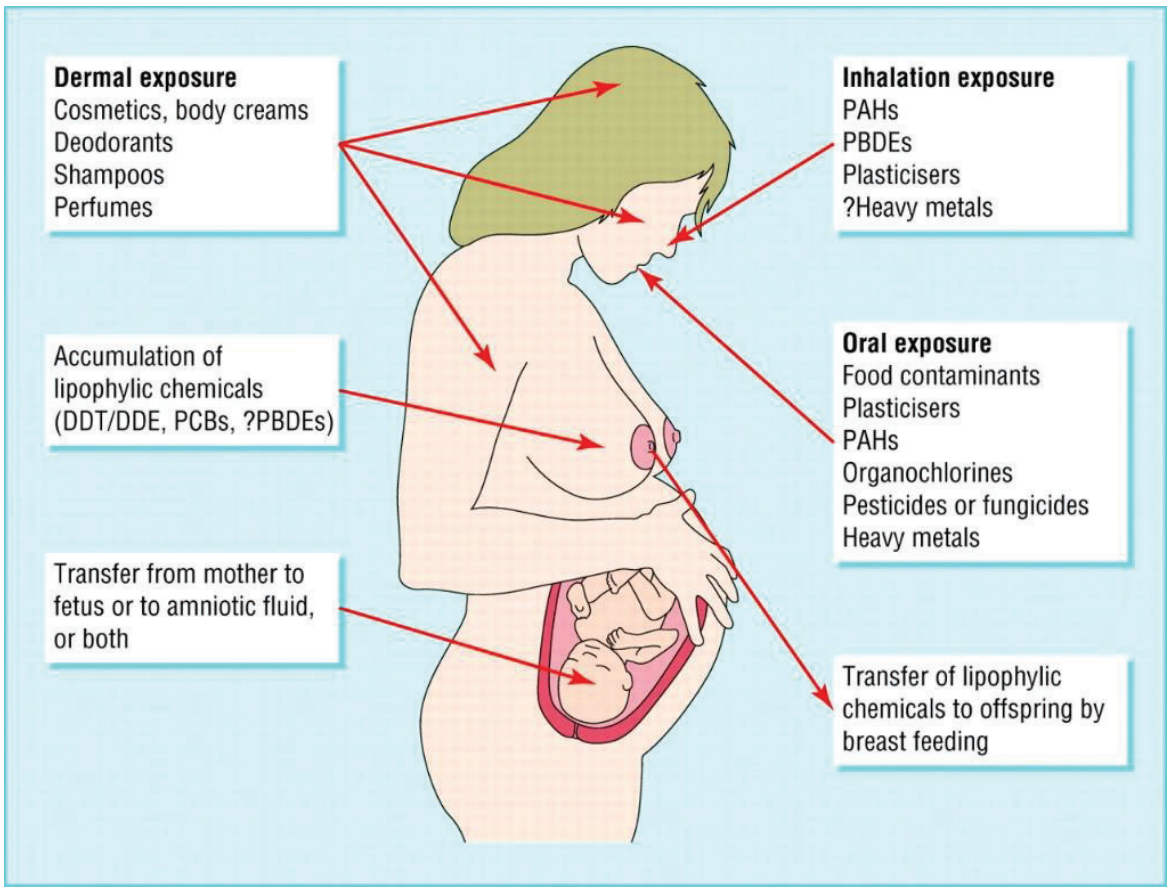

Figure 4. Routes of human exposure to some common environmental chemicals. Reproduced with permission from Sharpe and Irvine (2004) © 2004, BMJ Publishing Group Ltd.

A common source is contaminated food or water, which can contain pesticide residues like DDT, glyphosate, chlorpyrifos, persistent compounds like perfluoroalkyl and polyfluoroalkyl substances (PFAS), polychlorinated biphenyls (PCBs) and dioxins, or metals such as arsenic and mercury, while food packaging commonly contains bisphenol A (BPA), PFAS and phthalates. The home and indoor environment is another source: textile coatings and non-stick pans (PFAS), home furniture, electronics and building materials (flame retardants, PFAS, phthalates, PCBs), paints (lead), house dust (flame retardants, pesticides), drinking water (arsenic, lead, perchlorate, PFAS), personal care products and household cleaners (parabens, phthalates, triclosan), and even children's toys (phthalates) contain chemical mixtures. Furthermore, industrial solvents, lubricants and their waste, and air pollution (PCBs, dioxins, nitrogen dioxide, and particulate matter) can be prevalent in the outdoor environment (Bergman et al., 2013, Gore et al., 2015).

Some of the EDCs are also persistent organic pollutants (POPs) that remain intact for long periods of time in the environment, get widely distributed throughout the environment, bioaccumulate within individuals, become more concentrated (biomagnify) as they pass from one species to the next up the food chain, and are toxic to both humans and wildlife (Stockholm Convention, 2019). Fat serves as an important reservoir for persistent POPs, and the body burden of persistent EDCs/POPs reflects not only current exposure, but also past exposure from maternal transfer and exposure accumulated during one's lifetime. Furthermore, persistent EDCs affecting germ cells may be inherited by future generations long after they are eliminated from the body (Sharpe and Irvine, 2004, Gore et al., 2014, Gore et al., 2015).

The Stockholm Convention was adopted on 22 May 2001 and ratified by over 152 countries when the convention entered into force on 17 May 2004. Under the Stockholm Convention, countries agreed to protect human health and the environment from POPs by reducing or eliminating the releases into the environment (Stockholm Convention, 2019).

Table 1 below summarizes chemicals targeted by the Stockholm Convention. Countries party to the convention are required to take measures to eliminate and restrict the production and use of chemicals under Annex A and B, respectively, unless they register for specific exemptions.

Table 1. Stockholm Convention listings of persistent organic pollutants.

List of POPs	**Annex A** (Elimination)	**Annex B** (Restriction)	**Annex C** (Unintentional production)
The initial 12 POPs aka "dirty dozen" in Stockholm Convention (2004)	●Aldrin ●Chlordane ●Dieldrin ●Endrin ●Heptachlor ●Mirex ●Toxaphene ▲PCB ●/▲ Hexachlorobenzene (HCB)	●Dichlorodiphenyltrichloroethane (DDT)	■HCB ■Polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) ■PCB
Newly added POPs since 2009	● α-HCH ● β-HCH ● γ-HCH (lindane)[2] ● Chlordecone ▲Decabromodiphenyl ether (cDecaBDE)[1, 2] ● Dicofol ● Endosulfan[1, 2] ▲Hexabromobiphenyl ▲Hexabromocyclododecane [1,2] ▲Hexabromodiphenyl ether[2] and heptabromodiphenyl ether[2] ▲■Hexachlorobutadiene ●▲■ Pentachlorobenzene (PeCB) ●Pentachlorophenol[1,2] ▲ Perfluorooctanoate (PFOA)[1,2] ▲■Polychlorinated naphthalenes[1, 2] ▲Tetrabromodiphenyl ether and pentabromodiphenyl ether [2] ▲Short-chain chlorinated paraffins (SCCPs)[1, 2]	▲ Perfluorooctane sulfonate (PFOS)[1,2]	▲■Hexachlorobutadiene (HCBD) ● Pentachlorophenol ▲■Polychlorinated naphthalenes[1,2] ●▲■Pentachlorobenzene (PeCB)
Chemicals under review as of September 2021	Dechlorane Plus, Methoxychlor, PFHxS, UV-328		

Note: ● Pesticide ▲Industrial Chemical ■ Unintentional Production [1]Specific exemptions for production [2]Acceptable purposes for use

There is a moderate to a high correlation (Spearman Rho > 0.35) between maternal and child concentrations of POPs measured in blood across European countries. Children's concentrations significantly exceeded their mothers' in cohorts from the UK and Norway for hexachlorobenzene

(HCB) based on data from the Early-life Exposome project (HELIX). In addition, most of the mothers and their children exceeded the threshold level considered safe for mercury, perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) established by The Human Biomonitoring Commission of the German Federal Environment Agency (Haug et al., 2018).

Human milk surveys have been recommended as a biomonitoring tool to identify global temporal trends and spatial distributions of POPs, and to evaluate the effectiveness of the Stockholm Convention (van den Berg et al., 2017a). Some of the advantages of using human milk include easy availability, no requirement for trained personnel, cheap, non-invasive collection procedure, ability to collect large volumes, a good reflection of the body burden for persistent chemicals, and good correlation with blood and tissue concentrations (Todaka et al., 2010).

A global survey of POPs such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), PCBs, dichlorodiphenyltrichloroethane (DDT) by WHO indicates downward temporal trend for PCDDs, PCDFs and PCBs, and regional difference in the levels of POPs. The highest levels of DDTs were found in less industrialized countries while PCB levels were highest in East and West Europe (Figure 5). Figure 6, on the other hand, shows the results from 3rd (2000-2003) and 4th (2005-2010) surveys for (A) dioxin-like (DL)-PCBs in WHO toxic equivalencies (TEQs) (pg/g lipid) and (B) sum of DDT (μg/kg), in pooled human milk samples from different countries. The levels of PCDDs and PCDFs were highest in India and some European and African countries. The concentration of dl-PCBs exceeded the safety level in 46/52 countries surveyed including Norway (Figure 6A). DDT on the other hand was below the safety level in most of the developing and developed countries (Figure 6B).

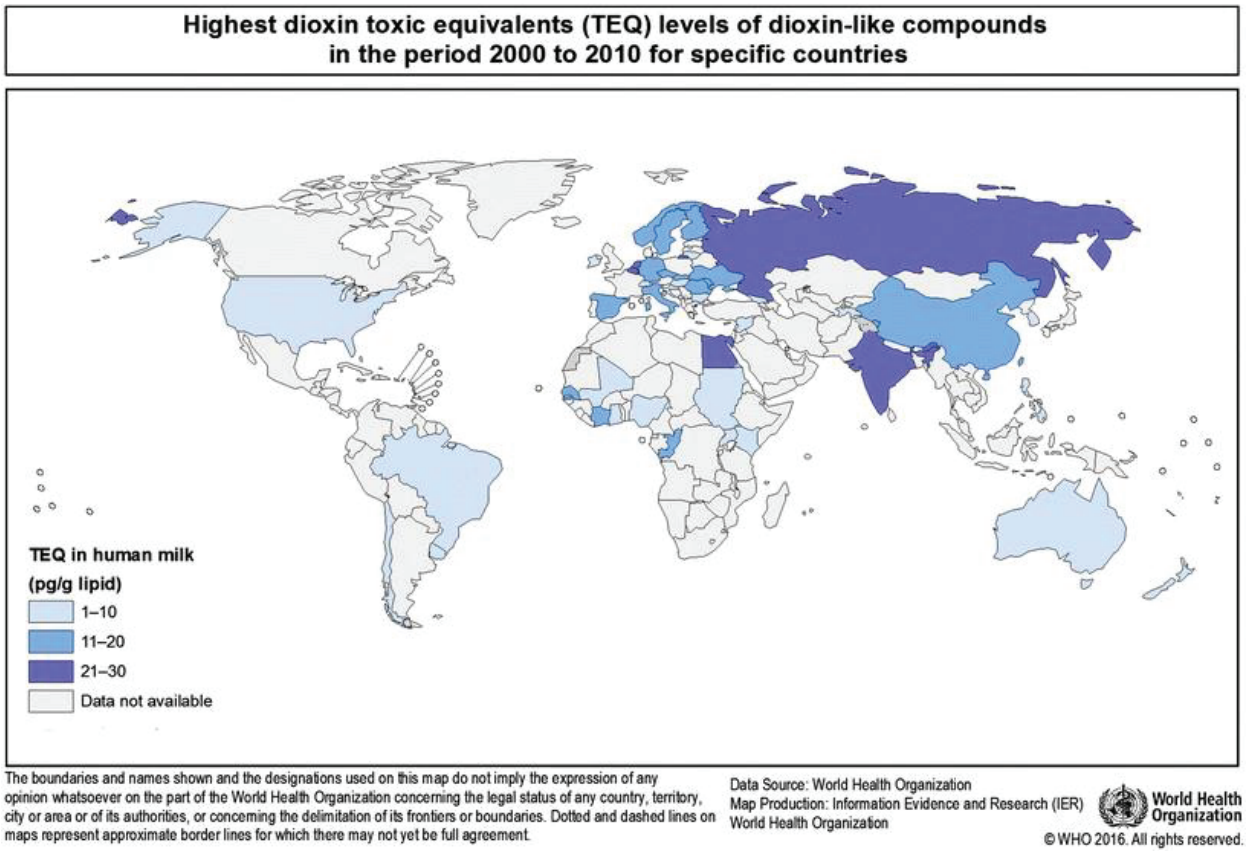

Figure 5. Participating countries in the WHO/UNEP human milk global surveys and levels of dioxin-like compounds expressed in TEQs.Reproduced from (van den Berg et al., 2017a) CC BY 4.0).

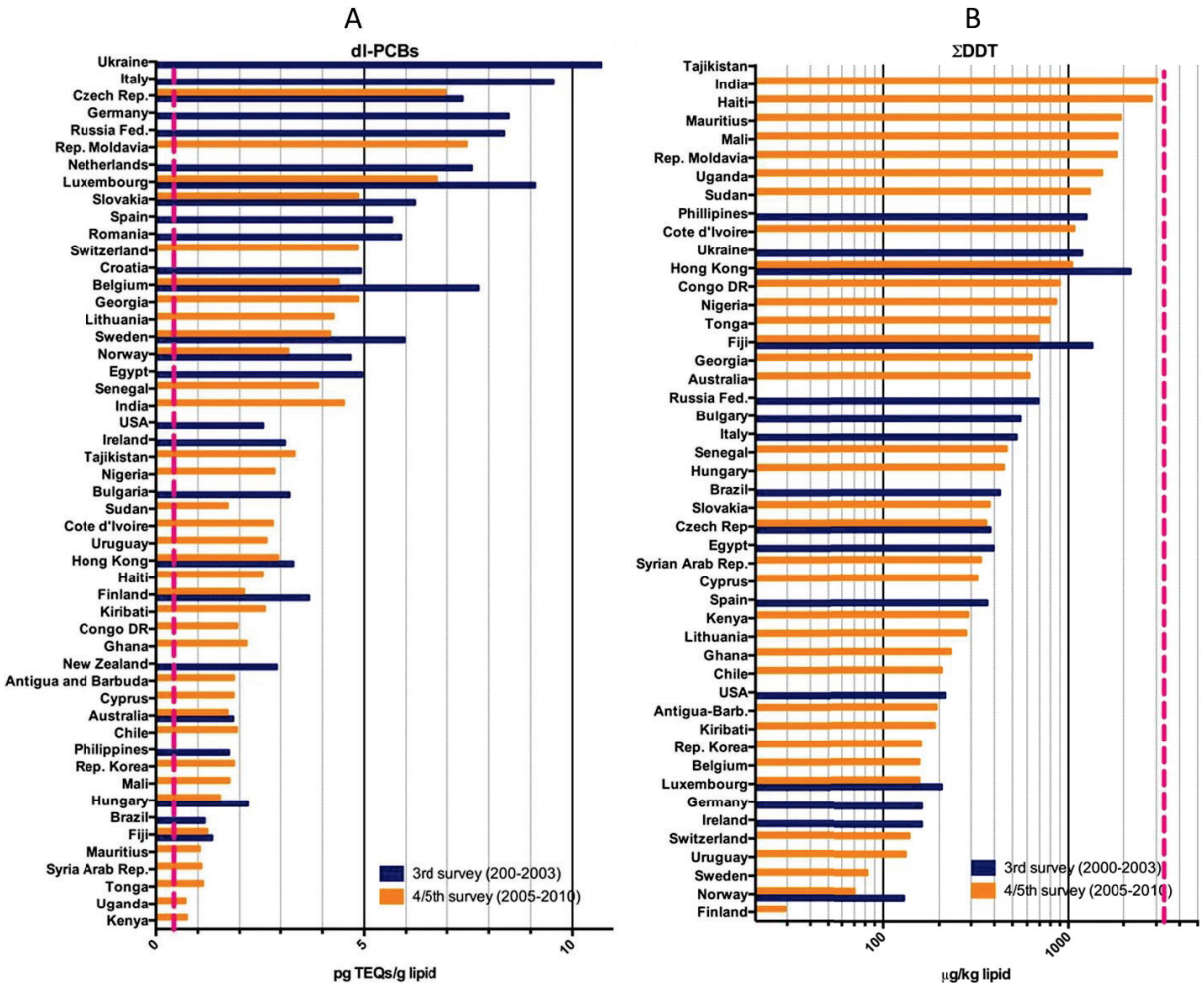

Figure 6. Results of the WHO/UNEP surveys for ***(A)*** *DL-PCBs in TEQs (pg/g lipid) and* ***(B)*** *the sum of DDT-like compounds in µg/kg lipid in pooled human milk samples from different countries.The dotted red line represents the calculated safe level of these compounds for the breastfed infant. Reproduced from (van den Berg et al., 2017a) CC BY 4.0.*

In Norway, apart from levels of brominated flame retardants (PBDEs), the levels of dioxins and dl-PCBs, other PCBs, and organochlorine pesticides (DDT, HCH, and HCB) have decreased by about 70 to 90 percent from 1986 to 2005 (Figure 7A-F) (NIPH, 2016).

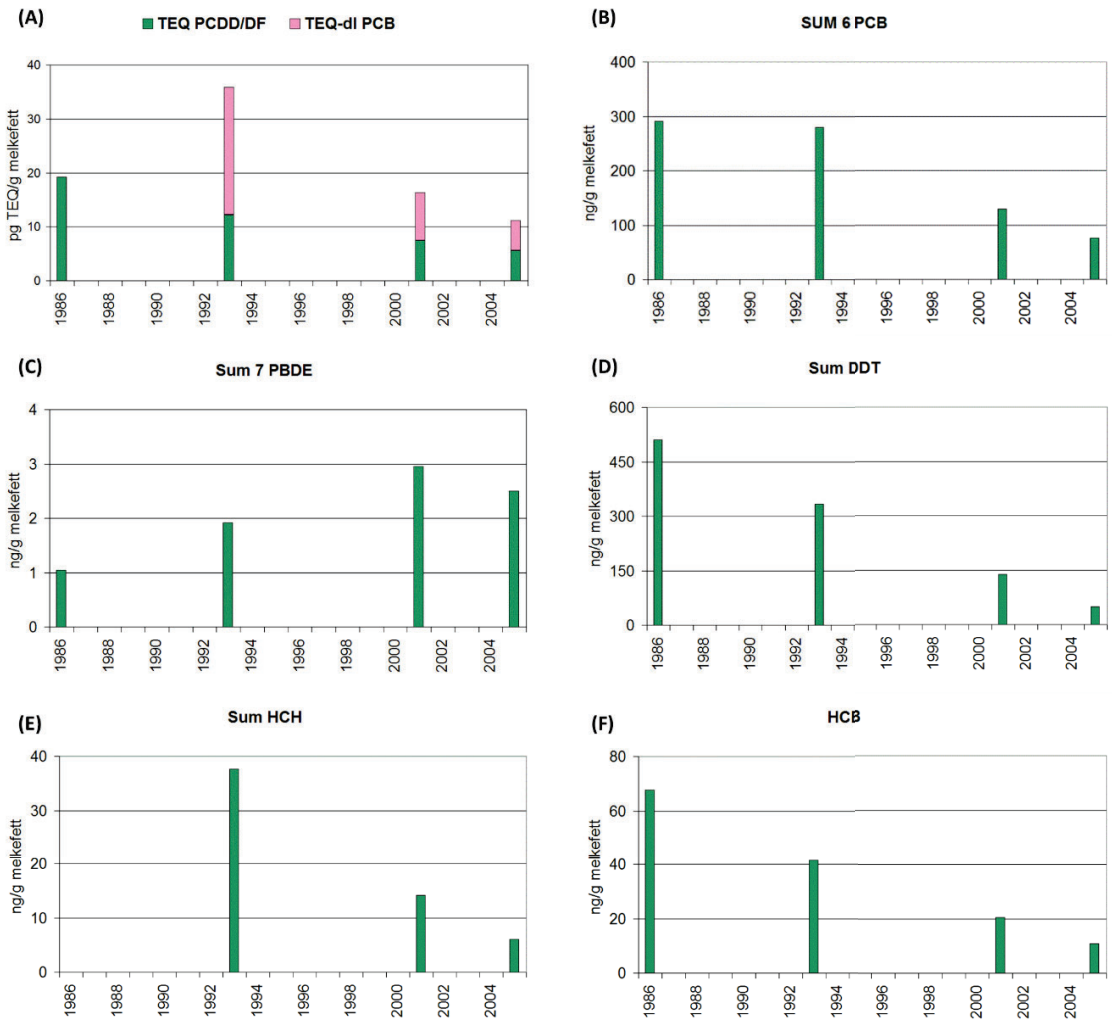

Figure 7. The concentration of dioxins and dl-PCBs, other PCBs, PBDEs, and organochlorine pesticides in human milk 1986-2005. Reproduced from NIPH factsheet (NIPH, 2016).

The European Chemical Agency (ECHA) estimated only about 500 chemicals out of the 100,000 chemicals on market by 2019 as sufficiently regulated. Most (approximately 70,000) have hardly any information about their hazards, and close to 10,000-20,000 chemicals have limited characterization for a subset of their hazard (European Environment Agency, 2019).

The EU started developing policies towards regulating EDCs and promoting scientific research after the EU Commission's Community Strategy of 1999. Currently, there are more than 40 pieces of legislation regulating chemicals in the EU. Some of the legislation that is relevant for regulation of EDCs includes: Registration, Evaluation, Authorisation and Restriction of

Chemicals (REACH), Pesticides or Plant Protection Products (PPP), Biocidal Products (BP), and Cosmetics regulation by SCCS. The commission also contribute to the work of Organisation for Economic Co-operation and Development (OECD) to develop standard international test guidelines for EDCs. Furthermore, the EU has funded several projects to identify, fill gaps, improve, and develop test guidelines on EDCs (European Commission, 2018). In fact, the PROTECTED project (PROTECTion against Endocrine Disruptors) that funded this Ph.D. also received from Horizon 2020 research and funding program.

Identification of EDCs is based on the scientific criteria described in the guidance document (EU) No 528/2012 and (EC) No 1107/2009 (European Chemical Agency et al., 2018). The status of EDCs identified (list I) and under evaluation (list II) under EU legislation as well as list of proposed potential EDCs (list III) is updated bi-annually by the national authorities of Belgium, Denmark, France, The Netherlands, and Sweden (https://edlists.org/the-ed-lists).

The burden of disease and dysfunction across the life course from exposure to fifteen EDCs, restricted to those with the highest probability of causation in the European Union, has been estimated to cost hundreds of billions of euros per year (Trasande et al., 2016). Moreover, the EDCs studied so far cover the tip of the iceberg and do not include new emerging EDCs.

1.2. Adverse developmental outcomes in children

Hormones play a critical role in cell differentiation during embryonic development and differentiation of tissues. Hormones also play different role (maintaining normal function) once the tissues are fully developed. Exposure to hormones or EDCs during sensitive windows of development can result in a permanent change in children or may first become visible decades later. Developmental origins of health and disease (DOHaD) hypothesis describes developmental programming whereby exposure to an adverse environment during the prenatal period can increase the risk of disease later in life such as coronary heart disease, type 2 diabetes, stroke, and hypertension (Barker et al., 2002).

The sensitive window can vary for each specific tissue's period of formation and development. Some tissues can have a longer sensitive window of exposure to EDCs if the tissue continues developing after birth into infancy, early childhood, and puberty or adulthood such as in the brain and the reproductive system (Bergman et al., 2013). Figure 8 below shows the sensitive periods

in human prenatal development. The embryonic period (shown in purple) is highly sensitive, while in general, the foetal period is less sensitive (shown in green). Tissues like the brain have a very long highly sensitive period spanning the embryonic, the foetal period as well as childhood and adolescence. Other organs such as genitalia are highly sensitive later in the embryonic period and then less sensitive for much of the foetal period (Figure 8).

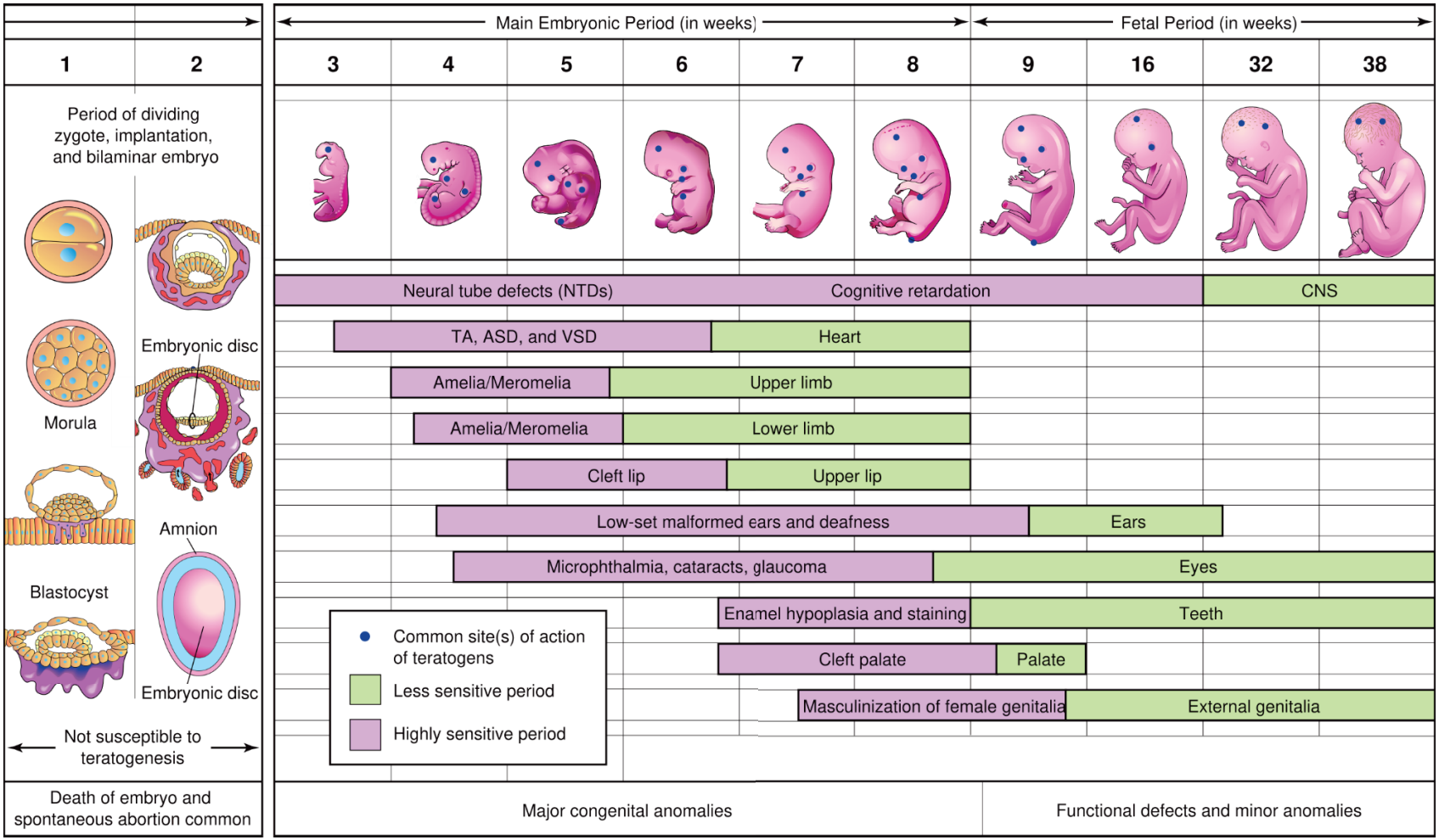

Figure 8. Sensitive periods in human prenatal development. Reproduced with permission from Moore, K. L., Persaud, T. V. N., & Torchia, M. G. (2015). Before We are Born E-Book: Essentials of Embryology and Birth Defects.

Evidence from experimental studies and epidemiologic data support a link between early-life exposure to EDCs and various endocrine-related adverse outcomes, with the impact being highest for exposure during sensitive windows of development such as prenatal development, early childhood and puberty (Bergman et al., 2013, Gore et al., 2014, Gore et al., 2015). Just like endogenous hormones, low doses of EDCs may be enough to elicit a response, can exhibit non-linear dose-response relationships, tissue-specific effects, life stage-specific effects, and permanent developmental effects. However, unlike endogenous hormones, lipophilic persistent EDCs can bioaccumulate in the body (Bergman et al., 2013).

Male reproductive disorders

The increasing trend in male reproductive disorders (cryptorchidism, hypospadias, reduced semen quality, subfertility, and testicular cancer) in developed countries has been attributed to exposure to environmental exposures related to modern lifestyle. Skakkebæk et al (2001) was the first to introduce the concept of testicular dysgenesis syndrome (TDS) (Figure 9). According to this hypothesis, the symptoms of TDS (cryptorchidism, hypospadias, reduced semen quality, subfertility, and testicular cancer) share several risk factors, and have their origin in foetal development (Virtanen et al., 2005). The hypothesis proposes that TDS results not only from genetic mutations, but most often from exposure to environmental chemicals, lifestyle factors, and epigenetic factors (Toppari et al., 2010, Juul et al., 2014, Skakkebaek et al., 2016).

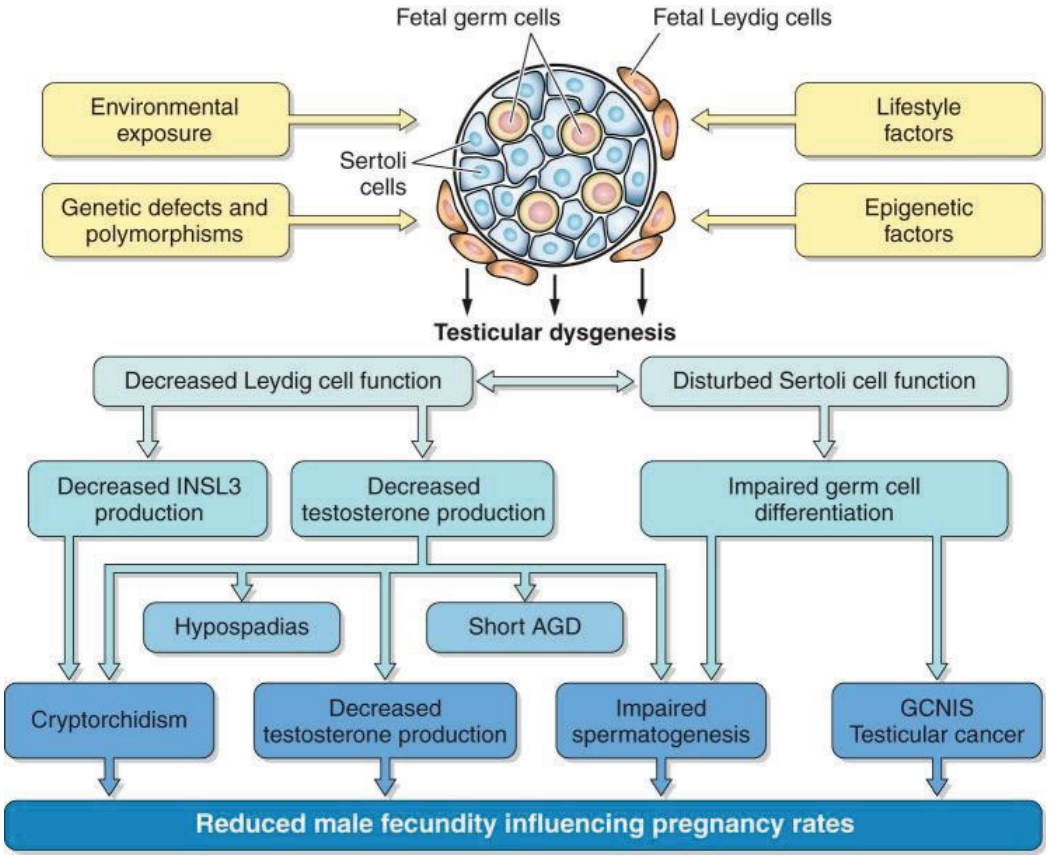

Figure 9. Testicular dysgenesis syndrome (TDS) hypothesis and conditions that might be linked to it: poor spermatogenesis, testicular cancer, hypospadias, cryptorchidism, and short anogenital distance (AGD). Reproduced with permission from Skakkebaek et al. (2016) © 2016 the American Physiological Society. Physiol Rev. 2016 Jan; 96(1): 55–97.

In experimental animal studies, the symptoms of TDS have been induced by exposure to anti-androgenic phthalates in rodents (Gray et al., 2000), and prenatal exposure to estrogenic diethylstilbestrol in mice (McLachlan et al., 1975). However, direct evidence of EDCs causing TDS symptoms in humans following deliberate prenatal EDC exposure cannot be established due to ethical reasons. Evidence from epidemiologic studies is limited and often inconsistent (Sweeney et al., 2015).

1.2.1. Cryptorchidism

Cryptorchidism is one of the most common urogenital abnormalities in newborn males. It represents a failure of either one or both of the testes to fully descend to a normal position at the base of the scrotum (Gurney et al., 2017). The prevalence of cryptorchidism appears to vary depending on the age of diagnosis, varying from birth to 1 year of age, and the diagnostic criteria used to assess the spectrum of disease severity. Overall, 1-9% of full-term baby boys are born with cryptorchidic testis while the prevalence estimates are higher in preterm and neonates (1-45%) (Sijstermans et al., 2008, Virtanen et al., 2007). The spontaneous descent of testes occurs during the first 3 months of life in approximately half of the cases, and during the first year of life for most of the cases following the natural course (Berkowitz et al., 1993, Gurney et al., 2017). This may partly explain the difference in reported prevalence estimates. Furthermore, discrepancies may also be due to differences in the inclusion criteria in relation to the types of cryptorchidism as different types of cryptorchidism may not share a similar etiology (Rajpert-De Meyts et al., 2016).

Temporal and geographical variations in the prevalence of cryptorchidism and hypospadias have also been observed (Figure 10) (Toppari et al., 2001, Skakkebaek et al., 2016). Some studies suggested an increasing trend for cryptorchidism, for example, in the UK (2.7%-4.1%) and Denmark (1.8-8.4%) since the 1950s, correlating with an increase in persistent chemicals in the environment (Juul et al., 2014). Other regions reported a stable prevalence such as eastern Canada since 1988 (Lane et al., 2017). Similarly, in Norway, the prevalence of cryptorchidism stabilized around 0.3% in the early 1970s based on the Medical Birth Registry of Norway (MBRN), but underreporting cannot be ruled out since cases only include diagnoses made within seven days of birth (Aschim et al., 2006, Brantsæter et al., 2016).

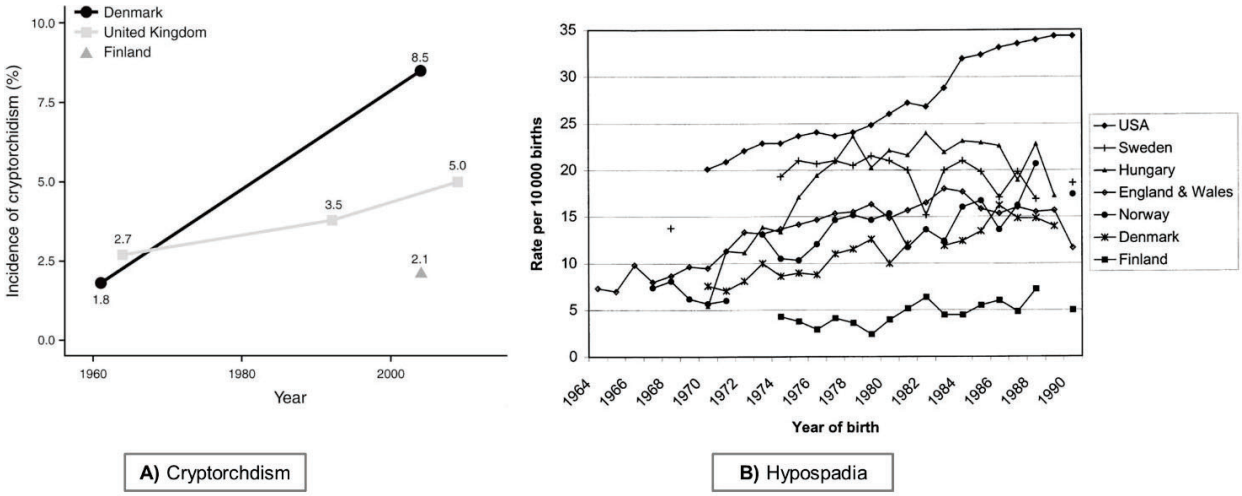

Figure 10. A) Incidence of cryptorchidism based on prospective clinical studies from the 1950s to 2000s (reproduced with permission from Skakkebaek et al., 2016. © 2016 the American Physiological Society). B) Incidence of hypospadias from 1960s to 1990s (reproduced with permission from Toppari et al., (2001) © Oxford University Press 2001)

While the exact cause of cryptorchidism in most cases remains unknown, epidemiologic studies have identified various risk factors associated with cryptorchidism. Established risk factors include being small for gestational age (low birth weight), preterm birth, family history of cryptorchidism, and genetics, and to a lesser extent maternal age, alcohol or caffeine consumption during pregnancy, pregnancy medications, recreational drug use, analgesics, parity, exposure to high levels of endogenous hormones and environmental endocrine disrupting chemicals (EDCs) (Gurney et al., 2017). Complications in pregnancy such as preeclampsia, gestational diabetes, and placental insufficiency have also been associated with cryptorchidism. Gestational diabetes, for example, potentially acts by decreasing maternal sex hormone-binding globulin concentration and causing fetal hyperinsulinemia that leads to fetal estrogen-androgen imbalance. Increased prevalence of cryptorchidism was reported in areas with high agricultural activity (Rajpert-De Meyts et al., 2016).

Cryptorchidism is one of the few established risk factors for testicular cancer. A meta-analysis by Lip et al.(2013) demonstrated that boys with cryptorchidism were three times more likely to develop testicular cancer in later life. In addition, cryptorchidism is strongly associated with subfertility and infertility (Gurney et al., 2017). Paternity studies show that the risk of infertility is doubled in boys with unilateral cryptorchidism compared to controls (Lee et al., 1996).

Hormonal and surgical treatment modalities exist for cryptorchidism. Cryptorchidism should be treated early to prevent further damage to spermatogenesis. The Nordic consensus for the treatment of cryptorchidism is surgery (orchiopexy) over hormonal treatment (not recommended) that should be performed between 6 and 12 months of age, or later upon diagnosis (Martin Ritzén et al., 2007).

Both genetic and hormonal factors play a vital role in the pathogenesis of cryptorchidism (Virtanen and Toppari, 2008). Genetic defects associated with cryptorchidism include androgen-receptor mutations, polymorphisms of the CAG repeat of the androgen receptor, 5α-reductase deficiency, insulin-like hormone 3 (INSL3) gene mutations, and polymorphisms of the estrogen receptor alpha gene. However, the role of endocrine or hormonal disorders involving hypothalamic-pituitary-gonadal axis and testosterone biosynthesis are likely more commonly involved in the etiology of cryptorchidism than genetic factors (Rajpert-De Meyts et al., 2016).

By the end of fetal week 9, interstitial cells in the testis region differentiate into Leydig cells. Leydig cells secrete testosterone and INSL3, which play an important role in testicular descent. Testicular descent occurs in two distinct phases (Figure 11), between 8-15 weeks and 25-35 weeks of gestation. INSL3 regulates the first phase, the transabdominal phase, where the testis is anchored at the internal inguinal ring by enlargement of the gubernaculum. The second phase of descent, the inguinoscrotal phase, is highly dependent on androgens. This phase is often compromised by endocrine disruption of the pituitary-gonadal axis or steroidogenesis and androgen-receptor mutations. Moreover, calcitonin gene-related peptides (CGRP), a neurotransmitter of the genitofemoral nerve plays a role in the inguinoscrotal phase of descent even though it is less well established in humans than in rodents (Hutson et al., 2015, Rajpert-De Meyts et al., 2016).

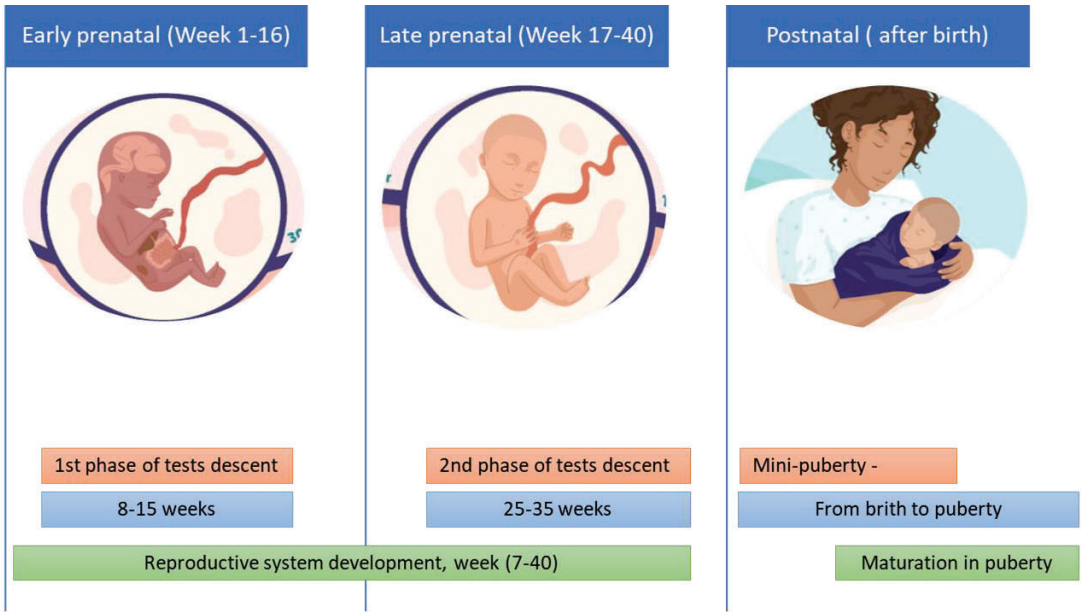

Figure 11. Potential sensitive windows of exposure affecting testicular descent.

Experimental studies support the hypothesis that disruption in the secretion or action of INSL3 or testosterone may affect testicular descent and lead to cryptorchidism (Fénichel et al., 2019). Reduced androgen to estrogen ratio, or interference with androgen and INSL3 secretion during prenatal life, are mechanisms causing cryptorchidism in animals (Virtanen and Adamsson, 2012). EDCs with estrogenic effects (bisphenol A, DDT, PCBs, phytoestrogens, and phenols), and anti-androgenic effects (DDE, phthalate, and vinclozolin) caused cryptorchidism in animal studies through some of these mechanisms (Skakkebæk, 2002, Virtanen and Adamsson, 2012).

In humans, epidemiological findings also suggest a link between EDC exposure and cryptorchidism. For example, prenatal exposure to diethylstilbestrol (DES) has been linked to cryptorchidism risk. Furthermore, several pesticides have been associated with cryptorchidism. However, the evidence in humans for a direct link between EDCs and cryptorchidism is still limited, or inconsistent (Bergman et al., 2013, Lamb et al., 2014). Yet, the estimated annual cost of male reproductive disorders from exposure to EDCs with sufficient epidemiological data is nearly €15 billion (Euro) per year in the EU (Hauser et al., 2015). Table 2 below summarizes the available studies, and these are discussed in detail in the discussion in relation to the findings from the studies in this thesis.

Table 2. Summary of studies on EDCs exposure and cryptorchidism risk

Country, study year	**Study design**	**Participants**	**Matrix**	**Exposure**	**Outcome**	**Result**	**Ref.**
Akwesasne, North America (1995-2000)	Cross-sectional study	703 adult Mohawks n= 257 men n= 436 women	Serum (fasting)	PCBs Chlorinated pesticides	Testosterone level	Significant inverse association between serum testosterone and - total PCBs, and - PCB congeners (PCBs 74, 99, 153, and 206) in men	(Goncharov et al., 2009)
Sonora, Mexico (2012-2014)	Cohort	Infants (first year of life) - 82 girls - 74 boys	Blood (3rd-trimester pregnancy)	PCBs (PCB-28, 74, 118, 138/158, 153, 170, 180) DDT	Anogenital distance	Inverse association among boys PCB 28 ($\beta = -0.005$; $p < 0.01$) PCB **74** ($\beta = -0.003$; $p = 0.01$) PCB 170 ($\beta = -0.005$; $p < 0.01$)	(Loreto-Gómez et al., 2018)
Sweden (1997-200)	Case-control study	n=44 cases/ n=45 controls	Blood (Mothers)	37 congeners of PCBs most abundant in human samples	Testicular cancer	Significant increased blood concentration of total and some PCBs for mothers of men with testicular cancer PCB-**74**/PCB-**114**, PCB-9/ 105/ 118/ 153/ 138/128/167//156/170/172/174/177/178/180/182/183/187/189/190, /208/ 209	(Hardell et al., 2004)
Copenhagen, Denmark Turku, Finland (1997-2001)	Case-control study	Danish (n= 39 cases/ 168 total) Finish (n=56 cases/112 total)	Placenta	Dioxins PCBs	Cryptorchidism	No significant association for - Individual PCB congeners - Dioxin WHO-TEq levels - PCB WHO-TEq levels - Total-TEq levels	(Virtanen et al., 2012)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Result	Ref.
Tokyo, Japan (2002-20009) Pisa, Italy (2006-2007)	Case-control study	Male Japanese n=95 cryptorchidism/ n= 98 hypospadias cases/141 controls Male Italian (n=58 cryptorchidism cases/129 controls)	Genomic DNA samples	384 single-nucleotide polymorphisms (SNPs) in 15 genes	Cryptorchidsm	Genetic polymorphisms in genes involved in EDCs metabolism (AhR and ARNT2) were associated with risk of cryptorchidism and hypospadia	(Qin et al., 2012)
United States of America (1959–1965)	Case-control study	n=230 cryptorchidism/ n= 201 hypospadias/ n= 593 controls	Serum (3rd-trimester)	The sum of PCBs	Cryptorchidism Hypospadias	No association with cryptorchidism for individual or sum of PCB congeners A trend of association between hypospadias and total PCBs levels (p=0.08)	(McGlynn et al., 2009)
Nice, France	Case-control study	n= 67 cryptorchidism n= 84 male controls	Cord Blood	ΣPCB DDE	Cryptorchidism	No association in cord blood	(Brucker-Davis et al., 2008)
		n= 56 cryptorchidism cases n= 69 male controls	Colostrum			No association with individual PCB congeners, for the sum of PCBs (OR = 2.74; 95% CI; 1.15–6.53, $p < 0.022$) DDE (OR = 2.16; 95% CI, 0.94–4.98, p = 0.071).	
Flanders, Belgium (1999)	Cross-sectional study	200 adolescents (boys and girls)	Serum	PCB congeners (138, 153, and 180) dl-compounds	Pubertal development	In boys, PCB-138 & 153 had inverse association with adult stage of genital development (p = 0.04) & male pubic hair growth(p = 0.04). In girls, dioxin positively increased not reaching the adult stage of breast development by 2.3 (p = 0.02).	(Den Hond et al., 2002)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Result	Ref.
Flanders, Belgium (2001-2006)	Cross-sectional study	1679 adolescents aged between 14 and 15 years	Blood and Urine	HCB DDE PCB congeners (138, 153 and 180)	Pubertal development Genital and pubic hair development	Significant positive association for exposure to HCB, DDE and the sum of PCB138, 153 and 180. - negative relationship for HCB and the occurrence of gynecomastia. In girls, higher serum PCB levels were significantly associated with a delay in menarche	(Den Hond et al., 2011)
The Faroe Islands Birth cohort (1986-1988)	Cohort	196 boys at 14 years of age	Umbilical cord	PCBs	Spermaturia and serum hormone concentrations	No definite associations with the development of puberty Occurrence of spermaturia not associated with PCB exposure, but with tanner stages and testicular size.	(Mol et al., 2002)
USA CPP cohort study (1959-1966)	Case-control study	n=219 cases n=564 controls	Serum (Maternal)	β-HCH HCB HCE	Cryptorchidism	β-HCH was associated (OR=1.6; 95% CI, 0.7-3.6) HCE (OR=1.2; 95% CI, 0.2-2.6) HCB (OR near 1)	(Pierik et al., 2007)
Germany	Case-control	n= 18 cryptorchidism cases n= 30 male controls	Fat tissue (from orchidopexy)	HCE, HCB DDE, DDT	Cryptorchidism	Higher median concentration (ng/g lipid) of β-HCH among cases (20.7 vs 11.2). HCB significantly associated (p =0.01) No significant difference for DDT (25.6/16.6) & DDE (264.5/170.15) between cases and controls.	(Hosie et al., 2000)
Granada University Hospital, Spain. Mother–child cohort (2000-2002).	Case-control study	n=50 cases n=114 controls	Placenta (at delivery)	Total effective xenoestrogen burden (TEXB) OCPs	Cryptorchidism Hypospadias	Lindane significantly associated with combined cryptorchidism and hypospadias cases (OR = 3.38; 95% CI, 1.36-8.38). DDT (OR = 2.63; 95% CI, 1.21-5.72) Mirex (OR = 2.85; 95% CI, 1.22–6.66) Endosulfan alpha (OR = 2.19; 95% CI, 0.99–4.82) TEXB (OR=2.82; 95% CI, 1.10–7.24)	(Fernandez et al., 2007)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Result	Ref.
Denmark (1997-2001)	Case-control study	n= 62 cases/ n= 68 controls	Breat milk	DDE, DDT β-HCH, HCB, α-endosulfan, cis-HCE, oxychlordane dieldrin	Cryptorchidism	Sum of pesticides significantly associated with cryptorchidism (p = 0.032). Except trans-chlordane (p = 0.012), no significant association for individual chemicals.	(Damgaard et al., 2006)
USA CPP cohort study (1958-1965)	Case-control study	n= 24 cases n=24 control (matched)	Serum (maternal, 1st trimester)	Estradiol	Cryptorchidism	Mothers of cryptorchid sons had significantly higher serum free estradiol (p = 0.010) and albumin-bound estradiol (p = 0.014).	(Bernstein et al., 1988)
USA Three US collaborative studies (1939-1975)	Cohort	n=1197 DES-exposed n=1038 unexposed	n.a	DES	Male urogenital abnormalities	DES increases risk of male urogenital abnormalities. RR=1.9; 95% CI, 1.1-3.4) for cryptorchidism, and RR= 2.5; 95% CI, 1.5-4.3 for epididymal cyst, and RR=2.4; 95% CI, 1.5-4.4 for testicular inflammation/infection	(Palmer et al., 2009)
USA CPP cohort study (1959-1965)	Case-control study	n=200 cases n=200 controls	Serum (Maternal, 3rd trimester)	Estradiol, estriol Testosterone Sex hormone-binding globulin, oestradiol/ testosterones	Cryptorchidism	No significant differences in concentration of testosterone, sex hormone-binding globulin, or oestrogens to androgens ratio. Total oestradiol and oestriol were significantly lower among all the cases (p=0.03 & 0.05, respectively).	(McGlynn et al., 2005)
UK Oxfordshire, John Radcliffe Hospital-Cryptorchidism Study group, 1992	Case-control study	n=28 cases n=108 controls	Serum (Maternal, 6-20 gestational weeks)	Oestradiol Testosereone	Cryptorchidism	Geometric mean concentrations of oestradiol and testosterone were 5% lower (95% CI -32% to +31%: p=0.74) and 25% lower (95% CI -45% to +1%: p=0.06) respectively in cases than in controls during the first phase of testicular descent.	(Key et al., 1996)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Result	Ref.
Cambridge, UK.	Case-control study	n= 83 cases n=129 controls	Symptoms of excess oestrogen exposure	Indicators (symptoms) of oestrogen exposure	Cryptorchidism	Symptoms (nausea, vomiting and hypertension) associated with excess oestrogen in pregnancy were similar in mothers of cases and controls.	(Davies et al., 1986)
British Columbia, Canada. (1982-1984)	Case-control study	n=244 cases n=488 cases	Symptoms of excess oestrogen exposure	Exogenous oestrogen endogenous oestrogen indicators	Cryptorchidism	Neither exogenous oestrogen, nor any of indirect indicators of endogenous oestrogen exposure (bleeding, nausea, vomiting) were significantly associated with risk of cryptorchidism.	(McBride et al., 1991)
San Francisco, USA. CHDS study. (1959-1967)	Case-Control study	n=75 cryptorchidism cases/ n=66 hypospadias cases/ n=283 controls	Serum (Maternal, each trimester & at delivery)	DDT DDE	Cryptorchidism Hypospadias	No significant association with Cryptorchidism DDE (OR=1.34; 95% CI, 0.51–3.48); DDT (OR=1.01; 95% CI, 0.44–2.28) or Hypospadias DDE (OR=1.18; 95% CI, 0.46–3.02); DDT (OR= 0.79; 95% CI, 0.33–1.89)	(Bhatia et al., 2005)
USA CPP cohort study (1959-1965)	Case-control study	n= 217 cryptorchidism cases/ n=197 hypospadias cases/ n= 557 controls	Serum (Maternal, 3rd trimester)	*trans*-Nonachlor Oxychlordane	Cryptorchidism Hypospadias	No notable association with both Cryptorchidism *trans*-Nonachlor (OR=1.22; 95% CI, 0.70-2.12); Oxychlordane (OR= 0.95; 95% CI, 0.55-1.64) and Hypospadias *trans*-Nonachlor (OR=1.08; 95% CI, 0.62-1.89); Oxychlordane (OR= 1.24; 95% CI, 0.69-2.22) comparing highest quartile with lowest	(Trabert et al., 2012)
USA CPP cohort study (1959-1966)	Case-control study	n=219 cryptorchidism cases n=199 n=552 controls	Serum (Maternal, 3rd trimester)	DDE/DDT	Cryptorchidism Hypospadias Polythelia	Modest-moderate association for DDE with cryptorchidism (OR=1.3; 95% CI, 0.7-2.4), hypospadias (OR=1.2; 95% CI, 0.6-2.4), and polythelia (OR=1.9; 95% CI, 0.9-4.0).	(Longnecker et al., 2002)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Result	Ref.
Danish-Finish study, (1997-2001)	Case-control study	n=62 cryptorchidism / n=68 controls	Breast milk	14 BDEs	Cryptorchidism	Levls in breast milk showed association Sum of BDEs (47, 153, 99, 100, 28, 66, and 154), $p < 0.007$	(Main et al., 2007)
		n=95 cryptorchidism / n=185 controls	Placenta			No association for levels in placenta	
USA, Michigan long-term Polybrominated Biphenyl cohort study (1973-1974)	cohort	n= 9 cryptorchidism cases/ N=5 hypospadias/ n=13 hernias/ n= 464 total sons	Serum (Maternal)	BDE-153	Male genitourinary conditions	No association for cryptorchdism or hypospadias BDE-153 (OR=0.7; 95% CI, 0.1-3.8) Possible association with other genitourinary conditions and in utero PBB exposure, e.g., hernia (p=0.04)	(Small et al., 2009)
Montreal, Toronto, and London, Canada. (2011-2014)	Case-control study	n=137 cases/ n=158 controls	Hair samples (Maternal)	8 BDE congeners (28, 47, 99, 100, 153, 154, 183, 209)	Cryptorchidism	Association with cryptorchidism for BDE-99 (OR = 2.53; 95% CI, 1.29-4.95), BDE-100 (OR = 2.45; 95% CI, 1.31-4.56), BDE-154 (OR = 1.88; 95% CI, 1.08-3.28)	(Goodyer et al., 2017)
Denmark and Finland. Joint prospective birth cohort, (1997-2002)	Case-control study	n=107 cryptorchidism cases n=108 controls)	Umbilical cord blood/serum	PFASs	Cryptorchidism	No significant association with cryptorchidism for PFOA (OR=0.46; 95% CI, 0.20–1.20) and PFOS (OR=0.83; 95% CI, 0.39–1.79) comparing levels (highest tertile vs lowest tertile)	(Jensen et al., 2013)
Denmark, National Patient Registry (1980–1996)	Case-control study	n= 270 cases/ n=75 hypospadias cases n=300 controls	Amniotic fluid (2nd trimester)	PFOS Fetal steroid hormone Insulin-like factor 3 (INSL3)	Cryptorchidism Hypospadias	No association between PFOS and cryptorchidism (OR=1.01; 95% CI, 0.66-1.53), for highest (> 1.4 ng/mL) vs. lowest tertile. However, PFOS was associated with lower INSL3 level (reduction of 40% (95% CI: -69, -11%) and increase in testosterone level of 18% (95% CI: 7, 29%).	(Toft et al., 2016)

Given the limited epidemiological evidence on EDCs and cryptorchidism, particularly on chemical mixtures, and the potential personal and economic costs, it is necessary to identify the chemicals and mechanisms involved, so that strategies to limit exposure may be developed. One of the aims of this thesis was, therefore, to investigate 27 potential EDCs measured in breast milk, as a proxy for perinatal exposure, and the risk of cryptorchidism. Furthermore, to investigate potential mechanistic pathways leading to cryptorchidism in an experimental study on zebrafish larvae.

Neurological and behavioural disorders

Neurological and behavioural disorders include attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD), as well as depression and other mood disorders, learning disabilities, executive function deficits, and conduct disorders (Gore et al., 2014). The increasing trends in these disorders has raised the alarm about the potential role of EDCs in neurodevelopment and behaviour (WHO/UNEP, 2013).

1.2.2. Autism

Autism spectrum disorder (ASD) refers to "a wide range of neurodevelopmental disorder that is characterized by difficulties with social communication, language difficulties, some degree of impaired social behaviour, repetitive behaviours, and limited range of interests and activities" (American Psychiatric Association, 2013). The economic burden of ASD is estimated to be around $ 2 million in both the US and the UK per individual, due to special education services needs and loss of parental productivity (Buescher et al., 2014).

Experimental studies have demonstrated that exposure to persistent organic pollutants (POPs) may cause neurological impairment, neurodevelopmental disorders, and neurodegenerative disease (Johansson et al., 1995, Zeliger, 2013). Most insecticides exert their effect by interfering with the nervous system of insects. Importantly, they exert neurotoxic effects on humans and other vertebrates as well, through common pathways in neurodevelopment (Briz et al., 2011, Brannen et al., 1998, Quaak et al., 2013, Stamou et al., 2013). Several mechanisms have been hypothesized by which POPs may increase the risk of ASD: alteration in the GABAergic, glutamatergic, serotonergic and dopaminergic system, oxidative stress, endocrine disruption, and epigenetic alterations (Quaak et al., 2013, Brannen et al., 1998, Shelton et al., 2014).

One in 160 children has ASD worldwide. However, the reported prevalence varies significantly across countries (WHO, 2013). In Norway, the nationwide prevalence of ASD for children aged 0-11 years was estimated to be 0.7% between 2008 and 2010 based on the Norwegian Patient Register (NPR) (Suren et al., 2012). Similar prevalence estimates (0.7-0.8%) were observed among the Norwegian Mother, Father and Child Cohort Study (MoBa) participants aged 9 years or older (Surén et al., 2014). Slightly higher prevalence rates were reported in the US (1.7%) (Baio et al., 2018), and in the UK (1.2%) (Baird et al., 2006).

The prevalence of ASD has increased substantially in recent times. Both genetic and environmental factors play a role in ASD's aetiology, but the exact causes are still poorly understood. The increase in prevalence may also be explained by improved awareness, better ascertainment, broader diagnostic criteria, development of services, or a true increase in incidence (Baird et al., 2006, Fombonne, 2009, Hinkka-Yli-Salomäki et al., 2014, Jensen et al., 2014).

The prevalence of ASD among boys is approximately four to five times higher than in girls (Hinkka-Yli-Salomäki et al., 2014, Lyall et al., 2017a), and this may point to both genetic and hormonal factors. Known risk factors for ASD include advanced parental age, preterm birth, and short inter-pregnancy interval. Other potential risk factors that require further exploration include endocrine disrupting chemicals, metabolic conditions, and folic acid deficiency (Johansson et al., 1995, Lyall et al., 2017a).

The epidemiologic evidence for an association between early-life exposure to POPs and ASD is inconsistent (van Wijngaarden et al., 2013, Kalkbrenner et al., 2014, Lyall et al., 2017b, Verner et al., 2015, Lyall et al., 2014, Rossignol et al., 2014, Jurewicz and Hanke, 2008, Ribas-Fitó et al., 2006). There is, however, a growing body of studies reporting associations between early-life exposure to environmental chemicals and other adverse cognitive and neurodevelopmental effects (Korrick and Sagiv, 2008, Marijke et al., 2012). Table 3 summarizes the available studies and these are again discussed in detail in the discussion in relation to the findings from the studies in this thesis.

Table 3. Summary of studies on EDCs exposure and the risk of autism spectrum disorders

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Results	Ref.
Greece (2015-2017)	Cross-sectional study	n= 39 ASD / n= 21 ADHD/ n= 32 SLD cases/ n= 18 controls	Serum (Children)	OCPs β-HCH, DDT, HCH isomers, cyclodienes, methoxychlor	ASD ADHD SLD	Higher concentration in cases than controls β-HCH, the sum of HCH isomers, DDT Mean ± SD β-HCH cases/control: 10.5 ± 7.7/ 6.1 ± 4.0 ng/g lipid. No significant difference for ADHD or SLD groups	(Makris et al., 2019)
Cincinnati, Ohio, USA. HOME Study (2003—2006)	Cohort	n=175 women	Serum (Maternal, 16 weeks)	8 Phthalates Bisphenol A 25 PCBs 6 OCPs 8 Flame retardants 4 PFASs	Autistic behaviours based on SRS score	Positively associated with more autistic behaviours. *Trans*-nonalchlor and & BDE-28 Negative association (fewer autistic behaviours) for β-HCH (β = –3.3; 95% CI: –6.1, –0.5), PCB-178 (β = –3.0; 95% CI: –6.3, –0.2), BDE-85 (β = –3.1; 95% CI: –5.9, –0.5), and PFOA (β = –2.0; 95% CI: –4.4, 0.4).	(Braun et al., 2014)
Finland FiPS-A study (1987-2005)	Case-control study	n=778 matched case-control pairs	Serum (Maternal, early pregnancy)	DDT/DDE PCBS	Autism, ID	Positively associated with autism DDE: OR=1.32; 95% CI, 1.02-1.71 Positively associated with ID DDE: OR=2.21; 95% CI, 1.32-3.69. No association between total PCBs and autism	(Brown et al., 2018)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Results	Ref.
California, USA EMA study (2000-2003)	Case-control study	n=545 ASD/ n=181 ID cases/ n= 418 controls	Serum (Maternal, mid-pregnancy)	2 OCPs 11 PCB congeners	ASD ID	Suggested increase in the risk of ASD and ID for DDE and *trans*-nonachlor. Positive association with ASD PCB-138/158 (OR=1.79; 95% CI, 1.10-2.92), PCB-153 (OR=1.82; 95% CI, 1.10-3.02) Positive association with ID PCB-138/158 (OR=2.41; 95% CI, 1.18-4.91), PCB-153 (OR=1.82; 95% CI, 1.10-3.02)	(Lyall et al., 2017b)
Norway THE HUMIS study (2002-2009)	Cohort	n= 55 ASD/ n=1199 total	Breast milk	27 POPs (14 PCBs, 5 OCPs, 6 BDEs, 2 PFASs)	ADHD	Positive association with ADHD β-HCH (OR = 1.75; 95% CI, 1.22- 2.53) and PFOS (OR = 1.77; 95% CI, 1.16- 2.72. Stronger association among girls. Negative association with ADHD DDT: OR=0.64; 95% CI, 0.42-0.97 HCB demonstrated a non-linear association with ASD.	(Lenters et al., 2019)
University of Texas Medical Center, USA.	Case-control study	n=50 PD / n=20 Alzheimer's / n= 43 Controls	Serum	16 OCPs	Parkinson's disease Alzheimer's disease	β-HCH increased the risk of PD (OR=4.39; 95% CI, 1.67-11.6).	(Richardson et al., 2009)
Faroe Islands (2005)	Case-control study	n= 79 PD cases/ n= 154 controls	Serum	Pesticides Metals PCBs	Parkinson's disease	Positive association with PD β-HCH: OR=1.44; 95% CI, 1.05-1.97. Doubling of risk among women: OR=2.59; 95% CI, 1.03-6.51. Positive association with Hg among only women Hg: OR=1.96; 96% CI, 1.10-3.48).	(Petersen et al., 2008)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Results	Ref.
California, USA EMA study (2000-2003)	Case-control study	n=545 ASD / n=181 ID cases/ n=418 controls	Serum (Maternal, 2nd trimester)	10 BDEs	ASD ID	Negative association of several BDE congeners with ASD BDE-153: OR=0.56; 95% CI, 0.38-0.84)). Positive association among girls for BDE-28 (OR=2.58; 95% CI, 0.86-7.79) and BDE-47 (OR=2.64; 95% CI, 0.97-7.19)	(Lyall et al., 2017c)
Finland FiPS-A study (1987-2005)	Case-control study	n=75 cases/ n=75 controls	Serum (Maternal)	PCBs OCPs (DDT, DDE, HCB) BDE-47	Autism	Positive association Total PCBs: OR=1.91; 95% CI, 0.57-6.39 DDE: OR=1.79; 95% CI, 0.52-6.21	(Cheslack-Postava et al., 2013)
Norway MoBA study (1998-2008)	Case-control study	n=400 ASD, n=821 ADHD cases / n=980 controls	Plasma (Maternal, gestational week 18)	19 PFASs	Autism ADHD	Positive association PFOA: OR=1.71; 95% CI, 1.20-2.45 (ASD) PFOA: OR=1.54; 95% CI,1.16- 2.04 (ADHD) Negative associations for other PFASs and total/ carboxylate/sulfonate PFASs mixture. OR=0.76; 95% CI, 0.64- 0.90	(Skogheim et al., 2021)
Denmark Danish National Birth Cohort (1996-2002)	Case-control study	n= 220 ASD / n= 220 ADHD cases/ n= 550 controls	Plasma (Maternal, early or mid-pregnancy)	16 PFASs	ASD ADHD	No association between 16 PFASs with ASD or ADHD. PFOA: RR=0.98; 95% CI, 0.73-1.31 (ASD) PFOS: RR=0.92; 95% CI, 0.69-1.22 (ASD) PFOA: RR=0.98; 95% CI, 0.82-1.16 (ADHD) PFOS: RR=0.87; 95% CI, 0.74-1.02 (ADHD)	(Liew et al., 2015b)
California, USA EMA study (2000-2003)	Case-control study	n=553 ASD/ n=189 ID cases/ n=433 controls	Serum (Maternal, gestational week (15-19))	8 PFASs	ASD ID	Negative association with ASD PFOA: OR=0.62; 95% CI, 0.41-0.93 PFOS: OR=0.64; 95% CI, 0.43- 0.97 Similar results for ID. Most PFASs were not associated significantly	(Lyall et al., 2018)

Country, study year	Study design	Participants	Matrix	Exposure	Outcome	Results	Ref.
Europe (Denmark, Greenland, Norway, Slovakia Spain, and Ukraine. (1996-2009)	Meta-analysis of nine population-based studies	n= 399 total ADHD cases n= 4,826 mother-child pairs (total)	Serum/ plasma/ breast milk (maternal)	PFAS	ADHD	No association between PFOA/PFOS and ADHD. Increased association in girls, children from nulliparous women, and low educated mothers.	(Forns et al., 2020)

2. Aim of the thesis

The motivation for our research was the increasing trend in endocrine related disorders worldwide that cannot be explained by genetics alone. We wanted to test the hypothesis that exposure to EDCs early in life, contributes to the increasing trend in endocrine-related adverse outcomes in children worldwide. We focused on reproductive (cryptorchidism) and neurodevelopmental disorders (autism) in children. We utilized the Norwegian Human Milk Study (HUMIS) cohort, a multi-centre birth cohort of mother-child pairs, and experimental (*in vitro* and *in vivo)* studies.

We selected 27 specific chemicals: 5 organochlorine pesticides (OCPs; β-HCH, HCB, p,p′-DDE, p,p′-DDT), 14 polychlorinated biphenyls (PCBs; PCB-105, PCB-114, PCB-118, PCB-156, PCB-157, PCB-167, PCB-189, PCB-74, PCB-99, PCB-153, PCB-170, PCB-180, PCB-194, and PCB-138), 6 polybrominateddiphenyl ethers ((P)BDEs; BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, and BDE-154), and 2 poly- and perfluoroalkyl substances (PFASs; PFOA, PFOS).

Research questions

1. Are specific potential endocrine disrupting chemicals on our list associated with cryptorchidism and autism spectrum disorders among Norwegian children?

2. Is there an association between the activation of hormonal receptors (androgen receptor and aryl hydrocarbon receptor) in breast milk and cryptorchidism?

3. Are any of the potential EDCs on our list associated with activation of hormonal receptors (androgen receptor and aryl hydrocarbon receptor), all measured in breast milk from mothers with sons in the HUMIS cohort?

Specific objectives

1. To investigate 27 potential EDCs measured in breast milk as a proxy for perinatal exposure and the risk of cryptorchidism in a prospective cohort.
2. To investigate the association between anti-androgenic activity, not derived from natural hormones, in maternal breast milk and cryptorchidism among boys in the HUMIS cohort, using a case-control study design.

3. To evaluate whether there is a link between aryl hydrocarbon receptor (AhR) activation and cryptorchidism, and further explore the association between 27 potential EDCs with AhR activation in breast milk samples in a case-control study derived from the HUMIS cohort.
4. To evaluate the association of maternal milk levels of 27 potential EDCs with the risk of ASD among Norwegian children in the HUMIS cohort, and further evaluate the identified chemicals for neurodevelopmental and behavioural toxicities using zebrafish embryos and larvae.

3. Material and methods

3.1 Study population

The Norwegian Human Milk Study (HUMIS) is a multi-centre birth cohort of mother-child pairs that were recruited between 2002 and 2009. Public health nurses recruited mothers during a routine home visit offered to all new mothers in Norway within approximately two weeks of giving birth (Eggesbø et al. 2009). A subset of mothers was recruited in 2002-2005 by a paediatrician at the maternity ward in Østfold hospital in Southern Norway, two consecutive term births for every preterm birth (Eggesbø et al. 2011). All mothers followed the same protocol and completed the same questionnaires regardless of recruitment procedure. They were requested to collect and store approximately 25 ml of breast milk each morning for eight successive days. Breast milk was sampled at a median of 33 days after delivery (10th–90th percentile: 18–56) in all counties, and greater than 90% were collected between two weeks and before the child turned 2 months of age, in line with the WHO recommendation (WHO, 2007). The median age (10th–90th percentile) in days at the time of breast milk sampling was 30 (24–36) and 29 (24–36) days, in Østfold and other counties, respectively. The samples were kept frozen and mailed to the Norwegian Institute of Public Health biobank in a 250 ml natural High-Density Polyethylene container. However, in Østfold county (n=560, 21%), study personnel collected milk samples and kept them frozen during transport. Further details have been published elsewhere (Eggesbø et al., 2011, Eggesbø et al., 2009). To date, among the 2,606 participants in the HUMIS study, only about half of the women have had their milk samples analysed for POPs, due to financial constraints. Various data subsets of the study participants were constructed based on eligibility criteria described in Figure 12 below.

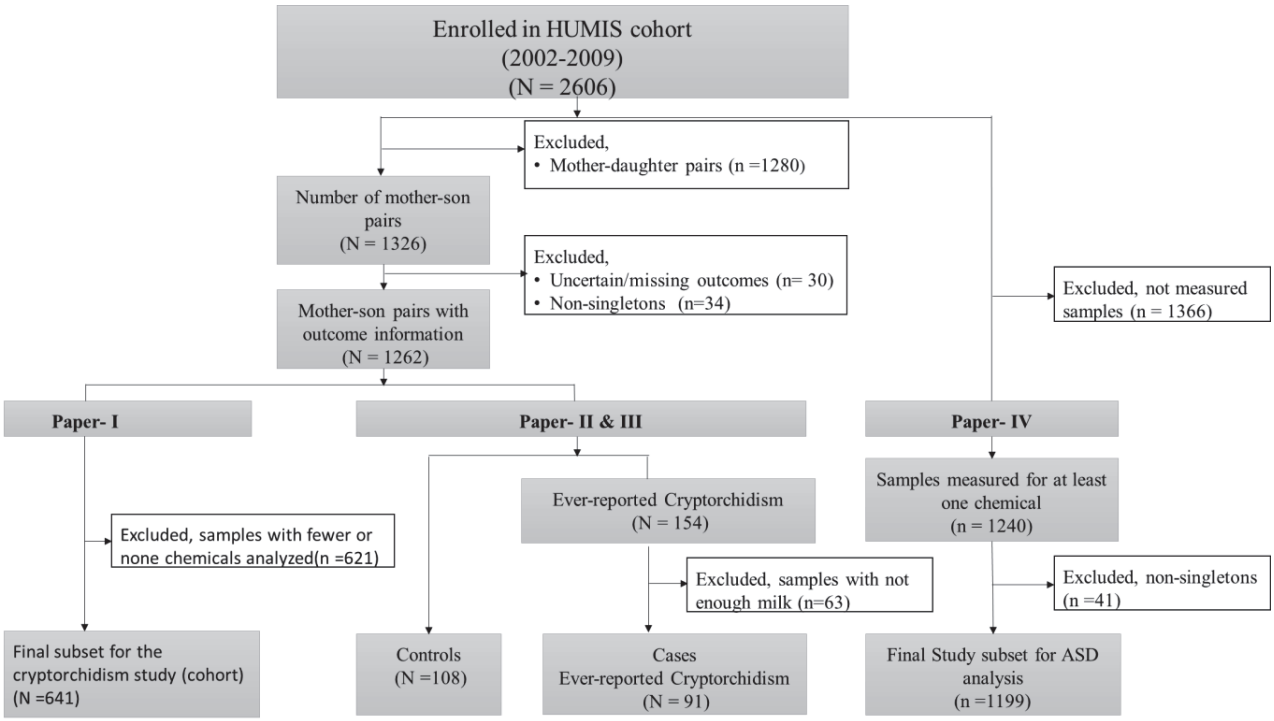

Figure 12. Flow chart showing the different study subsets included in this thesis.

3.2 Exposure

3.2.1. Potential EDCs in breast milk

The concentrations of 27 potential EDCs: 5 organochlorine pesticides (OCPs; β-HCH, HCB, p,p′- DDE, p,p′- DDT), 14 polychlorinated biphenyls (PCBs; PCB-105, PCB-114, PCB-118, PCB-156, PCB-157, PCB-167, PCB-189, PCB-74, PCB-99, PCB-153, PCB-170, PCB-180, PCB-194, and PCB-138), 6 polybrominated diphenyl ethers ((P)BDEs; BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, and BDE-154), and 2 poly- and perfluoroalkyl substances (PFASs; PFOA, PFOS) were quantified in 1199 breast milk samples (Figure 13). Breast milk lipid levels were quantified gravimetrically during chemical analysis, and breast milk concentrations of EDCs are lipid adjusted (ng/g), except for PFASs where concentrations are wet weight (ng/L). Concentration values below the limit of detection (LOD) were substituted by randomly generated values between zero and LOD. Supplementary Material of each paper describes the detailed analytical methods used.

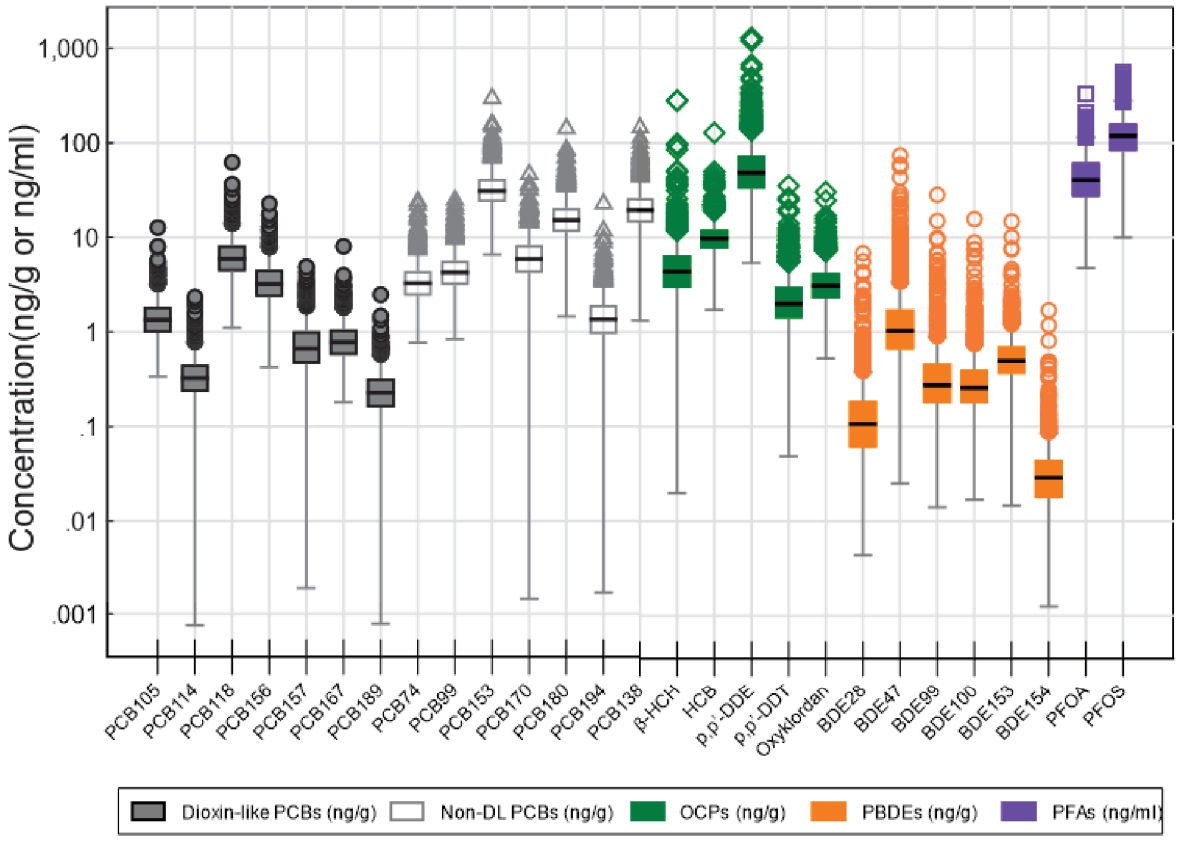

Figure 13. Boxplot distribution of 27 EDCs found in breast milk among 1199 mother-son pairs in the HUMIS cohort (2002-2009, Norway). Horizontal lines correspond to medians, and boxes to the 25th–75th percentiles; whiskers extend to data within the interquartile range times 1.5, and data beyond this are plotted as individual points (dots, triangles, diamonds, or squares). Wet weight concentrations are presented for PFASs (ng/L) and lipid adjusted concentrations for all other chemicals (ng/g lipid).

Abbreviations: *"BDE, brominated diphenyl ether; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; HCB, hexachlorobenzene; β-HCH, Beta-hexachlorocyclohexane; BDE, Brominated Diphenyl Ether; PCB, polychlorinated biphenyl; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate."*

3.2.2. Postnatal EDCs concentration

Postnatal exposures in the first 2 years of life (at 3, 6, 12, 18, and 24 months of age) were modelled using a two-compartment pharmacokinetic model (Stigum et al., 2015).

3.2.3. Anti-androgen receptor activity

Anti-androgen receptor (anti-AR) activity was measured using Chemically Activated LUciferase gene eXpression (CALUX®) bioassay in breast milk fractions and expressed in µg of flutamide equivalent, a well-known anti-androgen.

3.2.4. Aryl hydrocarbon receptor activity

Aryl hydrocarbon receptor (AhR) activity was measured using DR- CALUX® assay in the mothers' milk samples and expressed as pg 2,3,7,8-TCDD equivalent (TEQ)/g lipid or pg CALUX-TEQ/g lipid.

3.3 Outcomes

3.3.1. Cryptorchidism

Cryptorchidism was defined based on maternal reports from repeated self-administered questionnaires that are filled out at 1, 6, 12, 24 months after birth. For studies included in this thesis, cryptorchidism was defined as either:

1. Congenital cryptorchidism if reported at one month after birth,
2. Recurrent cryptorchidism if reported that the testis failed to descend at birth, but then spontaneously descended and then followed by re-ascending.
3. Persistent cryptorchidism if reported both at 12 months and 24 months, and may also include receipt of orchiopexy
4. Ever-reported cryptorchidism if reported at either 1-, 6-, 12- or 24 months after birth.

3.3.2. Autism spectrum disorder

ASD cases were identified by linking the HUMIS cohort to the Norwegian Patient Registry which contains specialist-confirmed diagnoses from government-owned hospitals and outpatient clinics based on the International Classification of Diseases (ICD-10). The register collected individual data beginning in 2008, and the linkage was performed in 2017 when children were around 13 years of age (median 12.7, IQR, 11.8–13.4 years).

ASD (previously termed pervasive developmental disorder in DSM-IV) cases were defined as F84.0 (childhood autism), F84.1 (atypical autism), F84.5 (Asperger syndrome), F84.8 (other pervasive developmental disorder), and F84.9 (pervasive developmental disorder, unspecified). There were zero

cases of F84.2 (Rett's syndrome), F84.3 (other childhood disintegrative disorder), or F84.4 (overactive disorder associated with mental retardation and stereotyped movements), which have also been included in case ascertainment in other publications (Suren et al., 2012).

3.4. Covariates

Potential confounders and additional covariates were extracted from self-administered questionnaires that mothers completed either at 1-, 6-, 12-, or 24 months postpartum. Other variables such as gestational age, birth weight, child's sex, and maternal smoking were taken from the Medical Birth Registry of Norway (Skjaerven et al., 2000).

3.5. Statistics

A detailed description of the statistical method and analysis for each study is available in each individual paper (Paper I-IV). Below is a description of the common statistical approach.

Univariate analysis: Pearson's chi-square test was used for univariate analysis of binary or categorical variables while T-test and Wilcoxon rank-sum test for continuous variables to test differences between the various outcomes and maternal and child characteristics that are presented in Table 1 of the respective papers.

Correlations *between chemicals were tested using Spearman's rank coefficient.*

Causal Assumptions**:** Directed Acyclic Graphs (DAGs) were used prior to analysis to select a minimal sufficient set of variables/ confounders for adjustment to allow unbiased effect estimations given the DAG for each paper included in this thesis. (http://www.dagitty.net/dags.html)

Single-pollutant analysis. *Multivariate logistic regression* was used to estimate the odds ratios with 95 % confidence intervals for the binary outcomes (cryptorchidism and ASD) while *Multivariate linear regression* was used to estimate coefficients with 95 % CI for the continuous outcome, AhR activity (pg CALUX-TEQ/g lipid).

Multi-pollutant analysis. Elastic net logistic regression was used to select key predictors among several correlated chemical co-exposures, and thereby reduce the potential for multicollinearity (Zou and Hastie, 2005, Lenters et al., 2018).

Imputation. Multiple imputation with predictive mean matching (White et al., 2011) was used to impute missing exposure and covariate data.

Potential effect modification for select covariates was also assessed in models including main effects and cross-product terms, with a Wald test *p*-value of <0.20 suggestive of an interaction.

Sensitivity Analysis. Sensitivity analysis was used to individually test the effect of further adjusting for selected covariates.

Software. Stata (version 15/16; Stata Corp LP, College Station, Texas) was used for all statistical analyses. $P < 0.05$ was regarded as statistically significant.

Table 4 below summarizes the objectives, study designs, and statistics used in each paper included in this thesis.

Table 4. summary of the objectives, study designs and statistics used in each paper.

Paper	Objective	Study design/ type	Statistics
I	To investigate 27 potential EDCs measured in breast milk as a proxy for perinatal exposure and the risk of cryptorchidism in a prospective cohort.	Cohort	Multivariate logistic regression Elastic-net logistic regression
II	To investigate the association between anti-androgenic activity, not derived from natural hormones, in maternal breast milk and cryptorchidism among boys using a case-control study from HUMIS cohort.	Case-control *in vitro*	Multivariate logistic regression
III	To evaluate whether there is a link between aryl hydrocarbon receptor (AhR) activation and cryptorchidism, and further explore the association between 27 potential EDCs with AhR activation in breast milk samples.	Case-control *in vitro*	Multivariate logistic regression Multivariate linear regression
IV	To evaluate the association of maternal milk levels of 27 potential EDCs with the risk of ASD among Norwegian children in the HUMIS study, and further evaluate the identified chemicals for neurodevelopmental and behavioural toxicities using zebrafish embryos and larvae.	Cohort *in vivo*	Multivariate logistic regression Elastic net logistic regression

3.6 Ethical consideration

The studies that make up this thesis were approved by the Norwegian Data Inspectorate and Regional Ethics Committee for Medical Research (S-02122). The study participants gave informed consent to participate prior to the study.

4. Main findings

Summary of the main findings

In this thesis, we evaluated the health effects of perinatal exposure to mixtures of chemicals (27 EDCs) among Norwegian children enrolled in HUMIS cohort. The summary of the findings of this thesis is presented below for each paper (Paper I – IV) and figure 14 shows how each objective and paper are interconnected.

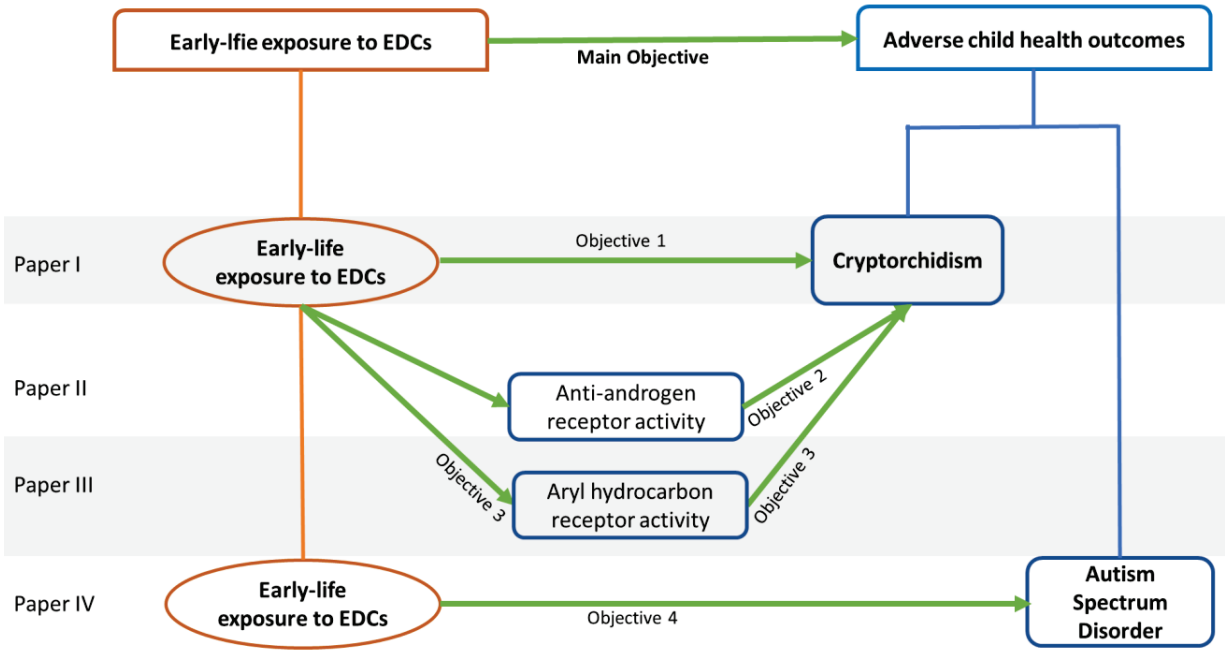

Figure 14. A flow chart of how the objectives and papers (I - IV) are interconnected.

Paper I

A case-cohort study of perinatal exposure to potential endocrine disrupters and the risk of cryptorchidism in the Norwegian HUMIS study. *(Desalegn et al., 2021). (Environment International. 2021. Volume 157, 106815).*

Background: We are exposed to an ever-growing number of chemicals every day. Some of these chemicals have the potential to interfere with endocrine hormones, including those that are important for testicular descent. This may lead to cryptorchidism among male infants and increased risk of infertility and testicular cancer later in life.

Objective: To study the association between early-life exposure to 27 potential EDCs measured in breast milk samples and cryptorchidism in infants enrolled in the HUMIS study (2002-009).

Method: We studied 641 male infants that had 27 chemicals measured in their mothers breastmilk samples including 14 polychlorinated biphenyls, 5 organochlorine pesticides, 6 polybrominated diphenyl ethers, and 2 perfluoroalkyl substances. We investigated the association between these chemicals and the risk of congenital, recurrent, persistent, and ever-reported cryptorchidism (defined based on maternal reports completed at 1, 6, 12, and 24 months after the birth of the child). Elastic net logistic regression was used to select the chemicals that best predicted cryptorchidism as it can deal with highly correlated exposures while logistic regression models were used to determine their effect estimates.

Results: The prevalence of cryptorchidism was 6.1% for congenital cryptorchidism and ranged between 2.2–2.4 % for cryptorchidism reported at 6, 12, or 24 months after birth. The ever-reported prevalence was 12.2 %. PCB-74 (OR=1.31, 95 % CI:1.001-1.703), PCB-114 (OR=1.36, 95 % CI:1.05-1.77), PCB-194 (OR=1.28, 95 % CI:1.03-1.53) and β-HCH (OR=1.26, 95 % CI:1.03-1.53) per an IQR increase in concentrations were identified as best predictors of congenital cryptorchidism per an IQR increase in concentrations. PCB-194 was in addition identified as a predictor for ever-reported cryptorchidism (OR=1.23, 95% CI: 1.01-1.51). None of the chemicals were selected for either recurrent or persistent cryptorchidism.

Conclusion: The reported prevalence of cryptorchidism declined by more than half in the six months after birth. Some PCBs (PCB-74, PCB-114, PCB-194) and the organochlorine pesticide residue, β-HCH, were associated with increased odds of congenital cryptorchidism. Single pollutant analysis demonstrated that some PCBs may falsely be associated with cryptorchidism due to confounding by highly correlated chemicals. Experimental studies are warranted to confirm our findings and shed light on potential mechanisms.

Paper II

Anti-androgenic compounds in breast milk and cryptorchidism among Norwegian boys in the HUMIS birth cohort. (Collet et al., 2021). *(Science of The Total Environment. 2022. Volume 803, 106815).*

Background: Reports indicate an increasing trend in the prevalence of cryptorchidism over the past decades, however, the reason remains poorly understood. Androgen hormone is one of the main hormones responsible for testis descent, and its disruption by EDCs has been linked with cryptorchidism development.

Objective: To evaluate the association between anti-androgenic receptor activity measured in breast milk samples as a proxy for early-life exposure and the risk of cryptorchidism among Norwegian boys enrolled in HUMIS study (2002-20009).

Method: A case-control study was conducted on 199 mother-child pairs (n=94 mothers with cryptorchidism boys and 105 random controls) in HUMIS study. Anti-androgen receptor activity in breast milk, which was not derived from natural hormones, was determined using the human cell-based in vitro anti-AR CALUX® assay and expressed as flutamide (μg) equivalent, a well-established anti-androgen drug. Polar, non-polar, and mixed breastmilk fractions were tested after extraction in samples from each of the mothers. Multivariable logistic regression was used to compare anti-androgen receptor activity between cases and controls.

Result: Norwegian children's daily exposure to anti-androgenic EDCs via breastmilk was estimated to be 78 μg flutamide eq./kg of body weight/day. The anti-androgenic receptor activity was the highest in the polar breastmilk fraction (1.48 ± 1.37 μg flutamide eq./g of milk) followed by the mixed and non-polar fractions, respectively. Antagonistic interactions were observed when combining the polar and non-polar breastmilk fractions. The anti-androgenic receptor activities in all three fractions were not significantly different between cryptorchidism cases and controls.

Conclusion: The estimated daily intake of anti-androgens via breast milk, and not derived from natural hormones among Norwegian boys in this study, is above the permitted daily exposure limit of 50 μg/d and thus of a concern. However, we found no evidence of an association between anti-androgenic receptor activity and cryptorchidism. Future studies should not be limited to studying androgen receptors, but also investigate other equally important androgen pathways such as synthesis, secretion, release, and metabolism to clarify the role of androgens in cryptorchidism development.

Paper III

A case-control study of aryl hydrocarbon receptor activity in human milk and the risk of cryptorchidism in the Norwegian HUMIS study. *(Manuscript)*

Background: The aetiology of cryptorchidism remains poorly understood, and early-life exposure to endocrine disrupting chemicals (EDCs) are suggested to play a role. One of the hallmarks of EDCs is that they affect several systems including the aryl hydrocarbon receptor (AhR).

Objective: To evaluate whether AhR activity in breast milk samples was associated with cryptorchidism.

Method: We conducted a case-control study based on 199 mother-child pairs (n=91 cases/108 controls) selected from the Norwegian Human Milk Study (2002-2009). AhR activity (pg 2,3,7,8-TCDD equivalent (TEQ)/g lipid) was determined using the DR- CALUX® assay using mothers' milk samples at median 33 days postpartum. We defined cases of congenital, recurrent, persistent, and ever-reported cryptorchidism based on maternal reports in questionnaires at 1-, 6-, 12-, and 24- months after birth. We used multivariate logistic regression to compare AhR activity levels between cases and controls, and separately, to estimate the association between 27 potential EDCs and AhR activity measured in breast milk.

Results: The median (interquartile range, IQR) AhR activation was 6.9 (5.2-9.5) pg CALUX-TEQ/g lipid. There were no significant associations between AhR activation in breast milk and any of the cryptorchidism definitions. Among the 27 chemicals measured in breast milk, AhR activity was associated with all dl-PCBs, three of the ndl-PCBs (PCB-74, PCB-180, PCB-194) and two of the organochlorine pesticides (OCPs; HCB, β-HCH), although not all were statistically significant. There were no associations between AhR activity and the PBDEs or PFASs measured in this study.

Conclusion: The estimated daily intake of total PCBs and dioxins express as AhR activity in breastmilk among Norwegian children enrolled in HUMIS study remains above the WHO's tolerable daily intake limits (1–4 pg/kg bw day). We found no association between AhR activity in breast milk and cryptorchidism among Norwegian boys. Consistent with a possible role in the observed AhR activity, all dl-PCBs were associated with AhR activity whereas the association was null for either PBDEs or PFASs

Paper IV

Early-life exposure to endocrine disrupting chemicals and autism spectrum disorder: A multi-pollutant analysis of a Norwegian birth cohort and evaluation of developmental neurotoxicity in zebrafish embryos and larvae. *(Submitted)*

Background: Early-life exposure to endocrine-disrupting chemicals (EDCs) may lead to neurodevelopmental disorders such as autism spectrum disorder (ASD) in children. However, the epidemiological evidence remains limited and inconclusive.

Objective: To evaluate the association of maternal milk levels of 27 potential EDCs with the risk of ASD in children, and further evaluate the identified chemicals for neurodevelopmental and behavioural toxicities using zebrafish embryos and larvae.

Methods: In breast milk from 1199 mothers enrolled in the prospective HUMIS Study between 2002 and 2009, we measured 27 chemicals representing polychlorinated biphenyls, organochlorine pesticides, polybrominated diphenyl ethers, and perfluoroalkyl substances. Twenty children had received a specialist-confirmed diagnosis of ASD by the age of 13 years. We used elastic net logistic regression and multivariable logistic regression, as a variable selection method and to determine effect estimates, respectively. We conducted a follow-up in vivo study in zebrafish larvae to replicate the neurodevelopmental toxicity of the identified chemical and to exclude possible confounding from unmeasured highly correlated chemicals.

Results: β-Hexachlorocyclohexane (β-HCH), the most persistent beta-isomer of the organochlorine pesticide hexachlorocyclohexane, was the only chemical associated with ASD, after adjusting for 26 other chemicals. Mothers with the highest levels of β-HCH in their milk had a significant increased risk of having a child with ASD (OR = 1.82, 95% CI: 1.20, 2.77 for an interquartile range increase in β-HCH concentration). Neurodevelopmental and social behavioural effects of β-HCH were confirmed in zebrafish embryos and larvae at relevant concentrations of β-HCH. Further analysis indicated perturbation of dopaminergic neuron development as a possible mechanism underlying the β-HCH-related neurotoxicity.

Conclusions: Perinatal exposure to β-HCH was associated with an increased risk of specialist-confirmed diagnoses of ASD in children. The neurotoxicity of β-HCH was replicated in zebrafish larvae and that perturbation of dopaminergic neurone development underlies β-HCH neurotoxicity.

5. Discussion

5.1. Summary and interpretation of the findings

5.1.1. Prevalence of cryptorchidism and autism

Cryptorchidism

The prevalence of cryptorchidism in the HUMIS cohort varied depending on the time after birth, presentation, and severity of cryptorchidism reported by mothers at 1-, 6-, 12-, 24- months after birth. The prevalence was 6.1% for congenital cryptorchidism and reduced to 2.2-2.4% after the first six months according to maternal reports at 6-, 12-, and 24-months. This is in line with previous knowledge whereby spontaneous descent of testes during the first 3 months of life occurs in approximately half of the cases, and during the first year of life for most of the cases (Berkowitz et al., 1993, Gurney et al., 2017). Spontaneous descent occurs in accordance with the normal physiology driven by the transient surge in gonadotrophins and consequent rise in testosterone levels (mini puberty), making testicular descent possible (Kuiri-Hänninen et al., 2019). Ever-reported cryptorchidism at any time point (1-, 6-, 12-, or 24- months) was 12.1% while about 8.0 % reported recurrent cryptorchidism.

The prevalence estimates in the HUMS cohort are high compared to the relatively stable prevalence (0.3-0.4%), reported in Norway since the early 1970s based on the Medical birth registry of Norway (MBRN) (Aschim et al., 2006, Brantsæter et al., 2016). However, prevalence estimates at birth are prone to underreporting since they exclude boys with recurrent cryptorchidism (normally descended testis at birth that subsequently ascended). However, a prospective clinical study previously reported a high prevalence of congenital cryptorchidism in Denmark (9%) (Boisen et al., 2004). Several other studies have also indicated an increasing trend in the prevalence of cryptorchidism and other male reproductive disorders, particularly in countries with Caucasian populations, attributed to changes in lifestyle factors (Boisen et al., 2004, Skakkebaek et al., 2016). Overall, the literature shows a wide geographical variation in the prevalence of cryptorchidism (1-9%) suggesting an uneven distribution of risk factors among populations. The prevalence appears also to vary depending on the age of the diagnosis, and the diagnostic criteria used to assess the spectrum of disease severity. Ascertainment of cryptorchidism later in life will thus exclude the 50% who have cryptorchidic testis at birth but

whose testicle descended during the first three months of life, while ascertainment at birth will exclude boys with normally descended testis at birth that subsequently ascended.

Autism spectrum disorder

The prevalence of ASD among the study participants in **Paper IV,** based on linkage at 2017, **was** 1.7%, double the prevalence in the whole HUMIS study population (0.8%): This is due to our study design where we have oversampled for child neurodevelopmental outcomes (Attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), and cognitive delay. The prevalence in the whole cohort is similar to the nationwide prevalence (0.7-0.8%) for children aged 0-11 years between 2008-2010 based on the Norwegian patient register (Suren et al., 2012), and among Norwegian children (aged 9 years or older) participating in MoBa study (Surén et al., 2014).

The World Health Organization estimates that one in every 160 children (0.6%) has ASD worldwide, although the prevalence varies across countries (WHO, 2013). For example, a higher prevalence of ASD was reported in the UK in 2001 (1.2%)(Baird et al., 2006), and in the USA in 2014 (1.7%) (Baio et al., 2018). There is an increasing trend in the prevalence of ASD worldwide according to recent reports. However, it is not clear whether the increase is entirely due to a true increase in incidence, or at least in part due to better ascertainment, broadening of diagnostic criteria, or due to improved awareness (Baird et al., 2006, Fombonne, 2009, Hinkka-Yli-Salomäki et al., 2014, Jensen et al., 2014).

The incidence of ASD among boys is approximately four to five times higher than in girls (Werling and Geschwind, 2013, Hinkka-Yli-Salomäki et al., 2014, Lyall et al., 2017a), and this may point to both genetic and hormonal factors, that are known to play a role in ASD's aetiology. Environmental factors such as EDCs may affect both our genes, through epigenetic changes, and the endocrine system. Thus, the ever-increasing production and release of chemicals worldwide in recent decades may be responsible for the increasing trend in ASD and other endocrine-related adverse outcomes in children.

5.1.2. Early-life exposure to EDCs and cryptorchidism

The main findings from **Paper I,** *a cohort study of perinatal exposure to potential endocrine disrupters and the risk of cryptorchidism in the Norwegian HUMIS study*, demonstrated that

early-life exposure to breast milk concentrations of some PCBs (PCB-74, PCB-114, PCB-194) and OCPs (β-HCH) were associated with increased risk of congenital cryptorchidism and ever-reported cryptorchidism (only for PCB-194), using elastic-net regression to control for the other potential EDCs (Desalegn et al., 2021). Additional PCBs were also associated with cryptorchidism in the single pollutant analysis. None of the 27 chemicals were selected as important predictors for recurrent and persistent cryptorchidism, although some PCBs were associated in the single pollutant analysis (Desalegn et al., 2021).

EDCs

In general, no adequate epidemiologic data to date establishes a clear association between EDCs exposure during the prenatal period and the risk of cryptorchidism. The support for a link between EDC exposure and cryptorchidism based on maternal or paternal occupational exposure to EDCs, geographical proximity to areas of intensive use of EDCs, and perinatal exposure to EDCs remain inconsistent (Gurney et al., 2017). However, evidence from animal studies clearly shows induction of cryptorchidism upon exposure to anti-androgenic (phthalate esters, p,p′-DDT, p,p′-DDE, vinclozolin, flutamide) and estrogenic (diethylstilbestrol, bisphenol A) compounds during the sensitive period of testicular descent. Different mechanisms are proposed to be involved, including disruption of androgen to estrogen ratio, and interference with androgen or INSL3 secretion during prenatal life (Skakkebæk, 2002, Veeramachaneni et al., 2007, Virtanen and Adamsson, 2012, Skakkebaek, 2016, Gurney et al., 2017).

PCBs

Early-life exposure to three PCBs (PCB-74, PCB-114, and PCB-194) out of 14 PCBs were selected as important predictors of cryptorchidism in elastic net logistic regression, although several additional PCB congeners were significantly associated with congenital or recurrent cryptorchidism in the unpenalized logistic regression (**Paper I**). This demonstrates the importance of using appropriate statistical models to handle highly correlated chemicals simultaneously.

PCB-74 was significantly associated with lower serum testosterone among females in the adult Native American population (Goncharov et al., 2009). Lower androgen production, or reduced androgen to estrogen ratio, are recognized mechanisms inducing cryptorchidism in animals (Virtanen and Adamsson, 2012). Furthermore, elevated prenatal level of PCB-74 was linked

with decreased anogenital distance, among Mexican boys in a cohort study (Loreto-Gómez et al., 2018, Moreno-Mendoza et al., 2020). Decreased anogenital distance is a surrogate biomarker of androgen action during fetal life and correlates with cryptorchidism. Maternal blood concentration of PCB-74 was among 19 PCB congeners significantly positively associated with testicular cancer (a symptom of TDS) in sons of mothers compared to mothers of controls (n=44 cases/45 controls) in a Swedish study (1997-200), supporting the hypothesis about TDS and the foetal aetiology of cryptorchidism and testicular cancer (Hardell et al., 2004).

PCB-114 is a dl-PCB congener, and its effect may be linked to its activation of AhR. AhR induces various biological responses including disruption of hormonal signaling pathways and reproductive defects (Ruegg et al., 2008). Furthermore, the cross-talk between AhR and estrogen receptor (ER) at transcription level exhibited an anti-estrogenic effect (Ruegg et al., 2008). Moreover, increased rate of estrogen degradation, suppression of estrogen-induced transcription by AhR, competition with of estrogen co-factors, and exhibiting a weak estrogenic or estrogenic effect may be potential mechanisms causing cryptorchidism in male infants exposed to PCB-114 (Ohtake et al., 2003, Safe and Wormke, 2003, Mortensen and Arukwe, 2008, Helle et al., 2016). These hypotheses were the basis of **Paper III**. We did not, however, find evidence of an association between AhR activity (pg CALUX-TEQ/g lipid) measured in breast milk samples and cases of ever-reported, congenital, recurrent, or persistent cryptorchidism and controls. However, the AhR activity in breast milk was associated with PCB-114 and other dl-PCBs (PCB-105, PCB-156, PCB-157, PCB-167, PCB-189). In contrast to our findings, a Japanese (n=95 cases/334 total) and an Italian (n=58 cases/187 total) cryptorchidism case-control study investigating 384 single-nucleotide polymorphisms (SNPs) of 15 genes demonstrated that genes involved in dioxin binding (AhR and ARNT2) were associated with the risk of cryptorchidism (Qin et al., 2012). Our study was of a similar size (n=91 cases/199 total) so differences in power cannot explain the discrepancy. Similar to our finding in **Paper III**, a joint Danish (n= 39 cases/ 168 total) and Finish (n=56 cases/112 total) case-control study found no significant association between the total WHO-TEQ (dioxins + PCBs) levels measured in placenta samples and congenital cryptorchidism (Virtanen et al., 2012). Increased maternal blood concentration of PCB-114 was also significantly associated with having sons with testicular cancer in the aforementioned Swedish study (Hardell et al., 2004).

PCB-194 was another important predictor of cryptorchidism identified in **Paper I**. Neither epidemiologic evidence nor mechanistic studies so far support the specific role of PCB-194 in cryptorchidism development. The U.S. Collaborative Perinatal Project (CPP, 1959-1965) found no support for an association of serum levels from 3rd trimester pregnancy of either PCB-194 among 10 individual PCB congeners measured or their sum, comparing the highest vs lowest quartile of 230 sons with cryptorchidism and 593 sons without cryptorchidism (McGlynn et al., 2009). Likewise, the joint Danish-Finish prospective cohort study (1997-2000), found no significant difference between placental levels of either PCB-194 or 36 other PCB congeners and risk of congenital cryptorchidism among the Danish and Finnish study based on measured placenta samples (Virtanen et al., 2012).

We did not find support for any association between 11 out of 14 individual PCB congeners (PCB-105, PCB-118, PCB-156, PCB-157, PCB-167, PCB-189, PCB-99, PCB-138, PCB-153, PCB-170, and PCB-180) and cryptorchidism in our study **(Paper I)**. Virtanen *et al*. (2012) also did not find a significant difference in placental levels of all the individual PCB-congeners above with congenital cryptorchidism in the joint Danish-Finish prospective cohort study, 1997-2000. In addition, total serum levels of six of the above 11 individual PCB congeners (PCB-105, PCB-118, PCB-138, PCB-153, PCB-170, PCB-180) were not associated with cryptorchidism comparing highest versus lowest quartile in the U.S. Collaborative Perinatal Project (McGlynn et al., 2009). Similarly, both colostrum and cord blood levels of four PCB congeners (PCB-118, PCB-138, PCB-153, PCB-180) were not associated with congenital cryptorchidism in a French prospective case-control study (Brucker-Davis et al., 2008).

Other male reproductive outcomes. The epidemiologic evidence for PCBs and other symptoms of TDS other than cryptorchidism is inconclusive. A range of PCB exposure levels was associated with reduced semen quality (sperm motility) and reduced testosterone (total/free/bound) levels consistently across human studies in the USA, Sweden, Poland, Ukraine, Netherland, India, and Taiwan (Meeker and Hauser, 2010). For example, the blood concentrations of 19 out of 37 PCB were significantly higher among mothers of patients with testicular cancer than in controls (n=44 cases/45 controls) in a Swedish study (197-2000) (Hardell et al., 2004). Delayed puberty was observed in the highest PCB contaminated areas in Flanders (Belgium), in both boys and girls demonstrating how PCB may interfere with sexual

maturation and human reproduction (Den Hond et al., 2002). In contrast, a Faroes birth cohort study examining prenatal levels of PCB exposure did not find an association with delayed puberty among 196 boys at 14 years of age, however, the smaller sample size might have reduced the statistical power to detect a significant difference (Mol et al., 2002).

OCPs

β-HCH was the only important predictor of cryptorchidism among the OCPs identified in **Paper I**. The odds of having congenital cryptorchidism among Norwegian boys in the HUMIS cohort was increased by 26 % for β-HCH (OR=1.26, 95% CI: 1.03-1.53) per IQR increase concentration compared to controls. In line with our finding, serum concentration of β-HCH was associated (with increased cryptorchidism comparing below the 10th and above the 90th percentiles in a larger case-control study sampled from the US CPP study population (OR=1.6, 95 % CI: 0.7-2.6) (n=219/ 564) (Pierik et al., 2007). Similarly, higher median concentrations of β-HCH were observed in fat samples obtained from orchidopexy among German children with cryptorchidism than among controls (20.7 vs 11.2) (Hosie et al., 2000). There is also indirect evidence for β-HCH from a Spanish nested case-control study that demonstrated placenta levels of lindane, an isomer of HCH, was significantly associated with combined cryptorchidism and hypospadias cases (OR = 3.38; 95% CI, 1.36-8.38) (n=50 cases/114 controls) (Fernandez et al., 2007). Contrary to our finding in **Paper I**, Damgaard *et al*., (2006) did not find evidence of an association for β-HCH when analysing breast milk levels of 27 OCPs individually in a Danish cryptorchidism case-control study (n=62/68). However, the median concentrations (ng/g lipid) of β-HCH were higher in cases than in controls (13.6/12.3), and higher than the concentration measured in our study.

The association between β-HCH and cryptorchidism may be explained by the chemical's estrogenic properties (Coosen and van Velsen, 1989). According to the estrogen hypothesis, early-life exposure to high levels of estrogen may lead to cryptorchidism and other male urogenital conditions (Sharpe and Skakkebaek, 1993). There are some studies supporting this hypothesis. Mothers of sons with cryptorchidism had significantly higher estradiol (free and albumin-bound) in serum collected during the first trimester of pregnancy in a matched case and control study from the US Collaborative Perinatal Study (n=24 cases/ 24 controls) (Bernstein et al., 1988). Furthermore, prenatal exposure to diethylstilbestrol (DES), a synthetic estrogen

agonist drug that used to be prescribed to prevent pregnancy complications during the 1940s-70s, increased the risk of cryptorchidism and other male urogenital abnormalities according to a collaborative follow-up of three U.S. cohorts (University of Chicago) initiated by the U.S. National Cancer Institute (Palmer et al., 2009). However, the majority of studies do not support the estrogen hypothesis for cryptorchidism development (McGlynn et al., 2005, Key et al., 1996, Davies et al., 1986, McBride et al., 1991).

We also investigated four other OCPs in breast milk in **Paper I** (HCB, p,p′-DDE, p,p′-DDT, and oxychlordane), however, none were selected as a risk factor of either congenital, recurrent, persistent, or ever-reported cryptorchidism, despite animal evidence for p,p′-DDE and p,p′-DDT (Veeramachaneni et al., 2007). The median breast milk concentrations, however, were slightly higher among cases than in controls (HCB: 9.8/8.2; DDE: 49.5/48.8; DDT: 1.8/1.4; and oxychlordane: 2.9/2.3). Similarly, breast milk levels of these four individual OCPs were not associated with congenital cryptorchidism in a Danish case-control study that investigated 27 persistent OCPs (n=62/68). However, similar to our finding in **Paper I**, most (17 of 21) of the studied OCPs were in higher median concentrations (ng/g) among cases than in controls: HCB: 10.6/8.8; DDE: 97.3/83.8; DDT: 4.6/4.0; and oxychlordane: 4.5/4.1 (Damgaard et al., 2006).

Likewise, serum levels of HCB were not significantly different in a larger U.S. case-control study (n=219/ 564) selected from the US CPP study that compared below the 10th and above the 90th percentiles (Pierik et al., 2007). Serum levels of DDT and DDE were not significantly different between cryptorchidism cases and controls (n=74 cases/283 controls) in the Child Health and Development study (1959-1967, San Francisco), a longitudinal cohort of pregnancies during the time DDT was produced and used in the United States. The median serum concentration was similar for cases and controls (Bhatia et al., 2005). Similarly, no significant difference in levels of DDE and DDT were found among German sons with cryptorchidism compared to the control's median concentrations in fat samples obtained from orchidopexy; DDE (264.5/170.15), DDT (25.6/16.6) (Hosie et al., 2000). There was also no support for an association between serum oxychlordane and risk of cryptorchidism (highest quartile versus lowest: OR=0.95, 95% CI: 0.55-1.64) among mothers of 217 sons with cryptorchidism, and 557 mothers of controls in the US CPP study. The median maternal serum values of oxychlordane (μg/L) among cases and controls was 0.29 vs 0.31 (Trabert et al., 2012).

Contrary to our findings in **Paper I**, Hosie *et al*., (2000) reported a significant difference for levels of HCB in fat samples obtained from orchidopexy among German sons with cryptorchidism than controls (median HCB case/control: 61.2 vs 20.1 ng/g lipid). Even though direct comparisons of HCB concentrations cannot be made, the levels in the fat samples from orchiopexy were 2-6 times higher than breast milk concentrations in our study (median HCB case/control: 9.8 vs 10.2 ng/g lipid). Likewise, Damgaard *et al*., (2006) found a significant difference for breast milk levels of a sum of eight of the most abundant persistent OCPs (β-HCH, HCB, DDE, DDT, oxychlordane,α -endosulfan, cis-HE, and dieldrin) and the risk of congenital cryptorchidism in the Danish cryptorchidism case-control study (n=62/68). For the concentration of DDE in colostrum, a French prospective case-control study found a positive association (OR=2.16, 95% CI: 0.94-4.98) with cryptorchidism (Brucker-Davis et al., 2008). Placenta levels of DDT were also significantly associated with combined cryptorchidism and hypospadias cases in a Spanish nested case-control study (OR = 2.63; 95% CI, 1.21-5.72) (Fernandez et al., 2007). Likewise, the large U.S. CPP cryptorchidism case-control study reported modest associations for serum DDE concentration (OR=1.3, 95% CI:0.90-2.4) when comparing (highest vs lowest quintiles: 85.6 vs 21.4 µg/l) with risk of cryptorchidism(n=219 cases/552 controls) (Longnecker et al., 2002). The median concentrations of OCPs in breast milk in our study were lower than most of the studies described above, median (IQR) ng/g lipid; HCB: 10.2(8.1-12.7); DDE: 48.8 (32.4-75.8); DDT: 2.0 (1.4-3.0); and oxychlordane: 3.1(2.3-4.1), which may explain why we did not observe any effects of these on cryptorchidism. The sum of eight most common persistent pesticides (DDE, DDT, β-HCH, HCB, α -endosulfan, cis-HE, oxychlordane, and dieldrin) were significantly associated with congenital cryptorchidism in a case-control study within a joint prospective longitudinal birth cohort study in Denmark and Finland (1997-2001) (Damgaard et al., 2006).

In Norway, farmers' occupational exposure to OCPs was associated with cryptorchidism in a retrospective study using the Norwegian Medical Birth Registry and exposure information obtained from the agricultural census (Kristensen et al., 1997). Similarly, maternal occupational exposure to OCPs has been associated with cryptorchidism amongst boys whose mothers worked in greenhouses in Denmark while pregnant (Andersen et al., 2008).

PBDEs

The levels of the five brominated flame retardants (BDE-47, BDE-99, BDE-100, BDE-153, BDE-154) were not associated with the risk of cryptorchidism in our study (**Paper I**). The median values between cases and controls were also similar (Supplementary Table in Paper I). Similarly, the prospective Danish-Finish study, 1997-2001, found no significant difference in individual congeners of BDEs in breast milk comparing boys with cryptorchidism and without (N=62/68). However, there was a significant difference in the breast milk levels of the sum for BDEs 47, 153, 99, 100, 28, 66, and 154 between cases and controls (median, 4.16 vs. 3.16 ng/g fat) (Main et al., 2007). In our study, the sum of these five congeners in breast milk was still quite similar between cases and controls (median: 2.2 vs. 2.3 ng/g lipid), but lower than levels in Danish and Finish study (median, 3.52 vs. 3.44 ng/g fat respectively). Main *et al*., (2007) also studied levels of BDEs in the placenta but did not find an association with congenital cryptorchidism (n=95 cases/185 controls), perhaps because a large number of congeners were below detection in the placenta, and levels were lower than in breast milk. In line with our study, the Michigan long-term Polybrominated Biphenyl cohort study involving mothers with accidental exposure during 1973–1974 found no association for *in utero* BDE-153 exposure and self-reported cryptorchidism (N= 9/464, OR=0.7, 95% CI:0.1, 3.8) (Small et al., 2009, Bonde et al., 2016).

In contrast to our study, a case–control study in Canada found an association between BDE levels in maternal hair and cryptorchidism for BDE-99 [OR = 2.53 (95% CI: 1.29, 4.95)], BDE-100 [OR = 2.45 (95% CI: 1.31, and BDE-154 [OR = 1.88 (95% CI: 1.08, 3.28)] among eight BDE congeners studied (n=137 cases/158 controls) . The median levels in maternal hair (ng/g) samples were much higher than the levels measured in our study in breast milk (ng/g) though not directly comparable: BDE-47 [10.0 vs 1.08], BDE-99 [7.94 vs 0.28], BDE-100 [7.40 vs 0.26), and BDE-154 [4.03 vs 0.03] (Goodyer et al., 2017).

PFOS and PFOA

PFOS and PFOA were not associated with cryptorchidism in our study (**Paper I**). Similarly, a nested case-control study (N=107 cases/108 controls) from the combined Danish-Finnish prospective birth cohort found no significant association between cord blood PFOA (OR=0.46, 95% CI 0.20–1.20) and PFOS (OR=0.83, 95% CI 0.39–1.79) (highest tertile vs lowest tertile)

and congenital cryptorchidism (Jensen et al., 2013). In another Danish study based on the National Patient Registry with a large sample size (N= 270 cases/300 controls), there was no evidence of an association between PFOS concentration measured in amniotic fluid from the second trimester and cryptorchidism comparing highest tertile vs. lowest (OR=1.01, 95% CI:0.66-1.53) (Toft et al., 2016).

However, there was indirect positive evidence of an association between PFOS in amniotic fluid and biomarkers that regulate testicle descent (steroid hormone and INSL3) (Toft et al., 2016). Insulin-like factor 3 regulates the transabdominal phase (first phase) of testicular descent occurring between 8-15 weeks of gestation (Hutson et al., 2015). High exposure to PFOS in amniotic fluid from the second trimester was associated with a 40% lower INSL3 level (Toft et al., 2016), signifying a need for sensitive effect biomarker to detect sub-clinical effects.

Table 2 presents a summary of available studies on EDC exposure and cryptorchidism.

5.1.3. Anti-androgenic receptor activity and cryptorchidism

The findings from **Paper II** demonstrated that anti-androgenic activity that is not derived from natural hormones was detected or present in breast milk samples among Norwegian mothers in the HUMIS cohort (2002-2009) (Collet et al., 2021). Low anti-androgenic receptor activity was measured in the non-polar fraction compared to the polar fraction of breast milk. The median anti-androgenic activity (µg flutamide eq./g of milk) of breast milk was 0.17 (0.14-0.23) for non-polar fraction, 1.24 (0.64-1.80) for the polar fraction, and 0.50 (0.27-0.70) for mixtures of polar and non-polar breast milk fractions.

Most of the activities from the non-polar fraction fell below the LOQ value of the anti-AR CALUX bioassay. As mentioned above, four of the 27 potential EDCs (14 PCBs, 5 OCPs, 6 BDEs, and 2 PFASs) were selected as strong predictors of cryptorchidism in **Paper I**. However, analysis in **Paper III** found no association between any of the 27 persistent EDCs and anti-androgenic receptor activity in either non-polar, polar, or mixed breast milk fractions. This was contrary to studies that have reported that some of the persistent chemicals are potent androgen receptor antagonists: DDE (Kelce et al., 1995), OCPs (Lemaire et al., 2004), and a POP mixture (McComb et al., 2019).

The anti-androgenic activities in the combined (polar and non-polar mixed fractions) exhibited lower activity than the sum of the individual activities from the non-polar and polar fractions. This may be due to interactions and shows the importance of considering mixture effects in risk assessments.

In **Paper II**, we found no significant difference in anti-androgenic activity, not derived from natural hormones, measured in either non-polar, polar, or reconstituted/mixed breast milk samples among mothers according to cryptorchidism status (94 cases/105 controls). Our finding is contrary to the suggested androgen hypothesis for cryptorchidism development (Virtanen and Adamsson, 2012). However, our investigation was limited to anti-androgen receptor activity, and we cannot rule out androgen activity related to other important pathways such as synthesis, secretion, or metabolism of androgen (La Merrill et al., 2020).

There is no strong epidemiological evidence that supports the role of androgen in cryptorchidism development. For example, levels of testosterone and non-protein bound testosterone collected during the first trimester of pregnancy did not differ in a 24 matched cryptorchidism case-control study drawn from the US Collaborative Perinatal Study (Bernstein et al., 1988). A study of third-trimester serum levels of testosterone (total, free, bioavailable) among 200 mothers of cryptorchid sons and 200 mothers of non-cryptorchid sons nested within the US Collaborative Perinatal Project found no significant difference(McGlynn et al., 2005). Furthermore, a French prospective case-control study at Nice University Hospital comparing cord blood levels of testosterone drawn prospectively just after delivery reported no association between reduced testosterone and cryptorchidism (52 cases/128 controls) (Fénichel et al., 2015). In animal experiments, however, the role of androgen in cryptorchidism is supported. Cryptorchidism has been induced by the administration of an androgen antagonist, flutamide, to pregnant rats (Mizuno et al., 2007, van der Schoot, 1992).

In **Paper I,** we showed that early-life exposure to breast milk levels of **PCB-74** increased the odds of having congenital cryptorchidism by 31% (95% CI: 0.1%-70%) per IQR increase in breast milk concentrations. Previous studies have demonstrated an inverse association between serum levels of testosterone and PCB-74 in the female native American population (Goncharov et al., 2009). Furthermore, an inverse association was observed between prenatal PCB-74 exposure levels and anogenital distance a marker of androgen action that correlates with cryptorchidism,

among Mexican boys (Loreto-Gómez et al., 2018, Moreno-Mendoza et al., 2020). Although anti-androgenic activity was not associated with cryptorchidism nor with PCB-74 levels, we cannot rule out other androgen pathways (e.g. synthesis, secretion, metabolism) (La Merrill et al., 2020), so these relations require further investigation.

Although no evidence of the role of anti-androgenic receptor activity in cryptorchidism development was found, it is worth noting that Norwegian children were exposed above the permitted daily exposure (PDE) limit calculated based on the European Medicines Agency (EMEA) directive for the androgen receptor antagonist flutamide (EMEA, 2014). A PDE of 0.05 mg/d (**50 µg/d**) has been set based on reproductive and developmental 28-day toxicity studies (Zacharia, 2017). Norwegian children's estimated daily intake (EDI) via breastfeeding in the HUMIS cohort is **78 µg flutamide eq./kg** of body weight/day, assuming 150 mL/kg of milk consumption for exclusively breastfed infants (Anderson and Sauberan, 2016) and using the measured average anti-androgenic activity of 0.52 µg flutamide eq./g of milk (Paper II).

5.1.4. Aryl hydrocarbon receptor activity and cryptorchidism

In **Paper I,** we demonstrated that the largest increase in odds of having congenital cryptorchidism was for **PCB-114;** 36% per IQR increase in breast milk concentrations (OR=1.36, 95 % CI:1.05-1.77) (Desalegn et al., 2021). PCB-114 is a dioxin-like PCBs, which acts via AhR receptor activation, resulting in various toxicological responses in the body amongst others on the reproductive system (Ruegg et al., 2008). Indeed, in **Paper III**, breast milk concentration of PCB-114 and other dl-PCBs (PCB-105, PCB-156, PCB-157, PCB-167, PCB-189) were significantly associated with AhR activity (pg CALUX-TEQ/g lipid) measured in breast milk samples.

AhR activation has been linked with mainly anti-estrogenic effects, and (weak) estrogenic effects via crosstalk with the estrogen receptor at transcription level, increased degradation of estrogen, competition with estrogen co-factors, and inhibition of estrogen-induced transcription (Ruegg et al., 2008, Ohtake et al., 2003, Safe and Wormke, 2003, Mortensen and Arukwe, 2008, Helle et al., 2016). In **Paper III**, we indirectly sought to test the estrogen hypothesis in cryptorchidism and male reproductive disorders (Sharpe and Skakkebaek, 1993) using AhR activity (pg CALUX-TEQ/g lipid).

We did not find an association between AhR activities measured in breast milk samples from mothers with cryptorchid sons and controls in **Paper III,** and this is contrary to the indirect evidence we expected according to the estrogen hypothesis. However, there is no strong epidemiologic evidence supporting estrogen hypothesis (Davies et al., 1986, McBride et al., 1991, Key et al., 1996, McGlynn et al., 2005). In line with our finding the total WHO-TEQ (dioxins + PCBs) values measured from the placenta were not associated with congenital cryptorchidism in a joint Danish-Finish study (Virtanen et al., 2012). In contrast to our findings in **Paper III,** genes involved in dioxin binding (AHR and ARNT2) were associated with the risk of cryptorchidism among Japanese (n=95 cases/334) and Italian (n=58 cases/187 total) study that explored 384 SNPs of 15 genes (Qin et al., 2012).

Our finding shows that the average EDI of dioxin and dl-compounds via breast milk among Norwegian children enrolled in the HUMIS study is 33.7 ± 17.9 pg TEQ/kg body weight per day (**Paper IV**). The EDI is significantly above what is considered toxicologically safe, TDI range of 1-4 pg TEQs/kg body weight (WHO, 2000). Yet many countries are above this level according to the WHO/UNEP surveys in human milk samples from different countries (Figure 6A) (van den Berg et al., 2017a). In Norway, a decreasing trend of dioxins and dl-PCBs levels in breast milk is reported ever since their ban based on monitoring from 1986-2005 (Figure 7) (NIPH, 2016). Measures to further reduce these levels are recommended to get it below the safety margin.

5.1.5. Early-life exposure to EDCs and autism spectrum disorder

The fourth paper (**Paper IV**), "*Early-Life Exposure to Endocrine Disrupting Chemicals and Autism Spectrum Disorder: A Multi-Pollutant Analysis of Norwegian Birth Cohort and Evaluation of Developmental Neurotoxicity in Zebrafish Embryos and Larvae*", found that breast milk level of β-HCH was associated with ASD among the Norwegian children enrolled in the HUMIS cohort. Of note, is that none of the other 26 POPs studied showed an association.

Findings from the epidemiologic study

Mothers with the highest levels of β-HCH exposure had a significant and marked increased risk of having a child with ASD (OR = 1.82, 95% CI: 1.20, 2.77 for an IQR increase, 2.9-6.5 ng/g lipid, in β-HCH concentration), which is almost twice the odds. β-HCH was selected using elastic

net regression, controlling for 26 other potential EDCs. In line with our finding, a cross-sectional survey in Greece (2015-2017) comparing ASD with non-ASD ((n=39/n=18) school children observed significantly higher mean serum concentration of β-HCH in children with ASD (mean ± SD: 10.5 ± 7.7 ng/g lipid) than in controls (6.1 ± 4.0, ng/g lipid) (Makris et al., 2019). The serum concentration of β-HCH corresponds to our measurement in the HUMIS study among ASD cases (19.7 ± 61.5 ng/g lipid) and controls (5.8 ± 10.3 ng/g lipid) though not directly comparable. Epidemiologic studies assessing the link between β-HCH and neurodevelopmental toxicities are few. Previously, our group also have demonstrated a positive association between β-HCH breast milk concentrations and ADHD (OR = 1.75, 95% CI: 1.22- 2.53 for an IQR increase) (Lenters et al., 2019).

In contrast to our findings, fewer autistic behavior patterns were observed among 45-year-old children born to women with detectable versus non-detectable serum concentration of β-HCH (n = 52; β = –3.3; 95% CI: –6.1, –0.5) in the Health Outcomes and Measures of the Environment (HOME) Study (2003—2006), a prospective birth cohort among 175 women in Cincinnati, Ohio (Braun et al., 2014). However, the HOME study is not comparable to the HUMIS study in many respects: statistical analysis was based on detectable vs. nondetectable serum concentrations of β-HCH unlike continuous exposure in the HUMIS study, 73 % of the cohort had maternal serum β-HCH level below LOD compared to 0.3% in the HUMIS cohort with breast milk concentration below LOD, and the diagnosis of ASD-related behaviors in the HOME study was based on the SRS completed by the mothers, unlike the specialist-confirmed diagnoses in the HUMIS (Braun et al., 2014).

The neurodegenerative properties of β-HCH have been demonstrated following occupational and residential exposure. A case-control study at the University of Texas Southwestern Medical Center demonstrated that elevated levels of serum β-HCH (median, 0.36 ng/mL) were associated with an increased risk of Parkinson's disease, dementia, and Alzheimer's disease (Richardson et al., 2009). Similarly, another case-control study in the Faroe Islands investigating dietary exposure to food contaminants found that median β-HCH serum concentrations differed significantly across Parkinson's disease status (Petersen et al., 2008).

Sensitivity analysis for potential effect modification of β-HCH by parity showed borderline significance in the model with adjustment for parity indicated that the risk of ASD from β-HCH exposure was higher among multiparous women than primiparous women.

Associations for modelled postnatal exposures to the toxicants did not reveal any additional risks tied to the exposure during the breastfeeding period, nor was there effect modification by duration of breastfeeding, an indication that the critical window might be during the prenatal period.

PCBs and OCPs

A population-based case–control study in California; The Early Markers for Autism study (EMA) reported that maternal serum levels of three PCBs (PCB-138, PCB-153, PCB-158) and two OCPs (DDE and trans-nonachlor) were positively associated with risk of ASD, and intellectual disability without autism. They compared cases with ASD (n=545) to controls from the general population (n=418). However, the findings in the EMA study reflect single pollutant analysis, unlike our study where we used elastic net to select predictors among several correlated exposures (Lenters et al., 2018, Agier et al., 2016). Since PCBs and OCPs are highly correlated, there is also a possibility for confounding. Like the EMA study, we found association for some of mono-*ortho*-substituted dioxin-like PCBs in the single-pollutant analyses but were not selected in the multiple-pollutant analyses. Unlike in our study, β-HCH was not analyzed in the EMA study due to low detection frequency (Lyall et al., 2017b), still any correlation between β-HCH and PCBs may also have confounded the results. A small pilot Finnish prenatal Study of POPs in maternal serum reported a non-significant association between total PCBs (PCB-118, PCB-138, PCB-153, PCB-156, PCB-170, PCB-180) and risk of childhood autism (n=75 cases/75 controls) (Cheslack-Postava et al., 2013).

PFASs

Our finding suggested a non-significant positive association for both PFOA (OR=1.23, 95% CI 0.63-2.42 per IQR increase) and PFOS (OR=1.69, 95% CI 0.86-3.31 per IQR increase), with ASD in the HUMIS study, but these toxicants were not selected as predictors using elastic net. Recently, a larger Norwegian case-control study (n=400 ASD cases/980 controls) from

Norwegian Mother, Father and Child cohort study showed a positive association with PFOA, but inverse associations were reported for other PFAS (Skogheim et al., 2021). A nested case–control study Danish National Birth Cohort investigating 16 PFASs measured in maternal plasma in early or mid-pregnancy for children with ADHD (n=220) or ASD (n=220) reported no associations (Liew et al., 2015b). On the other hand, the HOME study reported a negative association between PFOA and ASD-related behavior in 4-5-year old children (Braun et al., 2014) while Lyall et al. (2018) showed positive associations between prenatal maternal serum concentrations of PFASs and ASD in the EMA study. Among the PFAS, there is a strong suggestion for the role of PFOAs in ASD development.

Our group has previously reported an increased risk of ADHD among girls associated with PFOS exposure using a multi-pollutant analysis in the HUMIS study (Lenters et al., 2019), and also in a meta-analysis of nine European population-based studies (girls, n= 82 cases/1274 controls) (Forns et al., 2020). Both ADHD and ASD are neurodevelopmental disorders that often co-occur, share some phenotypic similarities such as difficulty in language and communication. However, they are characterized by distinct diagnostic criteria and may differ in underlying biological mechanisms.

PBDEs

We found no evidence of association between breast milk levels of PBDEs and the risk of ASD in our study. In contrast, the HOME study reported a positive association between PBDE-28 and ASD-related behavior among 4-5-year old children (Braun et al., 2014) while the EMA study in California showed an inverse association between PBDE and the risk of ASD in boys (Lyall et al., 2017c). As levels of PBDEs are ten-fold higher in the US compared to Norway, these results may not be generalizable to our setting. Due to the potential correlation between PBDEs and other POPs the lack of studies investigating POPs in a multipollutant model limits comparability with our study and is of general concern since reported associations may be confounded.

Table 3 summarises available studies that explore the association between EDC exposure and ASD and related neurodevelopmental outcomes.

Findings from our study on zebrafish embryos and larvae

Neurodevelopmental toxicity of β-HCH was further confirmed in zebrafish embryos and larvae, and perturbation of dopaminergic neurone network as a potential pathway for autism development was suggested.

Findings from exposed zebrafish larvae verified β-HCH's potential to interfere with neurodevelopment. We observed an increase in the average distance between free-swimming individual larvae, an indicator of lower social interaction (a behavioral pattern similar to ASD), in larvae exposed to β-HCH. We used a dosage that we expected would give a concentration of β-HCH comparable to the concentration measured in the breast milk of mothers with ASD children in the HUMIS study.

In addition, there were significantly more proliferative cells in the optic tectum, considered the equivalent of the human cortex and a marker of ASD at this stage of zebrafish development (Nevin et al., 2010, Courchesne et al., 2003, Schumann et al., 2010). Although a direct comparison cannot be made with humans due to toxicokinetic and toxicodynamic differences, neurodevelopmental and behavioral effects were seen at a concentration as low as 0.01 μM (2.9 ng/ml) of β-HCH in the larva, corresponding to concentration most children were exposed to in the HUMIS study.

We also observed a slow touch-evoked response of larvae at 290 ng/g of β-HCH. In addition, decreased capacity of the larvae to maintain an upright position was observed at 2.9 ng/g of β-HCH. Decreased ability to maintain an upright position was suggestive of the phenotype observed in *nipsnap1* mutant zebrafish larvae with defects linked with a reduced number of dopaminergic neurons (Princely Abudu et al., 2019). Previous studies have implicated alterations in the function of the dopaminergic system in ASD (Bowton et al., 2014, Pavăl, 2017, DiCarlo et al., 2019). Further experiments on embryos exposed to L-Dopa resulted in a reduction of the hypothalamus suggesting neurotoxicity as a result of oxidative stress due to high dopamine levels (Mosharov et al., 2009). These studies indicate a possible role of dopaminergic neurons mediating the effect of β-HCH. β-HCH's U-shaped response during the experiments may indicate activation of compensatory mechanisms at the highest concentrations.

The exact mechanism by which β-HCH contributes to ASD pathophysiology is not yet known, and multiple modes of action have been suggested in other animal models. Reviews of potential mechanisms linking OCPs and ASD in animal models (mouse, rat, zebrafish) show that inhibition of $GABA_A$ receptor binding, disruption of Ca^{2+} signaling and regulation, alteration in glutamate receptors, and disruption of thyroid states are associated with ASD (Shelton et al., 2012, Wu et al., 2019). β-HCH belongs to the OCPs class, and as such, it is likely to share some of the potential mechanisms that link OCPs and ASD.

5.2. Methodological consideration

In this section, methodological issues that may apply in the interpretation of the results, and subsequent inference of the findings in the thesis are discussed in brief.

5.2.1. Choice of the statistical methods

The table below summarizes the different statistical methods of the papers (I-IV) included in this thesis. Odds ratios were used as the effect measure for the logistic regression analyses while the β coefficients were used in the case of linear regression.

Table 5. Summary of the choice of statistical methods for each paper included in this thesis.

Paper	Study design/ type	Sample size (n)	Choice of the statistical method	Missing data handling
I	Cohort	641	Multivariate logistic regression Elastic-net logistic regression	MI
II	Case-control *in vitro*	199 (94 cases /105 controls)	Multivariate logistic regression	MI
III	Case-control *in vitro*	199 (91 cases /108 controls)	Multivariate logistic regression Multivariate linear regression	MI
IV	Cohort *in vivo*	1199	Multivariate logistic regression Elastic net logistic regression Analysis of Variance (ANOVA)	MI

Missing data: We used multiple imputation (MI) by predictive mean matching (Buuren and Groothuis-Oudshoorn, 2010, White et al., 2011) to impute both missing exposure and covariate data in Papers I-IV. The final model in Paper II was based on the complete case data as sensitivity analysis using the MI data showed no difference in magnitude and precision. Summary details on the missing data are presented in the corresponding papers and the associated supplementary material. In general, 100 multiply imputed data sets were used in each paper for all analyses. There were no material differences in the estimates from the multiply imputed data compared with estimates from the complete case data in any paper.

5.2.2. Internal validity

Selection bias

Selection bias is "a systematic error in a study that stems from the procedures used to select subjects and from factors that influence the study participation. It comes about when the association between exposure and disease differ for those who participate and those who don't participate in the study" (Rothman, 2012). Selection bias can be removed by adjustment for variables that separate the selection from the outcome given the exposure, if such variables exist (Greenland and Pearl, 2011).

Apart from preterm birth, which is overrepresented (9.3% vs 5%) in the HUMIS study, other socio-demographic characteristics (maternal age, parity, smoking at the start of pregnancy, birth weight, sex of the child) are representative of the general Norwegian population.

Oversampling of preterm birth may generate selection bias as it is among the key known risk factors for the outcome cryptorchidism (Gurney et al., 2017). However, no apparent selection bias from preterm birth affected our results as sensitivity analysis showed no significant difference in magnitude and precision when further adjusting for preterm birth in the model (Paper I-III). Preterm birth separates the selection from the outcome given the exposure and may therefore be used to adjust for selection bias. However, it also removes the indirect effect of preterm on cryptorchidism (Figure 15). Similarly, there was no evident selection bias in Paper IV as estimates from models with and without preterm birth were similar in both magnitude and precision (Figure 3 and Table S2, Paper IV).

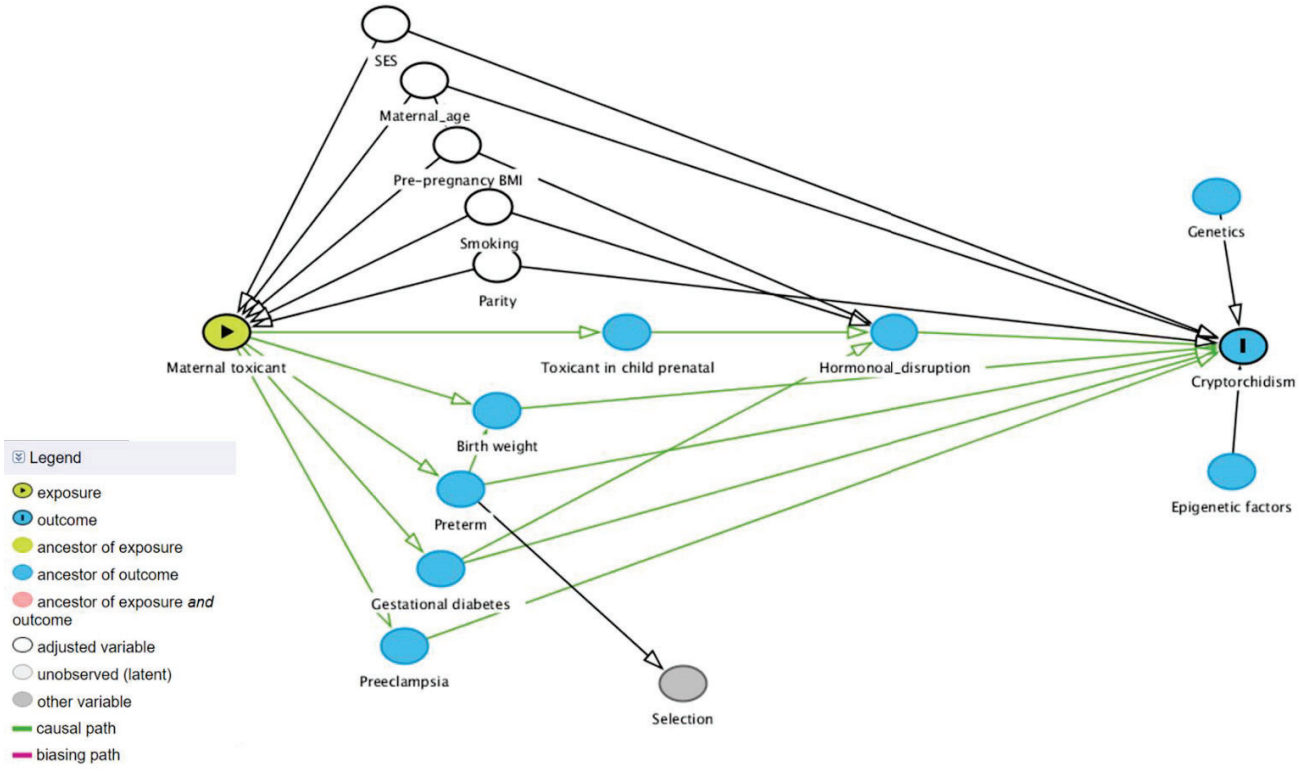

Figure 15. A simplified directed acyclic graph (DAG) for studying effect of prenatal exposure to potential EDCs on development of cryptorchidism among Norwegian sons in the HUMIS cohort, also showing selection (oversampling for preterm, and neurodevelopmental disorders).

The selection of study participants in **Paper I** and **IV** have been limited to samples with chemicals exposure already analyzed or measured (Figure 13). Not all participants have had their chemicals measured due to financial constraints, and chemical analysis varied also according to previous research questions and the project that funded it. Thus a small subset of the HUMIS study population from those with measured chemicals (n=1240) had been oversampled on preterm birth (n = 80), rapid growth (n = 77), and neurodevelopmental disorders (ADHD, ASD, and cognitive delay cases; *n* = 159). This could have led to selection bias as as the selection of neurodevelopmental disorders was not separated from the exposure given the outcome cryptorchidism, but could be removed by adjustment (Greenland and Pearl, 2011). Sensitivity analysis performed with adjustment for neurodevelopment disorders showed no significant difference in magnitude and precision of the estimates in Paper I. In Paper IV, oversampling of neurodevelopmental disorders (*n* = 159/1199 total), including the outcome ASD, was made to

increase the power of the study. As the selection was separated from the exposure status given the outcome ASD, it should not lead to bias in the odds ratio.

Other characteristics of study participants in Paper I (n=641) and Paper IV (n=1199) were representative of the entire study population enrolled in HUMIS study (n=2606): e.g., median maternal age at delivery (29-30), high maternal education (74-76%), obesity prior to pregnancy (10%), nulliparity (40-42%), small for gestational age (10-11%), and preterm births (9-10 %).

The selection of cases and controls in Paper II and III was limited to those with adequate banked breastmilk samples (>30 ml) to enable extraction and analysis. We expect the quantity or volume of milk provided to be well correlated with maternal milk production. Maternal milk production is associated with both maternal (age, parity, nutrition, anxiety and stress, acute illness, cigarette smoking, alcohol consumption, drugs) and child characteristics (birth weight, gestational age, nursing frequency) that are known to be associated with the outcome cryptorchidism (Institute of Medicine Committee on Nutritional Status During and Lactation, 1991, Gurney et al., 2017). This may have introduced selection bias similar to "healthy worker effect" bias in occupational studies that generally attenuates the effect (Arrighi and Hertz-Picciotto, 1994). In fact, the study participants in Paper II and III were characterized by healthy maternal (greater percentage of normal BMI, less preeclampsia, less smoking) and child characteristics (less preterm, less low birth weight) than the HUMIS study participants or the eligible cohort for cryptorchidism study. Therefore, we cannot rule out selection bias's role for the null findings in Paper II and III.

Loss to follow-up. Loss to follow-up is a type of selection bias almost inevitable in cohort studies that often leads to bias and affects the statistical power of the study (Rothman, 2012). Among the initial participants enrolled in the HUMIS cohort, 75% (n=2606) returned biological samples, and therefore constituted the HUMIS study (n=2606). In **Paper I-III**, repeated questionnaires were completed by the mothers after birth for outcome ascertainment. The maternal report was characterized by high response rates of 95%, 85%, 85%, and 86% at 1-, 6-, 12-, and 24-months after birth, respectively. The response rate figures fall above acceptable follow-up rates proposed by some authors (Altman, 2000). Simulations also showed that no important biases were introduced particularly when the loss to follow-up occurs randomly (Kristman et al., 2004).

Recall bias. The process for the selection of cryptorchidism cases and controls included in **Paper II** and **III** is shown in Figure 13. Recall bias or interviewer bias is common in most case-control

studies, however, these biases do not apply to our case-control studies drawn from the HUMIS study as the exposure information is based on toxicant measurement of breast milk samples. However, information about important confounders and covariates collected using questionnaires may introduce bias.

Live-birth bias. **"**Live-birth bias may arise in studies using pregnancy cohorts to investigate the impact of prenatal exposures on health outcomes that manifest only after births" (Liew et al., 2015a). The selection of the study participants in all papers included in this thesis was restricted to live-born children as the outcomes can only be ascertained after birth. Exclusion of those who did not survive till birth (stillbirths and spontaneous abortions) may introduce bias (livebirth bias) especially when exposure affects selection (Leung et al., 2021). In fact, previous studies have reported associations between spontaneous abortions and early life exposure to estrogenic and anti-androgenic EDCs such as DDE (Korrick et al., 2001), high levels of PCBs and dioxins (Tsukimori et al., 2008), and phthalate metabolites (Mu et al., 2015). Highly exposed mothers are more likely to experience spontaneous abortions and stillbirth and excluding this group may introduce differential misclassification bias that is biased toward the null or downward (Leung et al., 2021). Therefore, we cannot rule out the possibility that live birth bias influenced our findings.

Information bias

Another type of systematic error can be introduced when flawed information is collected about study participants. Such information bias can occur due to measurement errors related to the assessment of exposure, covariates, or outcome variables.

Measurement error in exposure assessment

The 27 potential EDCs (14 PCBs, 5 OCPs, 6 PBDEs, 2 PFASs) were analysed in four different laboratories (Norwegian Institute of Public Health (Oslo, Norway), the Department of Environmental Sciences, Norwegian University of Life Sciences (Ås, Norway), the Institute for Environmental Studies, Faculty of Earth and Life Sciences, VU University (Amsterdam, the Netherlands), and the Research Centre for Toxic Compounds in the Environment, Masaryk University (Brno, Czech Republic). Measurements values from different laboratories or different batches in the same laboratory can be subject to systematic errors in measurements. However, this measurement error is expected to be non-differential as it is unrelated to the occurrence of the

outcomes, and thus may have resulted in a bias of the effect estimates towards the null. Furthermore, a previous test for potential systematic difference between batches of samples analysed at different laboratories found limited evidence of 'batch effects' using principal component analysis (PCA) visualization and linear regression models; therefore no attempt was made to apply normalization methods to correct for batch effects (Lenters et al., 2019).

The precision of the analytical methods was previously shown to be sufficient when assessing the repeatability of chemical analysis in breast milk using replicates (Thomsen et al., 2010). Most of the analytical measurements were above the detection limit reducing the need for recalculating values below LOD, and the risk of misclassification bias. In **Paper IV**, exposure values were assessed after log transformation to reduce the influence of extreme values. In addition, the difference in the number of measured chemicals across class due to difference in funding led to differential statistical power among the chemicals, however, multiple imputation of missing chemical exposures improved the precision, while there was a negligible effect on the estimates.

In **Paper IV,** we estimated postnatal exposure of chemicals by using breast milk concentration of chemicals as a proxy for prenatal exposure and estimating postnatal concentration at 3, 6, 12, 18, and 24 months of age using a simple pharmacokinetic model (Stigum et al., 2015). However, measurement errors in postnatal concentration cannot be ruled out since we did not use physiologically based pharmacokinetic models (PBPK) that include various toxicokinetic parameters. The measurement error is expected to be non-differential, biasing the estimate toward no association, which may explain our null findings for postnatal exposure in **Paper IV**. The verification of the effect of β-HCH on developmental neurotoxicity in an experimental study on zebrafish embryos indicates that our results were not biased, and rules out strong effects from unmeasured/unknown confounders and provides support for a causal relation.

Measurement of anti-AR activity (Paper II) and AhR activity (Paper III) using CALUX® bioassay in breast milk extracts can also be subject to errors. Since the measurement error is not related to the outcome cryptorchidism, it is expected to be non-differential biasing the estimate toward null and could explain the null findings in Paper II and III.

Measurement error in outcome measures

The outcome measure in **Paper I-III**, cryptorchidism, was defined based on maternal reports from self-administered questionnaires at 1-, 6-, 12-, and 24- months after the birth of the child. The reliability of cryptorchidism diagnosis can be difficult even for trained observers. Thus, diagnosis based on maternal report likely has resulted in some misclassification of the outcome. However, this misclassification will be non-differential as the mothers did not have information about the chemical content of their breast milk, and thus maternal report is unrelated to the child's chemical exposure. Non-differential misclassification of the outcome cryptorchidism predictably biases the estimates towards the null or no effect. If there is no effect to begin with, then non-differential misclassification will not bias the effect estimate (Rothman, 2012). Furthermore, misclassification would be an issue if maternal reports were unreliable. The reliability of maternal reports has been validated for key birth pregnancy parameters among Norwegian mothers. Although cryptorchidism was not specifically included in the validation, the high reliability of maternal reports, in general, is likely to pertain to cryptorchidism as well (Skulstad et al., 2017). Moreover, information about the outcome cryptorchidism was inferred from multiple questions including performed or planned operation in a questionnaire, and repeated questionnaires at 1-, 6-, 12-, 24- months, increasing maternal awareness of the condition and providing the opportunity to include milder cases that mothers are more likely to recognize after some time. It also provided an opportunity to check for consistency across time.

Confounding

Confounding is also a type of systematic error that can bias the estimate in both directions. To identify potential confounders to control for in our studies, DAGs were constructed based on a priori information about the causal relationship between variables. DAGs can be used to identify variables for statistical adjustment, illustrate the source of bias, and back causal interpretation (Greenland et al., 1999).

The respective DAGs and the minimal sufficient adjustment set of covariates (confounders and/or mediators) for the total or direct effect are described in detail in the respective papers (**Papers I-**

IV). Misspecification of the DAG's causal structures due to omission or inclusion of improper covariates or pathways may bias the estimates.

Furthermore, potential residual confounding by factors that are unknown, unreported, unmeasured, or mismeasured confounders, such as emerging correlated chemicals associated with cryptorchidism (**Paper I-III**) or ASD (**Paper IV**), cannot be excluded. E-values is defined as "the minimum strength of association on the risk-ratio scale that an unmeasured confounder would need to have with both the treatment assignment and the outcome to fully explain away a specific treatment-outcome association, conditional on the measured covariates" (VanderWeele and Ding, 2017, Linden et al., 2020). In our studies, when sensitivity analysis was conducted for unmeasured confounding, the E-value for the point estimate in Paper I ranged between 1.8-2.1 for the selected chemicals, and a more robust E-value of 3.04 in Paper IV. Therefore, given the few known strong risk factors for both cryptorchidism and autism, it seems unlikely that a single unmeasured confounder could have moved the observed association to null by such magnitudes.

Since confounding from correlated chemical exposures may lead to a false positive association, elastic net variable selection method was applied in **Paper I** and **IV** (Agier et al., 2016)**.** The elastic net regression model outperforms conventional regression methods (Lenters et al., 2018) and other variable selection methods (Zou and Hastie, 2005) in the presence of highly correlated predictors. The result from experimental study in paper IV also confirms the unconfounded effect of β-HCH.

5.2.3. External validity

The generalizability of these findings to the general population of Norway may be affected by the study selection requirement for the mother's fluency in Norwegian, which may exclude immigrants or minority groups. Apart from fluency in Norwegian and the overrepresentation of

preterm birth at one recruitment site, other socio-demographic characteristics of the HUMIS study participants are representative of the general Norwegian population during the enrollment period. The generalizability of the findings in this thesis to other populations in the world is subject to a number of considerations. First, one of the main sources of exposure to persistent chemicals in Norway is seafood consumption which is high among Norwegian population. Socio-economic factors can modify the effect of the exposure, due to protective factors such as healthy diet and high Selenium. Moreover, Norwegian mothers are highly educated relative to other populations. Furthermore, postnatal exposure to children occurs via breastfeeding, and the duration of breastfeeding is very long in Norway (median 11 months in the HUMIS cohort).

5.2.4. Main strengths and limitations

Strengths

This thesis has several strengths. It combines both epidemiologic (*cohort, case-control studies*) and experimental (*in vivo, in vitro*) study designs to answer the research questions (Paper I-IV). The epidemiologic designs were used to assess associations while the experimental studies sought to verify potential mechanistic adverse outcome pathways.

The participants in the HUMIS study are enrolled from across Norway to represent the Norwegian population, and the maternal and child characteristics of the study subsets are, in general, representative of the entire enrolled cohort in the HUMIS, indicating that selection bias is unlikely.

The exposure breast milk represents early-life (both prenatal and postnatal) exposure to a mixture of many potential persistent EDCs. Large volumes of milk can be collected with little risk for the baby or the mother. As there is a good correlation between levels of persistent chemicals in breast milk, umbilical cord, and maternal serum (Cerrillo et al., 2005, Skaare et al., 1988), breast milk can be a suitable proxy for perinatal exposure as well as a good indicator of the maternal body burden of persistent compounds (**Paper I and IV**). Breast milk concentrations coupled with the duration of breastfeeding and maternal and child factors using pharmacokinetic models were used to estimate postnatal exposure in **Paper IV** up to 24 months of age which provides a better exposure assessment than the other methods in use (Stigum et al., 2015). Anti-androgenic receptor activity and aryl hydrocarbon receptor activity in breast milk were used, for the first time in our knowledge, in relation to cryptorchidism risk and represents the true endocrine disruptive properties of the breastmilk (**Paper II and III**).

An extensive list of potential confounders was available from repeated self-administered questionnaires at 1-, 6-, 12-, and 24-months after birth, and through linkage with the Medical Birth Registry of Norway. DAGs were also used to identify potential confounders for each research objective.

Twenty-seven chemicals, from four classes of chemicals (14 PCBs, 5 OCPs, 6 PBDEs, 2 PFASs) were investigated using elastic net regression as a variable selection method to reduce false-positive association from correlated co-exposures. The advantages of using elastic net (mulit-

pollutant modelling) compared with single pollutant models includes identification chemicals among correlated chemicals best predating the outcome, while also controlling for confounding in the model. However, it is not suitable for investigating the net mixture effect or non-linear dose-responses. There is no consensus on the preferred statistical methods to use when investigating health effects of exposures to multiple pollutants. The choice may depend on the purpose of the multipollutant analysis including: handling multicollinearity issues, ability to control confounding, identification of an important mixture component that drives the association, estimation of the net mixture effect, and supporting inclusion of interactions and non-linearity (Agier et al., 2016, Lazarevic et al., 2019).

The percentage of measured chemicals below the detection limit was low (<2%) for most of the chemical exposures (n=24/27) reducing the risk of misclassification bias.

The outcome ascertainment cryptorchidism in **Paper I-III** was defined based on repeated self-administered questionnaires to the mothers at 1, 6, 12 m, and 24 months after birth with several questions per questionnaire to cross-check the validity of their report. Four cryptorchidism definitions were made (congenital cryptorchidism, recurrent cryptorchidism, persistent cryptorchidism, ever-reported cryptorchidism) depending on the time points, and the spectrum of cryptorchidism severity included in the questionnaires. For example, measurement only at birth (congenital cryptorchidism) would exclude boys with a normally descended testis that subsequently ascends spontaneously (recurrent cryptorchidism). In **Paper IV**, the outcome (ASD) was based on a specialist diagnosis set at hospitals or specialist clinics via linkage to the Norwegian birth registry, previously shown as the best source of identifying children with ASD in the Norwegian population (Surén et al., 2014). Furthermore, children with neurodevelopmental disorders were oversampled for chemical analysis, increasing statistical power. The study sample in Paper IV was relatively large, including a subset of 1199 mother-child pairs and 20 ASD cases.

Limitations

This thesis also has several limitations. Due to financial constraints, only about half of the HUMIS samples were analyzed for chemicals, which has affected the statistical power. In addition, some classes of chemicals had more samples analyzed leading to different statistical power for the analysis of the different classes of chemicals. However, multiple imputation was

used to improve statistical power by bringing more measured information into the analysis and provide unbiased estimates. The non-significant difference findings in Paper II and III should be interpreted with caution, as the studies were not powered enough to detect significant differences given the small sample and effect size. Potential residual confounding from unknown, unmeasured, or mismeasured confounders may bias the estimate even though the questionnaire contained extensive lists of information.

The cryptorchidism ascertainment (**Paper I-III**) was based on maternal self-report which is not as robust as clinically diagnosed cases of cryptorchidism. However, the misclassification bias is non-differential since the mothers are unaware of their exposure status biasing the estimates towards the null. Furthermore, high reliability has been demonstrated in maternal reports from Norwegian mothers though cryptorchidism was not included (Skulstad et al., 2017).

5.3. Public health recommendations

It is of great concern that background concentrations of persistent chemicals banned decades ago in Norway have still the potential to give rise to cryptorchidism (PCB-74, PCB-114, PCB-194, and β-HCH) and autism spectrum disorder (β-HCH) in children exposed early in life.

PCBs were among the original group of 12 POPs covered by the Stockholm Convention in 2004 listed under Annex A (elimination) with specific exemptions and under Annex C (unintentional production) (Stockholm Convention, 2019). In Norway, PCBs started to be restricted in 1971 and were banned in 1979 (Skaare et al., 1988). Data from 1986-2005 shows a considerable decline in the concentration of PCBs in human milk samples among Norwegian mothers (NIPH, 2016). Even if the concentration of PCBs is declining, a recent study of six population-based birth cohort studies in Europe (n=1301) showed that Norwegian children had the highest blood concentration of PCBs (PCB-118, PCB-138, PCB-153, PCB-170, PCB-180) compared to other children in the UK, France, Spain, and Greece (Haug et al., 2018). This was explained by a longer duration of breastfeeding in Norway, and a higher rate of seafood consumption among the Norwegian population.

We found that PCB-74, PCB-114, and PCB-194 were associated with cryptorchidism (**Paper I**). Cryptorchidism is a key risk factor for testicular cancer, with at least three to four times increased

risk (Lip et al., 2013). Norway has one of the highest incidences of testicular cancer known in the world, and with an increasing trend (Cancer Registry of Norway, 2019). Furthermore, both cryptorchidism and testicular cancer are symptoms of testicular dysgenesis syndrome, share several risk factors, and have their origin in fetal development (Virtanen et al., 2005, Rajpert-De Meyts et al., 2016). Both are also linked with reduced male fertility, a condition linked to reduced quality of life. Therefore, it is important to identify and avoid all possible sources of PCB exposures as they continue to pose a threat decades after their ban in 1979 in Norway. Also, advice should be given with regard to marine foods that should be avoided by pregnant women, children, and adolescents until they have had the children, they plan to have in order to reduce the risk from exposure to POPs as low as possible.

When modelling the passage of pharmacological chemicals via milk, daily milk intake is often assumed to be 150 mL/kg for exclusively breastfed infants, although milk intake varies with age and weight of the infant (Anderson and Sauberan, 2016). Our findings in **Paper II** indicated that average anti-androgenic activity was equivalent to 0.52 µg flutamide eq./g of milk, and daily exposure to anti-androgenic flutamide eq. via breastfeeding can be estimated to be 78 µg flutamide eq./kg of body weight/day among Norwegian children. This is above the calculated permitted daily exposure (PDE) limit for the androgen receptor antagonist flutamide, a PDE of 0.05 mg/d (50 µg/d), based on no adverse effect levels (NOAELs) and lowest observed adverse effect levels (LOAELs) reported from reproductive and developmental 28-day toxicity studies (Zacharia, 2017). Exceeding the threshold during this sensitive period of life is worrying. Breastfeeding recommendations need to take this aspect into account along with the known beneficial effects of breastfeeding.

The average AhR activity, a measure of the total dioxin and dioxin-like compounds activity in breast milk, was 7.7 ± 4.1 pg CALUX-TEQ/g lipid (**Paper III)**. A declining trend (1986-2005) of the sum of dioxins and dl-PCBs levels in breast milk in Norway was previously reported (Figure 7) (NIPH, 2016). The average estimated daily intake (EDI) of dioxin and dl-compounds for the Norwegian breastfed child in the HUMIS study is calculated to 33.7 ± 17.9 pg TEQ/kg body weight per day, assuming consumption of 125 g milk/kg bw day and 3.5 % lipid composition of breast milk. This is above WHO derived TDI range of 1-4 pg TEQs/kg body weight (WHO, 2000, van Leeuwen et al., 2000), and also in line with the result of the dioxin and dl-compounds

(total TEQs (pg/g lipid) in WHO/UNEP 3rd (2000-2003) and 4th survey (2005-2010) in human milk samples from different countries (Figure 6A) (van den Berg et al., 2017a). Measures to further reduce exposure of children to dioxin and dl-compounds are thus necessary. The high AhR activities, however, were not significantly different between children with cryptorchidism cases and controls.

β-HCH was the only chemical associated with both cryptorchidism and autism among Norwegian children in the HUMIS study (**Paper I and Paper IV**). In zebrafish larva, neurodevelopmental and behavioral effects were observed at a low β-HCH concentration (2.9 ng/ml) which corresponds to the lowest exposure quartile in the breast milk among the study participants. However, direct comparisons can't be made because of toxicokinetic and toxicodynamic differences between species.

β-HCH used to be a component of the pesticide technical hexachlorocyclohexane (HCH), a blend of four isomers (70% α-HCH, 6% β-HCH, 15% γ-HCH, and 6% δ-HCH). Technical HCH was banned in most countries, and in the 1970s in the EU (EFSA, 2005). β-HCH is also a byproduct of lindane (γ-HCH) production. For each ton of lindane produced, 6-10 tons of β-HCH or α-HCH is created. Lindane was used in forestry and agriculture in Norway until it was banned in 1992 (coming into force in 1994) (Økland et al., 2005). In the EU, the use of lindane was allowed until 2008. Lindane (γ-HCH), β-HCH, and α-HCH were all included in the Stockholm Convention on POPs in 2009, aiming for the global elimination of these substances. However, β-HCH is still detected in the environment and the food chain owing to a long half-life of 7 years (Olsen et al., 2007, Bu et al., 2015, To-Figueras et al., 2000, Jung et al., 1997), transboundary air pollution, as well as from decomposition and leakage from landfills. Thus human exposure can be expected to remain substantial also in the years ahead (Økland et al., 2005). β-HCH is the most stable isomer against environmental degradation and also demonstrates the greatest chronic toxicity among the isomers (Jackovitz and Hebert, 2015).

The estimated daily intake of β-HCH among Norwegian children through breastfeeding in the HUMIS cohort is calculated to be *0.03 µg/kg of bw/day*, assuming consumption of 125 g milk/kg bw/ day and 3.5 % lipid composition of breast milk (van den Berg et al., 2017a). Based on a multi-generation study in male rats, the Dutch National Institute for Public Health and the Environment (RIVM), estimated TDI for β-HCH to be 0.02 µg/kg of bw/day. Thus, the average

consumption of β-HCH via breastfeeding in Norwegian children is above this threshold. However, no internationally agreed health-based guidance values are currently set for β-HCH (European Food Safety Authority, 2020). Low doses of EDCs such as the pesticides residue β-HCH may be enough to elicit a response, may exhibit non-linear dose-response relationships, and may produce permanent developmental effects (Bergman et al., 2013).

6. Conclusion

Early-life exposure to potential EDCs is associated with adverse child health outcomes among Norwegian children. PCBs (PCB-74, PCB-114, and PCB-194) and β-HCH were selected as important risk factors for congenital cryptorchidism while only β-HCH was selected for autism spectrum disorder (**Paper I and Paper IV**). No internationally agreed health-based guidance values are currently set for β-HCH, however, the EDI of β-HCH among Norwegian children during the exclusive breastfeeding exceeds RIVM's estimated TDI for β-HCH (0.02 µg/kg of bw/day).

Multipollutant analysis, using elastic net variable selection method, demonstrated the importance of using appropriate statistical models to handle highly correlated chemicals simultaneously to reduce false-positive associations, as we observed marked different results in the single pollutant analyses in both **Paper I and Paper IV**.

We found evidence for neither anti-androgenic receptor activity (**Paper II**) nor aryl hydrocarbon receptor activity's (**Paper III**) role in the development of cryptorchidism among Norwegian sons. Both anti-AR activity (µg flutamide eq./g of milk) not derived from natural hormones and AhR activities (pg CALUX-TEQ/g lipid) were not only present in breast milk samples, but also were above the permitted daily exposure limit among Norwegian children during exclusive breastfeeding period. This should be taken into consideration in the future risk-benefit assessment of breastmilk. Furthermore, anti-androgenic activity in a reconstituted breastmilk fraction mixed from non-polar and polar fraction was smaller than the sum of the individual fraction demonstrating a possible interaction, and the importance of basing risk assessments on or real-life mixtures (**Paper II**).

The average AhR activity in breast milk shows the declining trend of dioxin and dl-PCBs in Norway even if it remains above what is considered toxicologically safe (TDI range of 1-4 pg TEQs/kg body weight). All dl-PCBs, and some of the ndl-PCBs (PCB-74, PCB-180, PCB-194), and two of the OCPs (HCB, β-HCH) were associated with AhR activity (pg CALUX-TEQ/g lipid) in breast milk whereas the association was null for PBDEs and PFASs (**Paper III**).

An *in vivo* experimental study on zebrafish embryo and larvae verified the neurodevelopmental toxicity of β-HCH at concentrations corresponding to the lower quartile levels found in breast

milk among Norwegian mothers with autistic children in the HUMIS study (**Paper IV**). The potential role of dopaminergic neurons in mediating the effect of β-HCH was also demonstrated. The neurotoxicity of β-HCH, at levels infants are commonly exposed to, has important public health implications.

7. Suggestions for future research

Further mechanistic studies on PCBs (PCB-74, PCB-114 and PCB-194) and β-HCH, and their role in cryptorchidism development are necessary to confirm our findings and elucidate how these selected EDCs may lead to cryptorchidism development (**Paper I**).

Our attempt to confirm the androgen hypothesis (**Paper II**) and the oestrogen hypothesis indirectly using AhR activity (**Paper III**) in the aetiology of cryptorchidism development failed. However, the case-control studies for **Paper II and III** had several limitations, and future research needs to take this into account. First of all, investigation on larger sample size is required. In addition, there is evidence of selection bias

Furthermore, other pathways in the steroid hormones (synthesis, storage, release, metabolism) need to be investigated to rule out both the androgen and oestrogen hypothesis in cryptorchidism in humans.

Our study also points to the possibility that previous reports of single POPs being related to ASD may have been confounded by β-HCH (**Paper IV**). Future studies on POPs and ASD should include the assessment of β-HCH despite low background levels. Moreover, future epidemiologic studies analyzing highly correlated chemicals simultaneously should use the various variable selection methods to avoid false-positive associations.

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Papers I-IV

Paper I

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A case-cohort study of perinatal exposure to potential endocrine disrupters and the risk of cryptorchidism in the Norwegian HUMIS study

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ABSTRACT

Background: Exposure to endocrine-disrupting chemicals (EDCs) during the critical period of testicular descent may increase the risk of cryptorchidism and male fertility.
Objective: To investigate 27 potential EDCs measured in breast milk as a proxy for perinatal exposure and the risk of cryptorchidism in a prospective cohort.
Method: The Norwegian Human Milk Study (2002–2009) enrolled 2606 mother-infant pairs, of which 1326 were mother-son pairs. In a case-cohort design, we studied 641 male infants who had 27 EDCs already quantified in milk samples: 5 organochlorine pesticides, 14 polychlorinated biphenyls (PCBs), 6 brominated flame retardants, and 2 poly- and perfluoroalkyl substances. We defined cases of congenital, recurrent, persistent and ever-reported cryptorchidism based on questionnaires mothers completed when children were 1, 6, 12 and 24 months old. Variable selection via elastic net logistic regression identified the best cryptorchidism predictors while multivariable logistic regression models determined their effect estimates.
Results: The prevalence of reported congenital cryptorchidism was 6.1%, with half spontaneously descending within six months of birth, after which prevalence stabilized between 2.2 and 2.4%. The ever-reported prevalence of cryptorchidism at 1, 6, 12, or 24 months was 12.2%. Elastic net models identified PCB-74 (OR = 1.31, 95% CI: 1.001–1.703), PCB-114 (OR = 1.36, 95% CI: 1.05–1.77), PCB-194 (OR = 1.28, 95% CI: 1.03–1.53) and β-HCH (OR = 1.26, 95% CI: 1.03–1.53 (per interquartile range increase in concentration of EDCs) as best predictors of congenital cryptorchidism. No EDCs were selected for either recurrent or persistent cryptorchidism, and only PCB-194 was selected by elastic net for ever-reported cryptorchidism (OR = 1.23, 95% CI: 1.01–1.51), in contrast to unpenalized multivariable logistic regression, where most of the individual congeners of PCBs showed significant associations.
Conclusion: In the largest multi-pollutant analysis to date considering potential confounding from co-exposure to other chemicals, perinatal exposure to PCB-74, PCB-114, PCB-194 and β-HCH were associated with increased odds of congenital cryptorchidism. Many PCBs may falsely be associated with cryptorchidism when assessed individually, due to confounding by highly correlated chemicals. Experimental studies are warranted to confirm our findings.

1. Introduction

Cryptorchidism, undescended testes, is one of the most common urogenital abnormalities in newborn males (Batra et al., 2021). It represents the failure of either one or both testes to fully descend to a normal position at the scrotum's base and is strongly associated with

Abbreviations: BDE, brominated diphenyl ether; CPP, The U.S. Collaborative Perinatal Project; DAG, directed acyclic graph; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; HUMIS, The Norwegian Human Milk Study; IQR, interquartile range; LOD, limit of detection; LOQ, limit of quantification; NDL, (non)-dioxin-like; P, percentile; PBDE, Polybrominated Diphenyl Ether; PCB, polychlorinated biphenyl; PFASs, poly- and perfluoroalkyl substances; PFHxS, perfluorohexane sulfonate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; β-HCH, Beta-hexachlorocyclohexane.

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reduced male fertility and testicular cancer later in life (Gurney et al., 2017). The prevalence of cryptorchidism in children born full-term varies and can be influenced by the age of diagnosis (ranging from birth to one year) or the diagnostic criteria used to assess the spectrum of disease severity (retractile testis for example). Overall, 1–9% of full-terms baby boys are born with cryptorchidic testis, while the prevalence estimates are higher in preterm deliveries (1–45%) (Sijstermans et al., 2008, Virtanen et al., 2007). A spontaneous descent of testis occurs during the first 3 months of life in approximately half of the cases (Berkowitz et al., 1993, Gurney et al., 2017).

Prospective clinical studies have reported an increasing trend in the prevalence of congenital cryptorchidism in the UK (2.7–4.1%) and Denmark (1.8–8.4%) since the 1950s, which correlates with an increase in persistent chemicals in the environment (Juul et al., 2014, Virtanen and Toppari, 2008). In Norway, the prevalence of cryptorchidism, based on the Medical Birth Registry of Norway (MBRN), has been approximately 0.3% since the early 1970s, although registry-based estimates are prone to underreporting (Brantsæter et al., 2016).

While the exact cause of cryptorchidism remains unknown, identified risk factors include maternal smoking during pregnancy, birth weight, gestational age, family history of cryptorchidism, and genetics, and, to a lesser extent, maternal age, alcohol or caffeine consumption during pregnancy, pregnancy medications, recreational drug use, analgesics, parity (primiparous mothers) (Gurney et al., 2017). In addition, exposure to endogenous hormones and environmental endocrine disrupting chemicals (EDCs), have been suggested as risk factors for cryptorchidism (Gurney et al., 2017). EDCs with estrogenic effects (bisphenol A (BPA), DDT, some PCBs, phyto-estrogens, and phenols), and anti-androgenic effects (DDE, phthalate, and vinclozolin) have been directly linked with cryptorchidism in experimental animal studies (Skakkebæk, 2002, Virtanen and Adamsson, 2012). In humans, levels of some EDCs (BPA, phthalates, PFOS) were shown to correlate with insulin-like factor 3 (INSL3), regulator of testicular descent (Chevalier et al., 2015, Toft et al., 2016).

Several epidemiologic studies show an association between cryptorchidism in humans and, for example, prenatal exposure to diethylstilbestrol (DES) (Virtanen and Adamsson, 2012), maternal exposure to phthalates (Wagner-Mahler et al., 2011), BPA (Komarowska et al., 2015), and maternal residential pesticide (atrazine) exposure (Jørgensen et al., 2014; Agopian et al., 2013). Other studies found no association when they investigated individuals EDCs, but did find effects for a sum or combination of organochlorine pesticides (Damgaard et al., 2006), sum of PCB congeners (Brucker-Davis et al., 2008, Koskenniemi et al., 2015), and sum of PBDEs (Main et al., 2007), while others did not find any association for the sum of 11 PCBs in maternal serum from third trimester (McGlynn et al., 2009), placental levels of 37 PCBs or 17 dioxins (Virtanen et al., 2012). Furthermore, a meta-analysis that summarized 10 case-referent studies investigating different EDC exposures found no increased risk of cryptorchidism and EDC exposure (Bonde et al., 2016). Most of the studies to date have focused on a limited number or class of chemicals as exposure, used mainly congenital cryptorchidism as an outcome, or used single pollutant analysis, which is prone to potential confounding from co-exposure to other chemicals.

We therefore simultaneously investigated the association between exposure to 27 potential EDCs (four class of chemicals) measured in breast milk as a proxy for perinatal exposure and the risk of congenital, recurrent, persistent, and ever-reported cryptorchidism among Norwegian boys in the HUMIS birth cohort using both multi-pollutant analysis (elastic net penalized logistic regression) and single pollutant analysis (multivariable logistic regression)

2. Methods

2.1. Study population

The Norwegian Human Milk Study (HUMIS, 2002–2009) is a prospective multi-center birth cohort of 2,606 mother-infant pairs. It was established to measure levels of persistent organic pollutants (POPs) in breastmilk and to investigate possible health effects associated with high levels. The HUMIS cohort has previously been described in detail (Eggesbø et al., 2009). Briefly, public health nurses recruited new mothers between 2003 and 2009 during routine postnatal care home visits around two weeks postpartum in seven counties across Norway (see Supplementary Table S1 for details of the counties). 21.5% of the mother–child pairs were recruited in 2002–2005 by a pediatrician at the maternity ward in Østfold hospital in Southern Norway, two term births for every preterm birth (Eggesbø et al., 2009). Mothers from all counties followed the same protocol and completed the same questionnaires, regardless of the recruitment procedure.

Among the 2,606 participants enrolled in the HUMIS study, the present study included 1,262 mother-son pairs for prevalence estimates after excluding mother-daughter pairs, uncertain or missing outcomes, and non-singletons. For the study of cryptorchidism with potential EDCs exposure, we used a case-cohort design, restricting our analysis to a subset of 641 mother-son pairs where up to 27 chemicals had been measured in the mothers' breast milk samples (Fig. 1).

The study was approved by the Norwegian Data Inspectorate (ref. 2002/1398), and the Regional Ethics Committee for Medical Research (ref. S-02122). Informed consent was also obtained from all participating women prior to enrollment.

2.2. Outcome assessment

Cryptorchidism was mapped from self-administered questionnaires filled out by the mothers at 1, 6, 12, 24 months. For the purpose of this study, cryptorchidism was defined based on the timing of the presentation as either:

1. Congenital cryptorchidism
   - Cryptorchidism based on mother's report at one month after birth
2. Recurrent cryptorchidism
   - Cryptorchidism at birth that spontaneously descends and then re-ascends
3. Persistent cryptorchidism
   - Cryptorchidism reported both at age 1 and 2 years, including receipt of orchiopexy
4. Ever-reported cryptorchidism
   - Cryptorchidism reported at 1, 6, 12 or 24 months.

2.3. EDCs exposure assessment

The mothers were asked to collect 25 mL of breastmilk each morning on eight consecutive days two weeks after birth and before the child reached two months of age, in line with the WHO recommendation (WHO, 2007). Minor deviations in this sampling protocol, such as collection by breast pump, were accepted. The pooled milk samples were stored frozen in a 250 mL natural High-Density Polyethylene (HDPE) packaging container. The date and time of collection were recorded, as well as whether a breast pump had been used. When the packaging container had been filled, participants mailed it by regular mail, except in the county of Østfold (n = 171, 26.7% in this cryptorchidism study), where the milk samples were collected by study personnel and kept frozen during transport to the Norwegian Institute of Public Health biobank. The different modes of transport of milk samples was not expected to affect EDCs concentration as persistent chemicals are able to withstand severe conditions. The HDPE packaging container (Cat. No.: 967-21244, Thermo Scientific Nalgene®) was made from food-grade high-purity resins. Moreover, the containers were recently tested for potential migration of chemicals (Collet et al. 2020).

Milk was sampled at a median of 33 (10th–90th percentile: 18–57) days after delivery in all counties. More than 90% of the samples were sampled after two weeks and before the child turned 2 months of age.

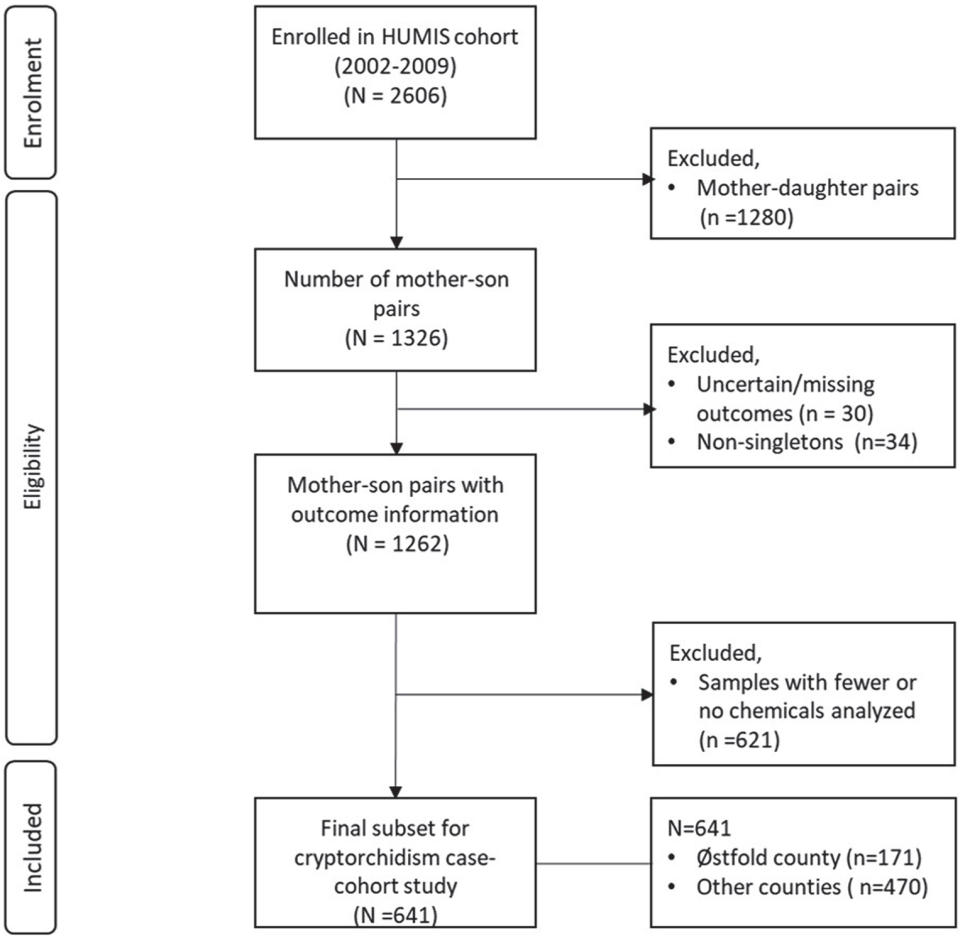

Fig. 1. Flow chart showing selection of the participants into the cryptorchidism study from the HUMIS cohort (2002–2009, Norway).

The median age (10th–90th percentile) in days at the time of breast milk sampling was 29 (10–65) and 34 (22–56) days in Østfold and other counties, respectively. The concentrations of 27 potential EDCs: 5 organochlorine pesticides (OCPs; β-HCH, HCB, p,p′-DDE, p,p′-DDT), 14 polychlorinated biphenyls (PCBs; PCB-105, PCB-114, PCB-118, PCB-156, PCB-157, PCB-167, PCB-189, PCB-74, PCB-99, PCB-153, PCB-170, PCB-180, PCB-194, and PCB-138), 6 polybrominateddiphenyl ethers ((P)BDEs; BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, and BDE-154), and 2 poly- and perfluoroalkyl substances (PFASs; PFOA, PFOS) were quantified in breast milk samples. Four laboratories took part in the chemical analyses for the PFASs, PBDEs, PCBs, and OCPs, as previously described (Forns et al., 2015, Polder et al., 2009, Thomsen et al., 2010, Forns et al., 2016, Cechova et al., 2017, Čechová et al., 2017): the Department of Environmental Exposure and Epidemiology, Norwegian Institute of Public Health (Oslo, Norway), the Department of Environmental Sciences, Norwegian University of Life Sciences (Ås, Norway), the Institute for Environmental Studies, Faculty of Earth and Life Sciences, VU University (Amsterdam, the Netherlands), and the Research Centre for Toxic Compounds in the Environment, Masaryk University (Brno, Czech Republic). Breast milk lipid levels were quantified gravimetrically during chemical analysis. See Supplementary Methods for detailed analytical methods. Exposure values falling below limit of detection (LOD) were replaced by randomly generated numbers between zero and LOD. The concentrations of the EDCs in breast milk are lipid adjusted (ng/g), except for PFASs where concentrations are wet weight (ng/L).

2.4. Covariates

We obtained information on potential confounders, mediators and other covariates from questionnaires that the mothers completed at 1, 6, 12, and 24 months postpartum and the Medical Birth Registry of Norway. Continuous variables included maternal age (years), pre-pregnancy BMI (kg/m^2), parity, birth weight (g), and gestational age (days). Preterm (yes/no), maternal education (low/medium/high), smoking status (no smoking/occasional smoking/daily smoker less than or equal to 10 cigarettes/daily smoker more than 10 cigarettes), gestational diabetes (yes/no), and preeclampsia (yes/no) were categorical variables. Information on child's sex, gestational age, birth weight, and maternal smoking during pregnancy were obtained from the Medical Birth Registry of Norway (Skjaerven et al., 2000).

2.5. Statistical analysis

2.5.1. Adjustment models

We estimated the effect of EDC exposure on the development of cryptorchidism for both unadjusted (crude estimate) and controlling for appropriate confounders (adjusted model) identified by a directed acyclic graph (DAG). Adjustment for the total effect included the following cofounders: maternal education (low, medium, high), maternal age (continuous), pre-pregnancy BMI (kg/m^2), smoking (yes/no), and nulliparity (yes/no) (Supplementary Fig. S1).

We used multiple imputation by chained equations with predictive mean matching (Buuren and Groothuis-Oudshoorn, 2010;(White et al., 2011) to impute missing exposure data (missing due to no chemical

analysis of milk samples: ≤3.3% for 13 chemicals, 13–18% for 12 chemicals, 25–28% for 2 chemicals, and missing covariate data (≤2.8%) up to the full sample size of 641. Details of the missing summary is presented in Supplementary Table S4. We generated 100 multiply imputed data sets, which we used for all analyses.

2.5.2. Single and multi-pollutant variable selection and effect estimation

We used separate ordinary least squares logistic regression models for the single pollutant analysis to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for congenital, recurrent, persistent and ever-reported cryptorchidism, controlling for the potential confounders identified by the DAG. For the multi-pollutant analysis, we used elastic net logistic regression (Zou and Hastie, 2005), a variable selection method to identify important predictors among a high number of correlated exposures, reducing the potential for multicollinearity (Lenters et al., 2018). High correlation between exposures is a challenge in conventional regression methods as they cannot differentiate between true predictors from correlated variables (Agier et al., 2016). Elastic net logistic regression model as a variable selection method that outperforms prediction of an outcome in the presence of strongly correlated predictors (chemical exposures) compared to conventional logistic regression (Lenters et al., 2018), or other regularization and variable selection methods such as Lasso (Zou and Hastie, 2005). The potential confounders were forced into the elastic net model unpenalized while the optimal level of penalization for the correlated 27 chemical exposures was determined using the default 10-fold cross-validation. The analysis was repeatedly performed in each of the multiply imputed datasets (n = 100) and an exposure considered selected if it was retained in at least 50% of the imputed sets. In the second step to obtain unbiased estimates, we then refit the selected subset of chemicals in an ordinary multi-pollutant logistic regression model. We used Stata (version 16.0; Stata Corp LP, College Station, Texas, USA) for all statistical analyses.

2.5.3. Sensitivity analyses

For the final adjusted model, we conducted a number of sensitivity analyses. We ran the model on the complete case dataset for comparison with the multiple imputation dataset. Furthermore, we assessed, individually, the effect of further adjusting for preterm birth (yes/no), use of breast pumps for milk collection (yes/no), timing of milk collection (days), and other reported congenital anomalies (including hypospadias, yes/no). For the elastic net selected EDCs, we also assessed potential effect modification by maternal education, nulliparity, and preterm birth in models including main effects and cross-product terms, with a Wald test *p*-value of <0.20 suggestive of an interaction.

3. Results

3.1. Maternal and son characteristics of the entire eligible HUMIS cohort and the cryptorchidism case-cohort study participants.

The maternal and son characteristics of the cryptorchidism study participants (n = 641) was representative of the entire HUMIS cohort (n = 2606) with respect to median maternal age (29 vs 30), high maternal education (74.1% vs 74.9%), nulliparity (43.5% vs 42%), small for gestational age (11.1% vs 10.0%), and preterm births (9.7% vs 9.3%) respectively (Table S1).

Among the entire eligible HUMIS cohort participants (n = 1262), the prevalence of reported congenital cryptorchidism was 6.1%, while the prevalence for recurrent cryptorchidism was 8%, 1.6% for persistent cryptorchidism, and 12.2% for ever-reported cryptorchidism at any one of the time points (1, 6, 12, 24 months) (Table S2). 56% of the congenital cryptorchidism cases descended spontaneously within the first 6 months of birth, after which the prevalence stabilized between 2.2 and 2.4%. There were twice as many cases of unilateral cryptorchidism (1.4%) than bilateral cryptorchidism (0.7%) according to the report at 12 months of age. Orchiopexy was planned or performed in three out of 12 babies with reported persistent cryptorchidism up to two years of age despite the recommendation for operation between six and 18 months of age (Batra et al., 2021). The rate of surgery in the recommended age range is also less than one-third in Australia & other populations (Schneuer et al., 2016).

Table 1 shows the socio-demographic characteristics for congenital, recurrent, persistent, and ever-reported cryptorchidism for the case-cohort study subset (n = 641). For ever-reported cryptorchidism, 73.6% of the mothers were between 25 and 35 years of age at delivery, 74% had higher education, close to 4% smoked cigarettes daily, and about 10% of the mothers were obese prior to pregnancy. Approximately 10% of the children were small for gestational age, and or preterm, due to the oversampling of preterm in Østfold County

Fig. 2 shows the boxplot distributions of the 27 EDCs measured in breast milk of mothers in the case-cohort cryptorchidism study, while numerical details (median, IQR, maximum) comparing levels from mothers with and without a cryptorchidic son is described in Supplementary Table S3. The highest breast milk concentrations within each of the four chemical classes were observed for PCB-118 (62.2 ng/g, among mono-ortho DL-PCBs), PCB-153 (296 ng/g, among non-DL PCBs), DDE (1280 ng/g), BDE-47 (73.6 ng/g) and PFOS (484.5 ng/L) (Supplementary Table S3).

The Pairwise Pearson correlations between the 27 EDCs showed 26.5% strong ($r_p \geq 0.75$), 15.1% moderate ($0.5 \leq r_p < 0.75$), and 10.6% low ($0.25 \leq r_p < 0.50$) correlations. In general, there was clustering by chemical class with moderate to strong correlation within chemical classes, low to moderate correlation between PCBs and OCPs, and weak or no correlations between BDEs and PFASs (see Fig. S2).

3.2. Association with cryptorchidism

In the ordinary least squares logistic regression, individual congeners of some of the seven DL-PCBs were significantly associated with congenital cryptorchidism (PCB-114, PCB-156, PCB-157), recurrent cryptorchidism (PCB-114, PCB-118, PCB-167), and ever-reported cryptorchidism (PCB-114). Likewise, some NDL-PCBs were also significantly associated with congenital cryptorchidism (PCB-74, PCB-194), recurrent cryptorchidism (PCB-153, PCB-170, PCB-180, PCB-138), and ever-reported cryptorchidism (PCB-194). None of the PCBs were associated with persistent cryptorchidism (Fig. 3). Among OCPs, β-HCH was the only one significantly associated with congenital cryptorchidism. There were no association for individual congeners of BDEs or PFASs with any of the cryptorchidism definitions. Details of the ordinary least squares regression results for each of the four outcome definitions with each of the 27 chemicals for crude estimate (unadjusted) and adjustment for confounders (total effect) are shown in Supplementary Tables S5–S8.

3.3. Variable selection result

The multipollutant analysis based on elastic net logistic regression selected PCB-74, PCB-114, PCB-194 and β-HCH in at least 50% of the imputed datasets (n = 100 imputations) as the best predictors among the 27 chemicals for congenital cryptorchidism. In the non-penalised multipollutant logistic regression model, the effect estimates were: OR = 1.31, 95% CI: 1.00–1.70 (p = 0.049) for PCB-74, OR = 1.36, 95% CI: 1.05–1.77 for PCB-114, OR = 1.28, 95% CI: 1.03–1.59 for PCB–194 and OR = 1.26, 95% CI: 1.03–1.53 for β-HCH per IQR increase in breast milk concentrations. No EDCs were selected for either recurrent or persistent cryptorchidism while PCB-194 (OR = 1.19, 95% CI: 1.01–1.41) was selected as the best predictor of ever-reported cryptorchidism in 52% of the imputed datasets (Table S9). The IQR varied from 81 to 124% change in concentration, see Supplementary Table S3 for units corresponding to IQR.

Table 1
Socio-demographic characteristics of mother-son pairs (N (%)[a] or median (IQR)[b]) by cryptorchidism case definitions included in the case-cohort study of EDCs and cryptorchidism in the HUMIS cohort (n = 641, 2002–2009, Norway).

		Total N = 641	Congenital cryptorchidism N = 26/485[c]	Recurrent cryptorchidism N = 11/185	Persistent cryptorchidism N = 9/500	Ever-reported cryptorchidism N = 77/641
Maternal age (years)						
	<25	101	5 (1.0%)	1 (0.5%)	2 (0.4%)	10 (1.6%)
	25–35	472	18 (3.7%)	8 (4.3%)	6 (1.2%)	58 (9.0%)
	>35	68	3 (0.6%)	2 (1.1%)	1 (0.2%)	9 (1.4%)
Maternal education (years)						
	<12 yrs	59	3 (0.6%)	0 (0.0%)	1 (0.2%)	9 (1.4%)
	12 years	92	4 (0.8%)	1 (0.5%)	3 (0.6%)	11 (1.7%)
	>12 years	475	19 (3.9%)	9 (4.9%)	5 (1%)	54 (8.4%)
	Missing	15	0 (0.0%)	1 (0.5%)	0 (0.0%)	3 (0.5%)
Birth weight (grams)						
	<2500	33	0 (0.0%)	3 (1.6%)	3 (0.6%)	6 (0.9%)
	2500–4000	437	22 (4.5%)	7 (3.8%)	5 (1%)	54 (8.4%)
	>4000	171	4 (0.8%)	1 (0.5%)	1(0.2%)	17 (2.7%)
Nulliparity		276	14 (2.9%)	7 (3.8%)	3 (0.6%)	28 (4.4%)
Gestational age (days)		641	283 (276–290)	282 (257–284)	271 (255–272)	280 (271–288)
Small-for gestational age		71	3 (0.6%)	1 (0.5%)	2 (0.4%)	8 (1.2%)
Preterm		62	1 (0.2%)	3 (1.6%)	3 (0.6%)	11 (1.7%)
Caesarean section		107	4 (0.8%)	2 (1.1%)	3 (0.6%)	10 (1.6%)
Smoking		78	3 (0.6%)	2(1.1%)	1(0.2%)	7 (1.3%)
Pre-Pregnancy BMI (kg/m^2)						
	Under weight (≤18.4)	22	0 (0.0%)	0 (0.0%)	0 (0%)	0 (0.0%)
	Normal (18.5–24.9)	386	17 (3.5%)	7 (3.8%)	4 (0.8%)	48 (7.5%)
	Overweight (25–29.9)	151	5 (1.0%)	4 (2.2%)	2 (0.4%)	15 (2.3%)
	Obese (≥30)	64	3 (0.6%)	0 (0.0%)	2 (0.4%)	11 (1.7%)
	Missing	18	1 (0.2%)	0 (0.0%)	1(0.2%)	3 (0.5%)
Preeclampsia		29	2 (0.4%)	0 (0.0%)	2 (04%)	8 (1.2%)

Note: BMI: body mass index; IQR: interquartile range.
[a] N (%)) is reported for binary or categorical variables from the total mothers who responded to the questionnaire for each cryptorchidism definition in the column.
[b] Median (IQR) is reported for continuous variables.
[c] The denominator in each column represents the number of mothers who responded to the specific questions related to cryptorchidism presentation.

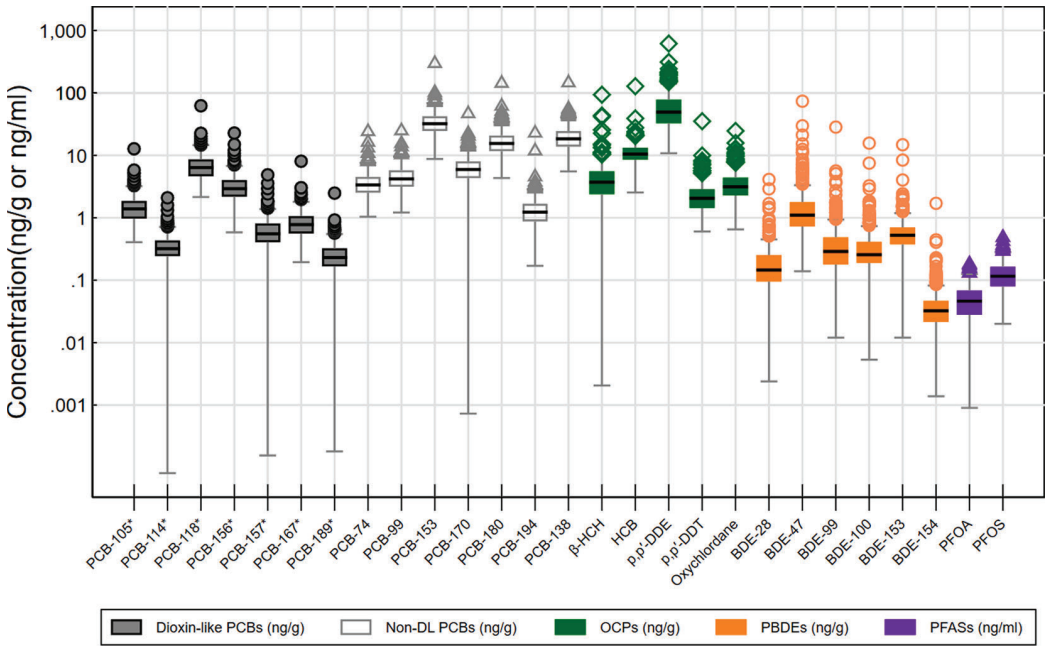

Fig. 2. Boxplot distribution of 27 EDCs found in breast milk among 641 mother-son pairs in the HUMIS cohort (2002–2009, Norway). Horizontal lines correspond to medians, and boxes to the 25th–75th percentiles; whiskers extend to data within the interquartile range times 1.5, and data beyond this are plotted as dots. Wet weight concentrations are presented for PFASs (ng/L) and lipid adjusted concentrations for all other chemicals (ng/g lipid). See Table S3 for numerical values. **Abbreviations:** BDE, brominated diphenyl ether; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; HCB, hexachlorobenzene; β-HCH, Beta-hexachlorocyclohexane; BDE, Brominated Diphenyl Ether; PCB, polychlorinated biphenyl; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate.

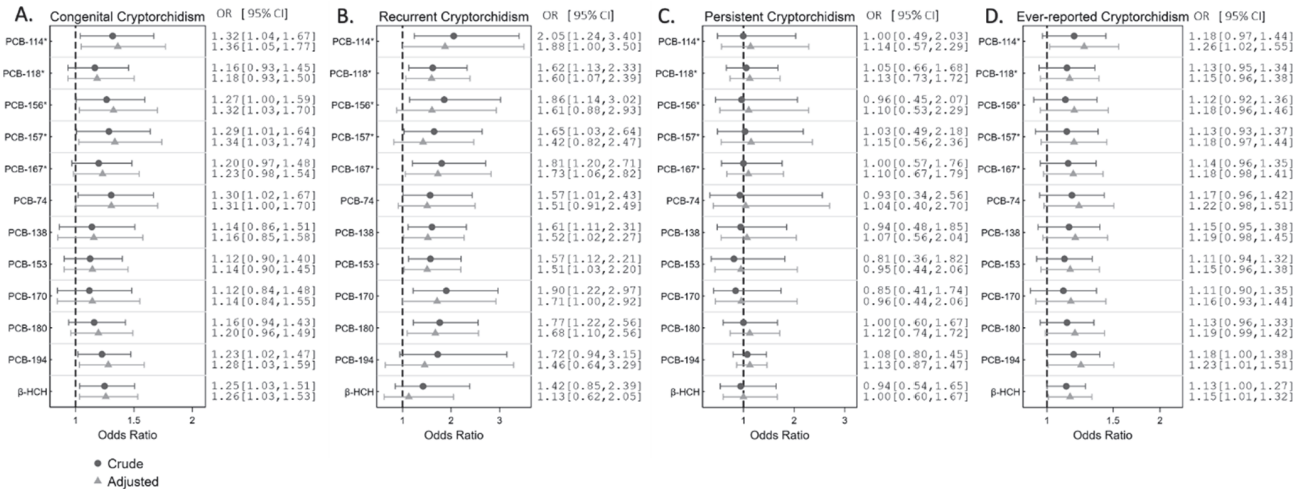

Fig. 3. Forest plot with odds ratio (OR) and 95% confidence interval (CI) per IQR increase in concentration of selected chemicals from 27 potential EDCs that were significantly associated with either congenital cryptorchidism, recurrent cryptorchidism, persistent cryptorchidism or ever-reported cryptorchidism in the case-cohort study in the HUMIS cohort (n = 641, 2002–2009, Norway). DL-PCBs are indicated with asterisk symbol (*) in the figure. Adjusted models included maternal age, education, pre-pregnancy BMI, parity, and smoking. Missing data was multiply imputed (n = 100 imputations).

3.4. Sensitivity analyses

Sensitivity analysis checking the additional effect of adjusting individually for preterm, congenital anomalies including hypospadias, timing of breast milk collection, and use of breast pump for milk collection did not materially alter effect estimates (Supplementary Fig. S3). We also did not detect significant effect modification for the selected EDCs by maternal education, preterm birth and nulliparity with congenital cryptorchidism (p-interaction > 0.30) or ever-reported cryptorchidism (p-interaction > 0.28). Effect estimates for congenital and ever-reported cryptorchidism from complete case analysis were similar to the multiple imputation analysis (Supplementary Fig. S4).

4. Discussion

In this case-cohort study, we found that infants with the highest exposure to breast milk concentrations of PCB-74, PCB-114, PCB-194 and β-HCH had increased odds of congenital cryptorchidism, and ever-reported cryptorchidism (PCB-194 only). The odds of having congenital cryptorchidism was increased by 28–36% for PCBs and 26% for β-HCH per IQR increase in breast milk concentrations. PCB-194 was associated with 23% increased odds of ever-reported cryptorchidism.

In the present study, only 3 PCBs (PCB-74, PCB-114, and PCB-194) were selected as the best predictors of congenital cryptorchidism among the PCBs using variable selection method although more individual PCB congeners were significantly associated with congenital or recurrent cryptorchidism in the unpenalized logistic regression. This highlights the importance of using multipollutant models in order to control for confounding by correlated exposures with shared exposure sources and similarities in kinetics with long half-lives (Zou and Hastie, 2005; Lenters et al., 2018). The epidemiological literature linking prenatal PCB exposure to the risk of cryptorchidism is inconclusive. Prenatal exposure to the sum of 12 dioxin-like PCBs from adipose tissue biopsies were positively associated with congenital cryptorchidism in the Danish-Finnish case-control study (2002–2006, n = 44 cases/38 controls) (Koskenniemi et al., 2015). Similarly, a French case-control study examining 151 cord blood (n = 67 cryptorchidism/84 controls) and 125 colostrum samples (n = 56/69) found a positive association between cryptorchidism and the total PCB concentrations (sum of 7 congeners) in colostrum, OR = 2.74, 95% CI (1.15–6.53), but not in cord blood, nor any association with individual congeners (Brucker-Davis et al., 2008). In the present study, less than 5% of the breast milk samples were collected within the first two weeks after delivery when colostrum is mainly produced. The U.S. Collaborative Perinatal Project (CPP) involving 12 U.S. medical centres between 1959 and 1965 did not find an association between eleven individual PCB congeners, or their sum, and cryptorchidism in a case-control study (n = 230/593) (McGlynn et al., 2009), nor did a nested case-control study within a joint Danish (n = 39/129) and Finish (n = 56/56) prospective cohort study using placental levels of 37 PCBs (n = 112/168) (Virtanen et al., 2012). Moreover, in the same Danish-Finish cohort using breast milk, Krysiak-Baltyn et al (2012) showed PCBs indicating protective effect within the Danish cohort but contrasting result within the Finish cohort.

More support is found in the literature for the role of organochlorines in the aetiology of cryptorchidism. In the present study, only β-HCH was associated with congenital cryptorchidism, among the organochlorines although the median concentrations of HCB, p,p'-DDE, p,p'-DDT, and oxychlordane were all higher in infants with cryptorchidism compared to infants without cryptorchidism (Supplementary Table S3). In a previous Norwegian study based on agricultural census, farmer's occupational exposure to OCPs was associated with cryptorchidism registered in the Medical Birth Registry (Kristensen et al., 1997). In accordance with this, a higher prevalence of cryptorchidism was also reported in Denmark amongst boys whose mothers were employed in greenhouses while pregnant (Andersen et al., 2008). In line with our study, β-HCH was associated with cryptorchidism in the US CPP study that compared serum concentration below the 10th and above the 90th percentiles (OR = 1.6, 95% CI: 0.7–2.6) (Pierik et al., 2007). Moreover, even though non-significant, children in Germany with cryptorchidism had higher median concentrations of β-HCH in fat samples obtained from orchidopexy compared to controls (Hosie et al., 2000). In Denmark, a case-control study (n = 62/68) reported an association between a combination of eight of the most abundant persistent OCPs in breast milk and congenital cryptorchidism (p = 0.032), although there was no significant difference when individually analysing 27 OCPs, including β-HCH (Damgaard et al., 2006). The exposure levels in breast milk in their study was two to three times higher than in our study in Norway (Supplementary Table S3). There is also literature suggesting associations between cryptorchidism and maternal serum level of other OCPs such as oxychlordane, p,p'-DDT, and p,p'-DDE (Trabert et al., 2012, Bhatia et al., 2005, Longnecker et al., 2002). However, not all studies support evidence of association between cryptorchidism and maternal serum levels of OCPs (Waliszewski et al., 2005, Bhatia et al., 2005, Axelsson et al., 2020).

We did not find any associations between brominated flame retardants and cryptorchidism, in line with previous studies (Small et al., 2009, Koskenniemi et al., 2015, Bonde et al., 2016), but in contrast to a case–control study in Canada that found an association with cryptorchidism and PBDEs measured in maternal hair samples (Goodyer et al., 2017). In the prospective Danish-Finnish study, Main et al. (2007) found an association to levels of flame retardants in breast milk, but not in placenta. Also, we did not find an association with PFOS and PFOA, in line with two previous studies (Jensen et al., 2013, Toft et al., 2016). However, PFOS exposure in amniotic fluid has been associated with hormones (steroid hormone and INSL3) that regulate testicle descent (Toft et al., 2016), suggesting that a more sensitive effect biomarker may be necessary to detect sub-clinical effects.

Experimental studies are necessary to confirm our findings of an association between PCB-74, PCB-114, PCB-194 and β-HCH and congenital cryptorchidism. Testicular descent occurs during prenatal life in two distinct phases, the first regulated by the insulin-like factor 3 (INSL3), and the second by androgens and calcitonin gene-related peptides (CGRP) (Hutson et al., 2015). Reduced androgen to estrogen ratio, and interference with androgen or INSL3 secretion during prenatal life, are mechanisms known to induce cryptorchidism in animals (Virtanen and Adamsson, 2012). Elevated serum PCB-74 was significantly associated with lower serum testosterone among the adult Native American population, particularly in females (Goncharov et al., 2009), and prenatal PCB-74 exposure was associated with decreased anogenital distance among boys in a cohort study in Mexico (Loreto-Gómez et al., 2018). The effect of PCB-114, a Dl-PCB congener, may be due to its aryl hydrocarbon receptor (AhR) activation, which exhibits anti-estrogenic or estrogenic effects based on tissue specificity (Warner et al., 2012). On the other hand, the effect of β-HCH on testicular descent may be due to its estrogenic properties (Coosen and van Velsen, 1989), while the probable mechanisms for PCB-194 are not clear.

In this Norwegian birth cohort, the prevalence of congenital cryptorchidism was 6.1%, and remained unchanged when established risk factors, such as preterm births and small for gestational age infants (Gurney et al., 2017), were excluded. Our prevalence estimates are higher than reported in the medical birth registry of Norway, which is, however, prone to underreporting (Brantsæter et al., 2016). More than half of the congenital cryptorchidism cases reported spontaneous descendent within six months of age in accordance with normal physiology, driven by the transient surge in gonadotrophins and consequent rise in testosterone levels (mini puberty), making testicular descent possible until five months of age (Kuiri-Hänninen et al., 2019). The reported prevalence after six months was stable (2.2–2.4%) in our cohort in accordance with the expected low probabilities of spontaneous testicular descent after 6 months of age (Shin and Jeon, 2020).

This case-cohort study has several advantages. The participants in the longitudinal HUMIS cohort are enrolled from across Norway to represent the general population. Chemicals were analysed in breast milk, which represents the real-life perinatal exposure to a mixture of many potential EDCs, and acts as a proxy for dose at the target tissue in the infants. Given the positive correlation between levels of persistent chemicals in breast milk, umbilical cord, and maternal serum, breast milk can be a suitable proxy for prenatal exposure and a good indicator of body burden for persistent compounds (Cerrillo et al., 2005, Skaare et al., 1988). It also represents both prenatal and postnatal exposure, and postnatal exposure may further delay testicular descent that has not occurred by the time of birth. Another strength of the study is the extensive assessment of 27 potential EDCs and the use of a variable selection method for the multi-pollutant analysis to reduce confounding from correlated co-exposure. The outcome ascertainment, cryptorchidism, was defined based on mother's report using repeated questionnaire at 1, 6, 12, and 24 months with several questions per questionnaire to cross-check validity of their report. In addition, having several time points to check cryptorchidism presentation increases the possibility of detecting any cryptorchidism and capture the spectrum of severity (Gurney et al., 2017). For example, measurement only at birth would exclude boys with a normally descended testis that subsequently ascends spontaneously; likewise, late measurements may lose less severe cases of cryptorchidism that descends shortly after birth. Another strength was substantial questionnaire data to enable a thorough assessment of potential confounding using DAGs. The prevalence of cryptorchidism in this study ranges between 1.6 and 8% depending on the time when the questionnaire was sent and severity of cryptorchidism. These prevalence estimates are high compared to the relatively stable prevalence of 0.3–0.4% reported since the early 1970s based on Medical birth registry of Norway (MBRN) (Brantsæter et al., 2016). However, prevalence estimates based on measurement at birth are known to be prone to underreporting since they may exclude boys with an ascending testicle or acquired cryptorchidism (normally descended testis at birth that subsequently ascended).

There are, however, some limitations to our study. One of the main limitations is the outcome ascertainment, which was based on maternal report and can be difficult even for trained observers. However, as mentioned above, multiple questionnaires were administered at different time points with detailed information including performed or planned operation to cross-check validity of their report. In addition, the measurement error arising from maternal report in our study is likely to be non-differential as mothers do not know their chemical exposure, biasing estimates towards the null. Reliability of maternal report among Norwegian mothers has also been validated for important birth and pregnancy parameters compared with registry data, although cryptorchidism was not included (Skulstad et al., 2017). Due to financial constraints measurement of chemical exposure was limited to 641 boys out of 1262, affecting the statistical power of the study. We obtained exposure information postpartum and not during first trimester, when testicles develop. Also, we cannot rule out the role of residual confounding from unmeasured or unknown confounders even though the questionnaire contained extensive list of information and we controlled for more classes of chemicals than any other study. Moreover, for some classes of potential EDCs, fewer samples were measured, leading to varying sample sizes, or had higher numbers of values below the limit of detection. However, multiple imputation was performed to reduce potential bias tied to missing values.

5. Conclusions

In this prospective cohort, PCB-74, PCB-114, PCB-194 and β-HCH were associated with congenital cryptorchidism. Altogether, 27 potential EDCs were analysed in this study making it the largest multi-pollutant study to date on cryptorchidism. We highlight the importance of using appropriate statistical models to handle highly correlated chemicals simultaneously to reduce false positive associations, as we observed marked different results in the single pollutant analysis compared to the elastic net logistic regression model. Further mechanistic studies are necessary to confirm our findings and elucidate how the selected EDCs may influence cryptorchidism.

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CRediT authorship contribution statement

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editing. **Nina Iszatt:** Data curation, Methodology, Formal analysis, Investigation, Writing – review & editing, Supervision. **Hein Stigum:** Methodology, Software, Formal analysis, Writing – review & editing, Supervision. **Tina K. Jensen:** Methodology, Writing – review & editing, Supervision. **Merete Eggesbø:** Conceptualization, Data curation, Methodology, Software, Project administration, Resources, Formal analysis, Investigation, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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Supplementary Material

A Case-Cohort Study of Perinatal Exposure to Potential Endocrine Disrupters and the Risk of Cryptorchidism in the Norwegian HUMIS Cohort

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Supplemental Methods

Chemical exposure assessment

To date, up to n = 1240 breast milk samples (specifically, aliquots from the samples pooled for each individual) have been assayed for some chemicals, and up to 1235 samples for any single chemical.

In the present cryptorchidism case-cohort study, we describe the methods for the 27 chemicals considered in the present analysis: those measured in at least 641 samples and detected in ≥50% of samples. Analytical protocols and quality control measures have been described in detail elsewhere. We provide the relevant references and briefly describe the protocols here.

PFASs: Two batches of breast milk samples were analysed for PFASs (PFOA, and PFOS) in at the Department of Environmental Exposure and Epidemiology (formerly the Department of Analytical Chemistry), Norwegian Institute of Public Health (NIPH) (Oslo, Norway), using high performance liquid chromatography/tandem mass spectrometry (LC-MS/MS) according to a previously described protocol (Forns et al. 2015; Haug et al. 2009; Thomsen et al. 2010a). In brief, after thawing and homogenization in a thermoshake incubator at 37 °C, 200 µL of breast-milk was transferred to a centrifugation tube, internal standards and acetonitrile added to make up a total volume of 600 µL for thorough precipitation of proteins, and mixed using a whirl mixer. The samples were then centrifuged, the supernatant transferred to a glass autosampler vial, and 500 µL 0.1M formic acid was added. Four hundred µL of the extract was injected into a column switching LC–MS/MS system. Calibration solutions were prepared in unprocessed cow's milk, which has been shown to be an acceptable surrogate matrix for breast milk in a thorough method validation (Thomsen et al., 2010). High quality of the determinations was ensured by analyzing samples (n = 8) from a previous inter-laboratory comparison study along with the samples (MTM research center, n.d.). The obtained concentrations were within ± 1 SD of the consensus value for all PFASs found above the limit of quantification (LOQ). The procedure blanks (n=15) did not contain any PFASs above the LOQ.

A third batch of samples was analyzed at the Institute for Environmental Studies (IVM), Faculty of Earth and Life Sciences, VU University (Amsterdam, the Netherlands), using LC-MS/MS coupled to a triple quadrupole mass spectrometer following a previously described protocol (de Cock et al. 2014; Forns et al. 2015). This method was based on the method by Haug et al. (Haug et al. 2009), with adaptations derived from Tao et al. (Tao et al. 2008). Briefly, after thawing and homogenizing the breast milk samples, 0.5 mL was taken for the analysis. After addition of the 13C4-PFOA and 13C4-PFOS internal standards (Wellington Laboratories) and 0.5 mL 1 M formic acid, the samples were sonicated for 30 min. Solid phase extraction was carried out using 1 cm3 , 30 mg Oasis WAX cartridges. After loading the whole sample mixture, the cartridges were washed with 1 mL 25 mM ammonium acetate pH4 and 0.5 mL 25% tetrahydrofuran in methanol. The PFASs were eluted from the cartridge with 0.4 mL 1% NH4OH in methanol and 0.4 mL 0.1 M formic acid was added to the eluate prior to injection onto a C8 trapping column in a column switching LC–MS/MS system. Like in the above method carried out at NIPH, the procedure blanks (n=28) did not contain any PFASs above the LOQ.

PBDEs: One batch of samples was measured for 6 PBDEs (28, 47, 99, 100, 153, 154) at NIPH using automated solid-phase extraction (SPE) extraction and a gas chromatography–mass spectrometry (GC– MS) system, as previously described (Thomsen et al. 2007; Thomsen et al. 2010b). The PBDE standards were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). A second batch was subsequently analyzed at NIPH. The same SPE extraction procedure was used (Thomsen et al. 2010b), while a GC–high resolution MS (GC-HRMS) with electron impact ionization was used for quantification (Forns et al. 2016; Frederiksen et al. 2010). The LOQ, which is based on the lowest level in the calibration curve, was ~0.02 ng/g lipids in the first round and ~0.01 ng/g lipids in the second round. A third batch of 10 PBDEs (28, 47, 66, 85, 99, 100, 153, 154, 183, 209) was measured at the Research Centre for Toxic Compounds in the Environment (RECETOX), Faculty of Science, Masaryk University (Brno, Czech Republic), as previously descried (Čechová et al. 2017b). The analytical method including detailed performance characteristics and method validation is described in (Čechová et al. 2017a). Briefly, labelled internal standards were added to freeze-dried milk samples. The samples were extracted using pressurized liquid extraction with hexane:dichloromethane:methanol 5:2:1, v/v/v. After the solvent reduction, lipid content was determined gravimetrically. Then, a two-step clean-up procedure based on dialysis in hexane followed by C18-silica and acidic alumina chromatography was performed. Instrumental analysis was performed by Gas chromatography coupled with high resolution mass spectrometry (GC-HRMS) [specifically a 7890A (Agilent, USA) GC coupled to a double-focusing magnetic sector HRMS AutoSpec Premier (Waters, UK)]. Procedural blanks and in-house reference materials were analyzed in the batch with every 7–11 breast milk samples.

PCBs and OCPs: The first batch of PCBs and OCPs was measured at the Norwegian University of Life Sciences (formerly the Laboratory of Environmental Toxicology, Norwegian School of Veterinary Science) (Oslo, Norway). The method included liquid/liquid, extraction, gravimetrical lipid determination and clean-up with sulfuric acid, and a GC-electron capture detector (GC-ECD) for OC pesticides and nondioxin like PCBs, and GC-low resolution MS (GC–LRMS) for the dioxin-like mono-ortho PCBs, as previously described (Brevik 1978; Eggesbø et al. 2009; Polder et al. 2008; Polder et al. 2009). A second batch of samples was measured (at the same time as the second batch analysis of PBDEs) at NIPH using the same SPE extraction procedure (Thomsen et al. 2010b), and GC-HRMS as described in (Caspersen et al. 2016; Forns et al. 2016).

A third batch of 10 PCBs and 26 OCPs was measured by RECETOX, Masaryk University. The analytical method is described in detail in (Čechová et al. 2017a). Briefly, as for PBDEs described above, labelled internal standards were added to freeze-dried milk samples. The samples were extracted using pressurized liquid extraction with hexane:dichloromethane:methanol 5:2:1, v/v/v. After the solvent reduction, lipid content was determined gravimetrically. Then, a two-step clean-up procedure based on dialysis in hexane followed by C18-silica and acidic alumina chromatography was performed. Instrumental analysis was performed by GC-MS/MS [specifically a Trace 1310 gas chromatograph with a 60-m × 0.25-mm × 0.25-µmHT8 fused-silica capillary column (SGE Analytical Science) coupled to a DFS double-focusing high resolution magnetic sector mass spectrometer (Thermo Scientific)]. Lipids: Breast milk lipid content was determined gravimetrically by the laboratories at the time of chemical analysis.

Potential batch effects: The subsets of samples analyzed were oversampled for outcomes which might be related to the outcomes assessed in the present study (all compounds except metals were oversampled ($n \leq 157$) for preterm birth, small for gestational age, or rapid growth; and metals for high fish eaters). We tested for systematic differences in measured levels between batches ('batch effects') using principal component analysis (PCA) visualization and linear regression models, adjusted for birth year, maternal age, parity and oversampling-stratum. We found limited evidence of batch effects, and therefore did not attempt to apply normalization methods to correct for batch effects (Lenters et al, 2019).

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Table S1. Comparison of maternal and child characteristics (N (%)[a] or median (IQR)[b]) of the present case-cohort study population examining potential EDC exposure and cryptorchidism, the eligible cohort for the study of cryptorchidism, and the total participants enrolled in the HUMIS cohort (2002-2009, Norway).

Maternal and child characteristics of the study population	Enrolled in HUMIS cohort[c] (N=2,606)	Eligible cohort for cryptorchidism study (N=1,262)	EDC-Cryptorchidism study participants (N=641)
Recruitment county			
Østfold county	560 (21%)	240 (19%)	171(26.7%)
Other counties (n=7)	2046 (79%)	1022(81%)	470(73.3%)
Maternal age (years)	30 (27-33)	30 (26-33)	29 (26-33)
Maternal Education level			
Low	223 (8.6%)	113 (9.0%)	59 (9.2%)
Middle	319 (12.2%)	158 (12.5%)	92 (14.4%)
High	1,952 (74.9%)	970 (76.9%)	475 (74.1%)
Missing	112 (4.3%)	21 (1.7%)	15 (2.3%)
Birth weight			
<2500	178 (6.8%)	56 (4.4%)	33 (5.1%)
2500-4000	1,853 (71.1%)	876 (69.4%)	437 (68.2%)
> 4000	574 (22.0%)	329 (26.1%)	171 (26.7%)
Missing	1 (0.0%)	1 (0.1%)	
Nulliparity	1,063 (42.5%)	531 (43.6%)	276 (43.8%)
Gestational age (days)	281 (273-288)	282 (274-288)	282 (273-288)
Small for gestational age	261 (10.0%)	113 (9.0%)	71 (11.1%)
Preterm	242 (9.3%)	101 (8.0%)	62 (9.7%)
Caesarean section	434 (16.7%)	205 (16.2%)	107 (16.7%)
Pre-pregnancy BMI			
Under weight	80 (3.1%)	39 (3.1%)	22 (3.4%)
Normal	1,536 (58.9%)	750 (59.4%)	386 (60.2%)
Overweight	565 (21.7%)	280 (22.2%)	151 (23.6%)
Obese	260 (10.0%)	127 (10.1%)	64 (10.0%)
Missing	165 (6.3%)	66 (5.2%)	18 (2.8%)
Smoking in pregnancy			
Never smoking	1,557 (59.7%)	747 (59.2%)	386 (60.2%)
Past smoker	682 (26.2%)	342 (27.1%)	179 (27.9%)
Occasional	63 (2.4%)	35 (2.8%)	19 (3.0%)
Daily smoker ≤ 10	208 (8.0%)	97 (7.7%)	48 (7.5%)
Daily smoker >10	32 (1.2%)	17 (1.3%)	7 (1.1%)
Missing	64 (2.5%)	24 (1.9%)	2 (0.3%)
Preeclampsia	115 (4.4%)	55 (4.4%)	29 (4.5%)
Gestational diabetes	15 (0.6%)	9 (0.7%)	4 (0.6%)

Note: [a]N (%)) is reported for binary or categorical variables from the total in each column while [b]median (IQR) is reported for continuous variables. IQR, interquartile range. [c]Cohort includes male and female children. [d]Other counties enrolled in HUMIS cohort include Rogaland (31%), Troms (21%), Telemark (10%), Nordland (5%), Finnmark (5%), Oppland (5%), and Akershus (2%).

Table S2. Univariate analysis[1] of mother-son characteristics and cryptorchidism among HUMIS cohort participants (n=1262, 2002-2009, Norway)

	Congenital Cryptorchidism			Recurrent Cryptorchidism			Persistent Cryptorchidism			Ever-reported Cryptorchidism		
	Total [2]	Cases		Total	Cases		Total	Cases		Total	Cases	
	N=907	N=55 (6.1%)	P-value	N=312	N=25 (8%)	P-value	N=886	N=14 (1.6%)	P-value	N=1,262	N=154 (12.2%)	P-value
Maternal age			0.55			**0.43**			**0.51**			0.68
<25	128 (14.1%)	8 (14.5%)		52 (16.7%)	2 (8.0%)		123 (13.9%)	2 (14.3%)		186 (14.7%)	21 (13.6%)	
25-35	671 (74.0%)	43 (78.2%)		230 (73.7%)	21 (84.0%)		659 (74.4%)	9 (64.3%)		923 (73.1%)	117 (76.0%)	
>35	108 (11.9%)	4 (7.3%)		30 (9.6%)	2 (8.0%)		104 (11.7%)	3 (21.4%)		153 (12.1%)	16 (10.4%)	
Maternal education[3]			**0.04**			0.56			**0.09**			**0.05**
Low	80 (8.8%)	4 (7.3%)		27 (8.7%)	1 (4.0%)		61 (6.9%)	1 (7.1%)		113 (9.0%)	20 (13.0%)	
Middle	114 (12.6%)	13 (23.6%)		37 (11.9%)	2 (8.0%)		94 (10.6%)	4 (28.6%)		158 (12.5%)	24 (15.6%)	
High	702 (77.4%)	38 (69.1%)		242 (77.6%)	21 (84.0%)		719 (81.2%)	9 (64.3%)		970 (76.9%)	106 (68.8%)	
Missing	11 (1.2%)	0 (0.0%)		6 (1.9%)	1 (4.0%)		12 (1.4%)	0 (0.0%)		21 (1.7%)	4 (2.6%)	
Birth weight			**0.05**			0.21			**0.01**			0.24
<2500	36 (4.0%)	0 (0.0%)		16 (5.1%)	3 (12.0%)		39 (4.4%)	3 (21.4%)		56 (4.4%)	10 (6.5%)	
2500-4000	633 (69.8%)	46 (83.6%)		208 (66.7%)	17 (68.0%)		608 (68.6%)	8 (57.1%)		876 (69.4%)	110 (71.4%)	
> 4000	238 (26.2%)	9 (16.4%)		88 (28.2%)	5 (20.0%)		238 (26.9%)	3 (21.4%)		329 (26.1%)	34 (22.1%)	
Missing							1 (0.1%)	0 (0.0%)		1 (0.1%)	0 (0.0%)	
Nulliparity	386 (43.1%)	28 (50.9%)	0.23	139 (45.4%)	11 (45.8%)	**0.97**	384 (45.0%)	6 (42.9%)	**0.87**	531 (43.6%)	65 (43.3%)	0.95
Small for gestational age	84 (9.3%)	6 (10.9%)	0.66	34 (10.9%)	2 (8.0%)	**0.63**	77 (8.7%)	2 (14.3%)	**0.45**	113 (9.0%)	17 (11.0%)	0.34
Preterm	65 (7.2%)	1 (1.8%)	0.11	25 (8.0%)	4 (16.0%)	**0.13**	68 (7.7%)	4 (28.6%)	**0.003**	101 (8.0%)	17 (11.0%)	0.14
Caesarean section	144 (15.9%)	6 (10.9%)	0.3	48 (15.4%)	3 (12.0%)	**0.62**	142 (16.0%)	2 (14.3%)	**0.86**	205 (16.2%)	21 (13.6%)	0.35
Pre-Pregnancy BMI			0.87			**0.35**			**0.41**			**0.09**
Under weight	24 (2.6%)	1 (1.8%)		9 (2.9%)	1 (4.0%)		25 (2.8%)	0 (0.0%)		39 (3.1%)	1 (0.6%)	
Normal	560 (61.7%)	33 (60.0%)		182 (58.3%)	16 (64.0%)		525 (59.3%)	7 (50.0%)		750 (59.4%)	90 (58.4%)	
Overweight	210 (23.2%)	12 (21.8%)		75 (24.0%)	7 (28.0%)		207 (23.4%)	3 (21.4%)		280 (22.2%)	34 (22.1%)	
Obese	89 (9.8%)	7 (12.7%)		33 (10.6%)	0 (0.0%)		83 (9.4%)	3 (21.4%)		127 (10.1%)	22 (14.3%)	
Missing	24 (2.6%)	2 (3.6%)		13 (4.2%)	1 (4.0%)		46 (5.2%)	1 (7.1%)		66 (5.2%)	7 (4.5%)	
Smoking at the end of pregnancy			0.91			**0.95**			**0.95**			0.68
no smoking	681 (75.1%)	37 (67.3%)		234 (75.0%)	22 (88.0%)		666 (75.2%)	12 (85.7%)		939 (74.4%)	118 (76.6%)	
occasional	4 (0.4%)	0 (0.0%)					4 (0.5%)	0 (0.0%)		5 (0.4%)	0 (0.0%)	
daily smoker≤ 10	51 (5.6%)	2 (3.6%)		21 (6.7%)	2 (8.0%)		48 (5.4%)	1 (7.1%)		72 (5.7%)	7 (4.5%)	
daily smoker >10	2 (0.2%)	0 (0.0%)		1 (0.3%)	0 (0.0%)		0 (0.0%)	**0 (0.0%)**		2 (0.2%)	0 (0.0%)	
Missing	169 (18.6%)	16 (29.1%)		56 (17.9%)	1 (4.0%)		168 (19.0%)	1 (7.1%)		244 (19.3%)	29 (18.8%)	
Preeclampsia	34 (3.7%)	2 (.6%)	0.96	14 (4.5%)	1 (4.0%)	**0.9**	33 (3.7%)	3 (21.4%)	**<0.001**	55 (4.4%)	11 (7.1%)	**0.07**

[1] Univariate analysis of maternal and child characteristics and cryptorchidism were tested using Pearson's chi-square test for binary or categorical variables, and Wilcoxon rank-sum test for continuous variables. [2] The total in each cryptorchidism definitions represents the number of mothers who responded to the specific questions about cryptorchidism presentation. [3] Maternal education is presented as a proxy for socio-economic status.

Table S3. Comparison of distribution of potential EDCs measured in breast milk (median (IQR)) of mothers who ever-reported cryptorchidism, and did not report cryptorchidism, and in the total case-cohort study participants from the HUMIS cohort (n=641, 2002-2009, Norway)

	EDCs	Cryptorchidism (n=77)					No cryptorchidism (n=564)					Total (N=641)				
		N	Median	IQR		Max	N	Median	IQR		Max	N	Medain	IQR		Max
			p50	p25	p75			p50	p25	p75			p50	p25	p75	
DL-PCBs	PCB105 (ng/g)	62	1.39	0.96	1.95	5.24	475	1.37	0.97	1.85	12.72	537	1.37	0.97	1.85	12.72
	PCB114 (ng/g)	62	0.31	0.22	0.46	2.34	475	0.33	0.25	0.46	2.09	537	0.33	0.25	0.46	2.34
	PCB118 (ng/g)	77	6.34	4.10	8.80	36.46	564	6.04	4.43	8.30	62.23	641	6.06	4.42	8.34	62.23
	PCB156 (ng/g)	62	3.16	2.06	4.83	14.96	475	3.20	2.32	4.45	22.73	537	3.20	2.28	4.45	22.73
	PCB157 (ng/g)	62	0.67	0.48	1.10	3.57	475	0.67	0.46	0.96	4.87	537	0.67	0.47	0.99	4.87
	PCB167 (ng/g)	59	0.79	0.50	1.15	3.03	465	0.78	0.59	1.07	8.05	524	0.78	0.58	1.08	8.05
	PCB189 (ng/g)	62	0.21	0.13	0.34	0.92	475	0.22	0.16	0.31	2.48	537	0.22	0.16	0.31	2.48
Sum of DL-PCBs		59	12.51	8.86	16.81	44.09	465	12.75	9.56	17.62	115.17	524	12.74	9.5	17.48	115.17
	PCB74 (ng/g)	62	3.02	2.33	4.74	24.00	475	3.31	2.45	4.41	18.68	537	3.30	2.44	4.42	24.00
	PCB99 (ng/g)	62	4.68	3.28	6.14	11.86	475	4.19	3.32	5.72	24.74	537	4.27	3.31	5.80	24.74
	PCB153 (ng/g)	77	33.01	22.25	45.17	156.74	564	31.49	24.62	41.82	296.03	641	31.68	24.53	42.33	296.03
	PCB170 (ng/g)	62	6.50	4.02	8.49	20.82	475	6.17	4.50	8.18	46.49	537	6.20	4.47	8.18	46.49
	PCB180 (ng/g)	77	15.84	9.55	22.91	71.92	564	15.28	11.69	20.10	142.46	641	15.38	11.37	20.20	142.46
	PCB194 (ng/g)	62	1.39	0.92	2.12	22.72	475	1.32	0.94	1.84	11.76	537	1.33	0.94	1.87	22.72
	PCB138 (ng/g)	77	20.13	13.59	29.18	87.30	564	19.56	14.48	25.57	145.06	641	19.58	14.47	26.14	145.06
Sum of NDL-PCBs		62	86.43	59.3	115.89	238.42	475	82.4	64.54	109.65	679.66	537	82.76	64.22	111.24	679.66
OCPs	β-HCH (ng/g)	77	4.69	3.67	7.00	93.67	564	4.41	2.91	6.51	43.39	641	4.43	2.95	6.60	93.67
	HCB (ng/g)	77	9.75	8.08	12.11	41.97	564	10.22	8.15	12.71	127.86	641	10.21	8.13	12.67	127.86
	ppDDE (ng/g)	77	49.46	28.58	94.32	617.26	564	48.77	32.73	74.09	1280.00	641	48.78	32.40	75.75	1280.00
	ppDDT (ng/g)	52	1.83	1.29	2.87	35.16	406	2.00	1.41	2.96	9.94	458	1.97	1.40	2.95	35.16
	Oxychlordane	58	2.93	2.23	4.22	11.20	426	3.11	2.28	4.08	24.60	484	3.10	2.27	4.12	24.60
Sum of OCPs		37	60.29	46.82	126.3	692.85	310	70.53	50.73	100.88	360.02	347	70.41	50.52	105.01	692.85
BDEs	BDE28 (ng/g)	72	0.12	0.06	0.20	1.37	548	0.12	0.07	0.20	6.82	620	0.12	0.07	0.20	6.82
	BDE47 (ng/g)	72	1.08	0.66	1.78	8.57	549	1.06	0.69	1.79	73.63	621	1.08	0.68	1.78	73.63
	BDE99 (ng/g)	73	0.26	0.18	0.47	1.81	549	0.28	0.18	0.48	28.31	622	0.28	0.18	0.47	28.31
	BDE100 (ng/g)	73	0.25	0.19	0.41	1.71	549	0.26	0.18	0.41	15.67	622	0.26	0.18	0.41	15.67
	BDE153 (ng/g)	73	0.46	0.33	0.66	1.91	548	0.50	0.36	0.70	14.78	621	0.49	0.36	0.70	14.78
	BDE154 (ng/g)	73	0.03	0.02	0.05	0.19	548	0.03	0.02	0.04	1.70	621	0.03	0.02	0.05	1.70
Sum of BDES		72	2.23	1.58	3.68	13.03	547	2.37	1.7	3.51	137.01	619	2.35	1.68	3.56	137.01

PFAS	PFOA (ng/L)	68	39.83	24.02	61.74	152.03		488	40.00	25.32	60.12	182.55		556	40	25.00	60.12	182.55
	PFOS (ng/L)	68	110	77.31	146.78	430		488	110	77.49	160	484.54		556	110	77.49	159.03	484.54
Sum of PFAs		68	151.68	106.9	210	495		488	157.29	108.5	223.44	570		556	155.54	107.92	220	570

Lipid adjusted concentrations are reported for all chemicals (ng/g) except PFASs where wet weight concentrations are presented (ng/L).

BDE brominated diphenyl ether; DDE dichlorodiphenyldichloroethylene; DDT dichlorodiphenyltrichloroethane; HCB hexachlorobenzene; HCH hexachlorocyclohexane; HUMIS The Norwegian Human Milk Study; IQR interquartile range; LOD limit of detection; Max maximum; NDL non-dioxin-like; P percentile; BDE brominated diphenyl ether; PCB polychlorinated biphenyl; PFASs poly- and perfluoroalkyl substances; PFOA perfluorooctanoate; PFOS perfluorooctane sulfonate; β-HCH beta-hexachlorocyclohexane.

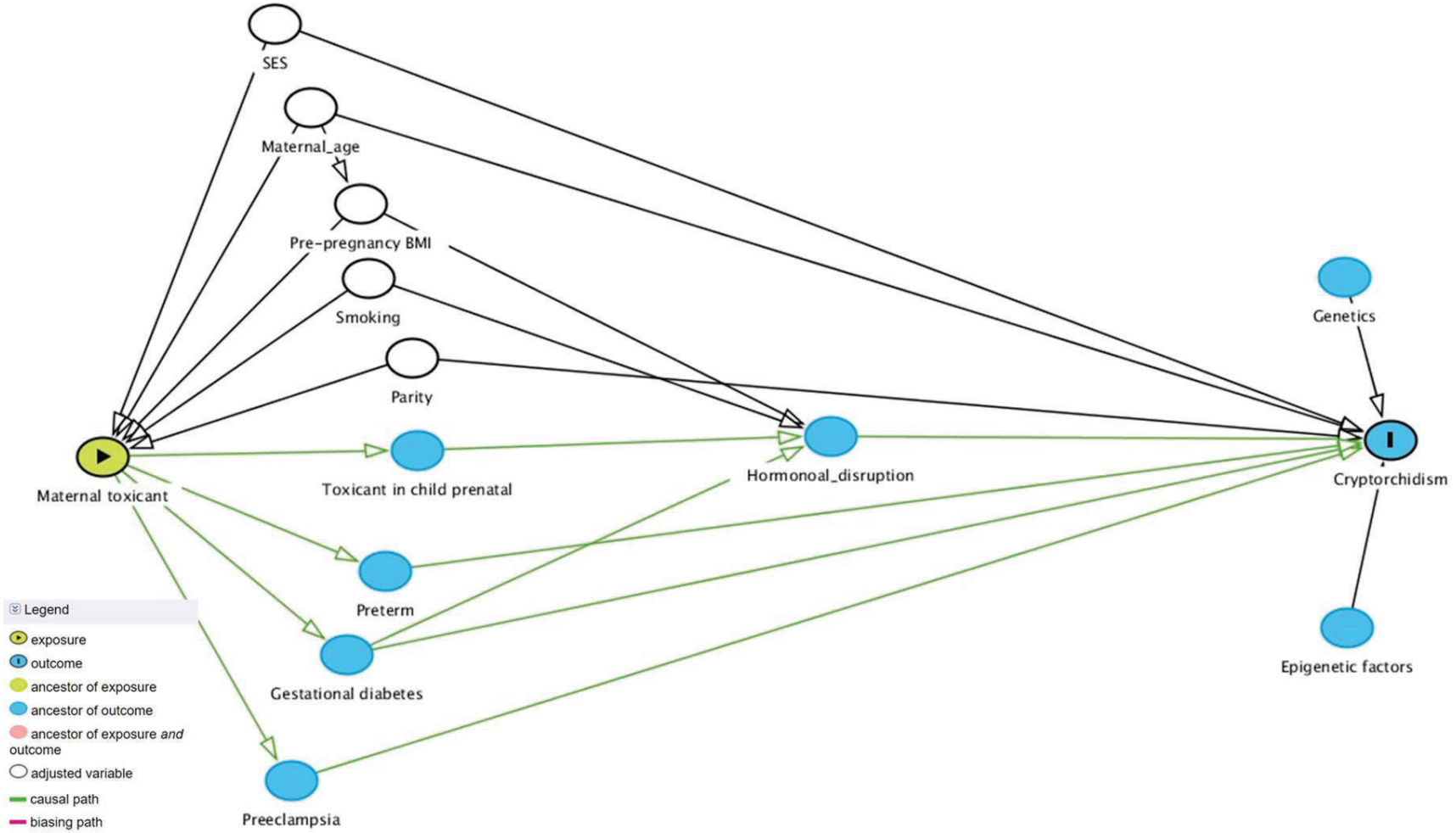

Figure S1. A simplified Directed acyclic graph (DAG) for studying effect of prenatal exposure to potential EDCs on development of cryptorchidism among Norwegian sons in the HUMIS cohort. The total adjustment set included confounders: maternal education (low, medium, high) as a proxy for socio-economic status, maternal age (continuous), pre-pregnancy BMI (kg/m2), smoking (yes/no), and nulliparity (yes/no) (Shown as adjusted in the figure above, white circles). The biasing pathways change from pink to black when the confounders are shown as adjusted (white circles). Potential mediators of cryptorchidism in the DAG included: preterm (yes/no), diabetes (no/yes) and preeclampsia (no/yes). The DAG was created using DAGitty. http://www.dagitty.net/dags.html

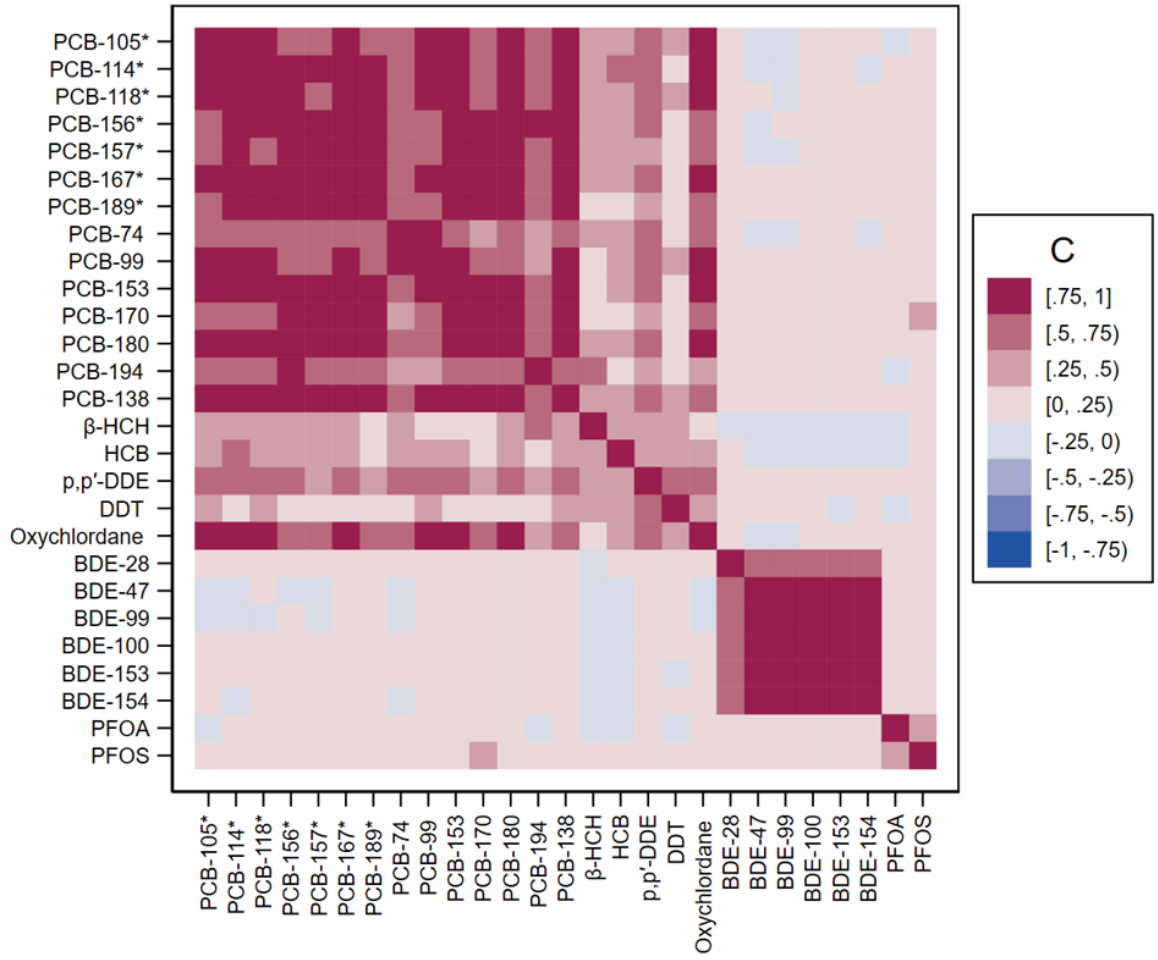

Figure S2. Heat map of Pearson correlation coefficient matrix for potential EDCs measured in breast milk samples of mothers in the cryptorchidism case-cohort study, HUMIS cohort (n=641, 2002-2009, Norway). Dark red or blue indicates a positive or a negative correlation, respectively. The intensity of the colour reflects the magnitude of the correlation coefficient. There is clustering within classes of EDCs with moderate ($0.5 \leq rp < 0.75$) to high ($rp \geq 0.75$) correlation for dioxin-like PCBs, non-dioxin-like PCBs, OCPs, BDEs and PFAs. Across different classes of EDCs, BDEs and PFASs have weak or no correlation ($-0.25 \leq rp < 0.25$) with other class of EDCs. 26.5% and 15.1% of Pearson pairwise correlations coefficients (rp) were high ($rp \geq 0.75$) or moderate ($0.5 \leq rp < 0.75$) respectively. Almost half (48%) of the correlations were weak/none ($-0.25 \leq rp < 0.25$). * The asterisk with PCBs indicates DL-PCBs.

Table S4. Table summarizing the missing in the exposure and confounding variables used for the cryptorchidism case-cohort study in the HUMIS cohort (n=641, 2002-2009, Norway)[a]

Variable	Missing (N)	Not missing (N)	Percent Missing	Min	Max
EDCS					
β-HCH	0	641	0.0 %	0.0	93.7
HCB	0	641	0.0 %	1.7	127.8
p,p'-DDE	0	641	0.0 %	5.4	1280.1
PCB153	0	641	0.0 %	6.6	296.0
PCB180	0	641	0.0 %	2.8	142.5
PCB118	0	641	0.0 %	1.1	62.2
PCB138	0	641	0.0 %	1.3	145.1
BDE99	19	622	3.0 %	0.0	28.3
BDE100	19	622	3.0 %	0.0	15.7
BDE47	20	621	3.1 %	0.1	73.6
BDE153	20	621	3.1 %	0.0	14.8
BDE154	20	621	3.1 %	0.0	1.7
BDE28	21	620	3.3 %	0.0	6.8
PFOS	85	556	13.3 %	20.0	484.5
PFOA	85	556	13.3 %	0.9	182.6
PCB157	104	537	16.2 %	0.0	4.9
PCB170	104	537	16.2 %	0.0	46.5
PCB74	104	537	16.2 %	1.0	24.0
PCB105	104	537	16.2 %	0.4	12.7
PCB189	104	537	16.2 %	0.0	2.5
PCB99	104	537	16.2 %	0.8	24.7
PCB114	104	537	16.2 %	0.0	2.3
PCB156	104	537	16.2 %	0.6	22.7
PCB194	104	537	16.2 %	0.2	22.7
PCB167	117	524	18.3 %	0.2	8.1
Oxychlordane	157	484	24.5 %	0.5	24.6
p,p'-DDT	183	458	28.5 %	0.0	35.2
Covariates					
Maternal age	0	641	0.0 %	17.0	44.0
Maternal Education	15	626	2.3 %	1.0	3.0
Smoking	2	639	0.3 %	0.0	4.0
Nulliparity	11	630	1.7 %	0.0	4.0
Pre-pregnancy BMI	18	623	2.8 %	1.0	4.0
Birth Weight	0	641	0.0 %	1048.0	5100.0
Small for gestational age	0	641	0.0 %	0.0	1.0
Preterm	0	641	0.0 %	0.0	1.0
Gestational Diabetes	0	641	0.0 %	0.0	1.0

[a] Based on the ever-reported cryptorchidism dataset

Table S5. Crude and adjusted estimates for the single pollutant associations between EDC exposures and congenital cryptorchidism in case-cohort study in the HUMIS cohort (n=26 cases/485, 2002-2009, Norway) [a]

Exposure	Crude				Adjusted [b]			
	OR	95% CI		*p*-value	OR	95% CI		*p*-value
PCB105	1.20	0.96	1.50	0.11	1.22	0.96	1.56	0.10
PCB114	1.32	1.04	1.67	**0.02**	1.36	1.05	1.77	**0.02**
PCB118	1.16	0.93	1.45	0.18	1.19	0.94	1.50	0.16
PCB156	1.27	1.00	1.59	**0.05**	1.32	1.03	1.70	**0.03**
PCB157	1.29	1.01	1.64	**0.04**	1.34	1.03	1.74	**0.03**
PCB167	1.20	0.96	1.48	0.11	1.23	0.98	1.54	0.08
PCB189	1.15	0.92	1.43	0.21	1.20	0.95	1.51	0.13
PCB74	1.30	1.02	1.67	**0.03**	1.31	1.00	1.70	**0.05**
PCB99	1.17	0.82	1.66	0.38	1.17	0.79	1.73	0.43
PCB153	1.12	0.90	1.40	0.30	1.14	0.90	1.45	0.26
PCB170	1.12	0.84	1.48	0.45	1.14	0.84	1.55	0.40
PCB180	1.16	0.94	1.43	0.17	1.20	0.96	1.49	0.11
PCB194	1.23	1.02	1.47	**0.03**	1.28	1.03	1.53	**0.03**
PCB138	1.14	0.86	1.51	0.36	1.16	0.85	1.58	0.36
β-HCH	1.25	1.03	1.51	**0.02**	1.26	1.03	1.53	**0.02**
HCB	1.06	0.89	1.26	0.51	1.04	0.86	1.26	0.66
DDE	1.22	0.94	1.59	0.14	1.24	0.93	1.65	0.15
DDT	0.86	0.41	1.81	0.66	0.79	0.33	1.89	0.57
Oxychlordane	1.10	0.82	1.48	0.51	1.09	0.78	1.53	0.62
BDE28	1.03	0.89	1.19	0.66	1.03	0.89	1.20	0.68
BDE47	1.00	0.90	1.11	0.99	1.00	0.90	1.11	0.97
BDE99	0.98	0.86	1.13	0.82	0.98	0.87	1.12	0.81
BDE100	0.99	0.86	1.13	0.85	0.98	0.86	1.13	0.81
BDE153	0.99	0.85	1.17	0.94	0.99	0.85	1.16	0.90
BDE154	0.97	0.80	1.17	0.75	0.97	0.80	1.17	0.73
PFOA	1.11	0.71	1.73	0.66	1.01	0.61	1.68	0.98
PFOS	0.71	0.40	1.27	0.24	0.65	0.35	1.20	0.16

OR odds ratio; CI confidence interval

[a] Odds ratios were obtained from single pollutant logistic regression models and based on the multiply imputed dataset (n=100 imputed datasets).

[b]The model was adjusted for maternal age, education, pre-pregnancy BMI, smoking, and parity (total effect). The coefficients represent the odds ratio of congenital cryptorchidism for an interquartile range increase in the measured concentration of EDCs in breast milk.

Table S6. Crude and adjusted estimates for the single pollutant associations between EDC exposures and recurrent cryptorchidism in the case-cohort study in the HUMIS cohort (n=11 cases/185, 2002-2009, Norway) [a]

Exposure	Unadjusted				Adjusted [b]			
	OR	95% CI		*p*-value	OR	95% CI		*p*-value
PCB105	1.51	1.01	2.25	**0.05**	1.42	0.89	2.28	0.14
PCB114	2.05	1.24	3.40	**0.01**	1.88	1.00	3.50	**0.05**
PCB118	1.62	1.13	2.33	**0.01**	1.54	1.04	2.29	**0.03**
PCB156	1.86	1.14	3.02	**0.01**	1.61	0.88	2.93	0.12
PCB157	1.65	1.03	2.64	**0.04**	1.42	0.82	2.47	0.21
PCB167	1.78	1.19	2.68	**0.01**	1.67	1.05	2.67	**0.03**
PCB189	1.67	1.08	2.59	**0.02**	1.45	0.86	2.47	0.16
PCB74	1.57	1.01	2.43	**0.05**	1.51	0.91	2.49	0.11
PCB99	1.66	1.14	2.57	**0.01**	1.53	1.00	2.37	0.05
PCB153	1.57	1.12	2.21	**0.01**	1.46	1.01	2.12	**0.04**
PCB170	1.90	1.22	2.97	**<0.01**	1.71	1.00	2.92	**0.05**
PCB180	1.77	1.22	2.56	**<0.01**	1.68	1.10	2.56	**0.02**
PCB194	1.72	0.94	3.15	0.08	1.46	0.64	3.29	0.36
PCB138	1.61	1.11	2.31	**0.01**	1.52	1.02	2.27	**0.04**
β-HCH	1.42	0.85	2.39	0.18	1.13	0.62	2.05	0.69
HCB	1.30	0.72	2.36	0.39	1.06	0.57	1.99	0.85
DDE	1.03	0.84	1.26	0.81	0.97	0.72	1.31	0.83
DDT	1.92	1.03	3.57	**0.04**	1.88	0.91	3.88	0.08
Oxychlordane	1.68	1.07	2.64	0.03	1.45	0.85	2.48	0.17
BDE28	1.05	0.66	1.67	0.83	0.96	0.55	1.65	0.87
BDE47	0.89	0.59	1.33	0.56	0.88	0.61	1.29	0.52
BDE99	0.74	0.39	1.41	0.37	0.77	0.42	1.40	0.39
BDE100	0.89	0.59	1.34	0.59	0.88	0.60	1.27	0.49
BDE153	0.72	0.31	1.67	0.44	0.57	0.23	1.44	0.23
BDE154	0.88	0.55	1.41	0.60	0.86	0.54	1.35	0.50
PFOA	1.56	0.62	3.94	0.33	1.40	0.53	3.73	0.49
PFOS	1.58	0.83	3.04	0.17	1.47	0.73	2.94	0.28

OR odds ratio; CI confidence interval

[a] Odds ratios were obtained from single pollutant logistic regression models and based on the multiply imputed dataset (n=100 imputed datasets).

[b] The model was adjusted for maternal age, education, pre-pregnancy BMI, smoking, and parity (total effect). The coefficients represent the odds ratio of recurrent cryptorchidism for an interquartile range increase in the measured concentration of EDCs in breast milk.

Table S7. Crude and adjusted estimates for the single pollutant associations between EDC exposures and persistent cryptorchidism in the case-cohort study in the HUMIS cohort (n=9 cases/500, 2002-2009, Norway) [a]

Exposure	Unadjusted				Adjusted[b]			
	OR	95% CI		*p*-value	OR	95% CI		*p*-value
PCB105	1.10	0.70	1.74	0.68	1.18	0.76	1.82	0.46
PCB114	1.00	0.49	2.03	0.99	1.14	0.57	2.29	0.71
PCB118	1.05	0.66	1.68	0.82	1.13	0.73	1.72	0.59
PCB156	0.96	0.45	2.07	0.92	1.10	0.53	2.29	0.79
PCB157	1.03	0.49	2.18	0.93	1.15	0.56	2.36	0.70
PCB167	1.00	0.57	1.76	1.00	1.10	0.67	1.79	0.71
PCB189	0.93	0.46	1.90	0.84	1.03	0.52	2.03	0.93
PCB74	0.93	0.34	2.56	0.88	1.04	0.40	2.70	0.93
PCB99	0.88	0.40	1.94	0.75	0.99	0.43	2.25	0.98
PCB153	0.81	0.36	1.82	0.61	0.95	0.44	2.06	0.90
PCB170	0.85	0.41	1.74	0.65	0.96	0.44	2.06	0.91
PCB180	1.00	0.60	1.67	1.00	1.12	0.74	1.72	0.59
PCB194	1.08	0.80	1.45	0.64	1.13	0.87	1.47	0.37
PCB138	0.94	0.48	1.85	0.87	1.07	0.56	2.04	0.84
β-HCH	0.94	0.54	1.65	0.84	1.00	0.60	1.67	1.00
HCB	0.46	0.16	1.32	0.15	0.45	0.14	1.42	0.17
ppDDE	0.69	0.28	1.67	0.41	0.74	0.28	1.90	0.53
ppDDT	1.08	0.81	1.43	0.61	1.07	0.80	1.42	0.65
Oxychlordane	0.85	0.35	2.03	0.71	0.97	0.41	2.28	0.94
BDE28	0.73	0.31	1.73	0.48	0.68	0.28	1.67	0.40
BDE47	0.93	0.62	1.39	0.72	0.90	0.58	1.41	0.64
BDE99	1.00	0.85	1.16	0.96	0.98	0.79	1.20	0.82
BDE100	0.99	0.81	1.22	0.94	0.97	0.78	1.21	0.82
BDE153	0.99	0.72	1.34	0.93	0.99	0.77	1.27	0.96
BDE154	1.01	0.88	1.15	0.89	1.00	0.85	1.16	0.96
PFOA	0.50	0.15	1.66	0.26	0.51	0.14	1.86	0.30
PFOS	0.72	0.21	2.53	0.60	0.76	0.20	2.89	0.67

OR odds ratio; CI confidence interval

[a] Odds ratios were obtained from single pollutant logistic regression models and based on the multiply imputed dataset (n=100 imputed datasets).

[b] The model was adjusted for maternal age, education, pre-pregnancy BMI, smoking and parity (total effect). The coefficients represent the odds ratio of persistent cryptorchidism for an interquartile range increase in the measured concentration of EDCs in breast milk.

Table S8. Crude and adjusted estimates for the single pollutant associations between EDC exposures and ever-reported cryptorchidism in the case-cohort study in the HUMIS cohort (n=77 cases/641, 2002-2009, Norway) [a]

Exposure	Crude				Adjusted[b]			
	OR	95% CI		*p*-value	OR	95% CI		*p*-value
PCB105	1.15	0.96	1.37	0.13	1.17	0.97	1.41	0.11
PCB114	1.18	0.97	1.44	0.09	1.26	1.02	1.55	**0.03**
PCB118	1.13	0.95	1.34	0.16	1.15	0.97	1.38	0.11
PCB156	1.12	0.92	1.36	0.25	1.18	0.96	1.46	0.12
PCB157	1.13	0.93	1.37	0.21	1.18	0.96	1.44	0.10
PCB167	1.14	0.96	1.35	0.12	1.18	0.98	1.40	0.11
PCB189	1.11	0.93	1.30	0.25	1.14	0.95	1.36	0.17
PCB74	1.17	0.96	1.42	0.21	1.22	0.98	1.51	0.07
PCB99	1.18	0.96	1.48	0.11	1.23	0.99	1.55	0.07
PCB153	1.11	0.94	1.32	0.22	1.15	0.96	1.37	0.13
PCB170	1.11	0.92	1.38	0.25	1.16	0.94	1.46	0.15
PCB180	1.13	0.96	1.33	0.14	1.19	0.99	1.41	0.07
PCB194	1.18	1.00	1.38	**0.05**	1.23	1.01	1.51	**0.04**
PCB138	1.15	0.95	1.38	0.16	1.19	0.97	1.44	0.10
β-HCH	1.13	1.00	1.27	**0.05**	1.15	1.01	1.32	**0.04**
HCB	1.00	0.83	1.19	0.98	1.01	0.85	1.20	0.92
DDE	1.05	0.94	1.18	0.35	1.05	0.94	1.18	0.36
DDT	1.12	0.95	1.30	0.17	1.11	0.95	1.31	0.20
Oxychlordane	1.05	0.87	1.28	0.57	1.08	0.90	1.34	0.38
BDE28	0.96	0.84	1.10	0.55	0.96	0.84	1.10	0.53
BDE47	0.95	0.84	1.07	0.42	0.95	0.84	1.07	0.39
BDE99	0.96	0.85	1.07	0.42	0.95	0.84	1.07	0.36
BDE100	0.96	0.85	1.08	0.49	0.95	0.84	1.08	0.46
BDE153	0.85	0.66	1.10	0.21	0.87	0.68	1.11	0.27
BDE154	0.96	0.85	1.09	0.52	0.96	0.84	1.09	0.51
PFOA	1.02	0.74	1.34	0.98	1.13	0.78	1.50	0.65
PFOS	1.02	0.78	1.34	0.88	1.07	0.81	1.42	0.61

OR odds ratio; CI confidence interval

[a] Odds ratios were obtained from single pollutant logistic regression models and based on the multiply imputed dataset (n=100 imputed datasets).

[b] The model was adjusted for maternal age, education, pre-pregnancy BMI, smoking, and parity (total effect). The coefficients represent the odds ratio of ever-reported cryptorchidism for an interquartile range increase in the measured concentration of EDCs in breast milk.

Table S9. EDCs selected by elastic net logistic regression in the case-cohort study in the HUMIS cohort (2002-2009, Norway)

	Selected EDCs[1]												
Congenital cryptorchidism	**β-HCH**	**PCB-194**	**PCB-114**	**PCB-74**	PCB-157	DDT	PCB-156	PCB-105	HCB	PFOA	PCB-167	PCB-189	PCB-170
[2]Percentage (selection)	**96 %**	**95 %**	**58 %**	**54 %**	24 %	34 %	17 %	14 %	10 %	7 %	4 %	1 %	1 %
Recurrent cryptorchidism	PCB-194	PCB-99	PCB-180	PCB-189	BDE-47	BDE-153	HCB	PCB-170	BDE-100	BDE-99	PCB-153	DDE	PFOA
Percentage (selection)	8 %	7 %	7 %	7 %	6 %	6 %	6 %	6 %	6 %	6 %	6 %	3 %	1 %
Persistent cryptorchidism	PCB-114	PCB-74	PCB-194	PFOS	PCB-170	PCB-189	PFOA	PCB-105	BDE-28	PCB-180	HCB	PCB-118	BDE-153
Percentage (selection)	14 %	12 %	8 %	7 %	6 %	5 %	5 %	4 %	3 %	3 %	2 %	1 %	1 %
Ever-reported cryptorchidism	**PCB-194**	PCB74	β-HCH	BDE-153	DDT	PCB-114	PCB-199	HCB	BDE-47	Oxychlordane	BDE-100	BDE-99	PFOA
Percentage (selection)	**52 %**	16 %	14 %	12 %	11 %	7 %	4 %	4 %	3 %	2 %	2 %	1 %	1 %

[1] The variable selection result is based on elastic net logistic regression, using the multiply imputed dataset (n=100 imputation data set). The potential confounders (maternal age, education, pre-pregnancy BMI, parity, and smoking) were forced into the model unpenalized while the optimal level of penalization for the 27 EDCs was determined using the default 10-fold cross-validation. [2] An EDC was considered selected if it was retained in at least 50 % of the imputed sets in this study.

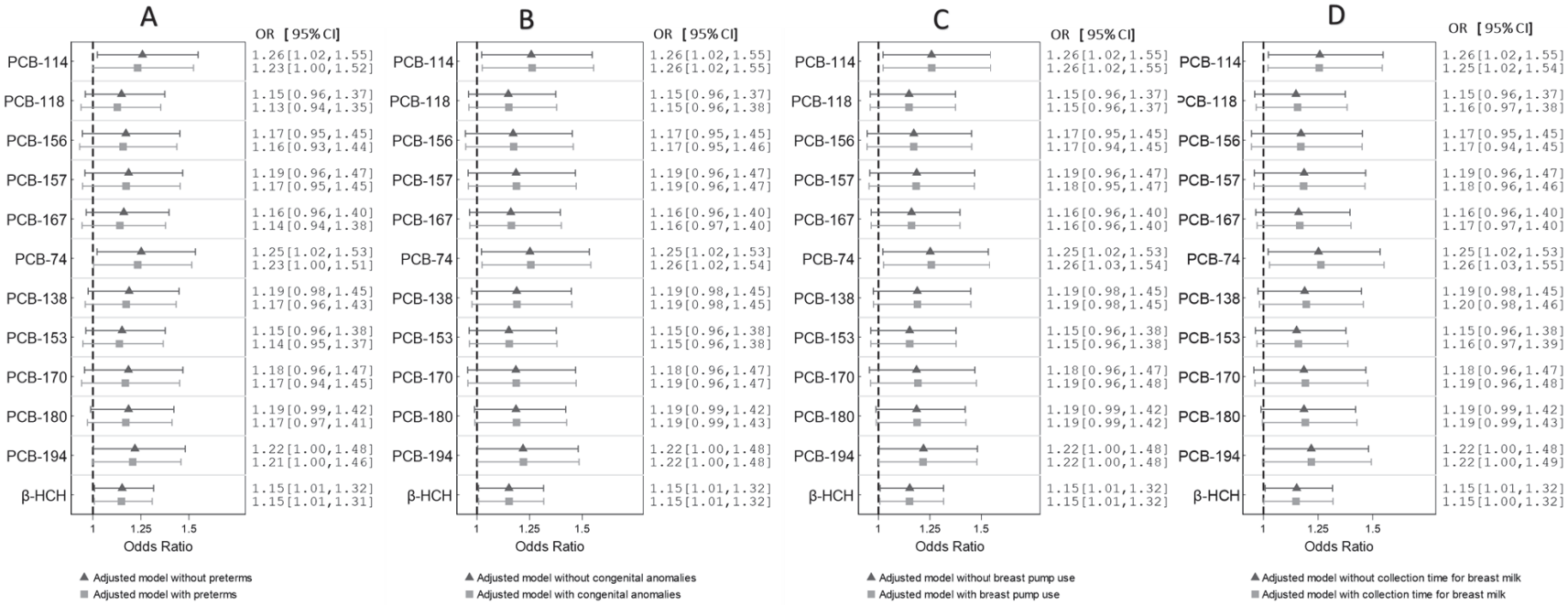

Figure S3. Forest plots of the association between selected EDCs and ever-reported cryptorchidism and sensitivity analysis of the additional adjustment for A) preterm, B) congenital anomalies including hypospadias, C) the use of breast pump for milk collection, and D)timing of breast milk collection in the case-cohort study in the HUMIS cohort (n=77 cases/641, 2002-2009, Norway). Odds ratios were obtained from single pollutant logistic regression models and based on the multiply imputed dataset (n=100 imputed datasets). The model was adjusted for maternal age, education, pre-pregnancy BMI, smoking and parity (total effect). The coefficients represent the odds ratio and 95% confidence interval for ever-reported cryptorchidism per interquartile range increase in the measured concentration of EDCs in breast milk. The estimates were further adjusted for A) preterm (yes/no), B) congenital anomalies including hypospadias (yes/no), and C) the use of breast pump for milk collection (yes/no).

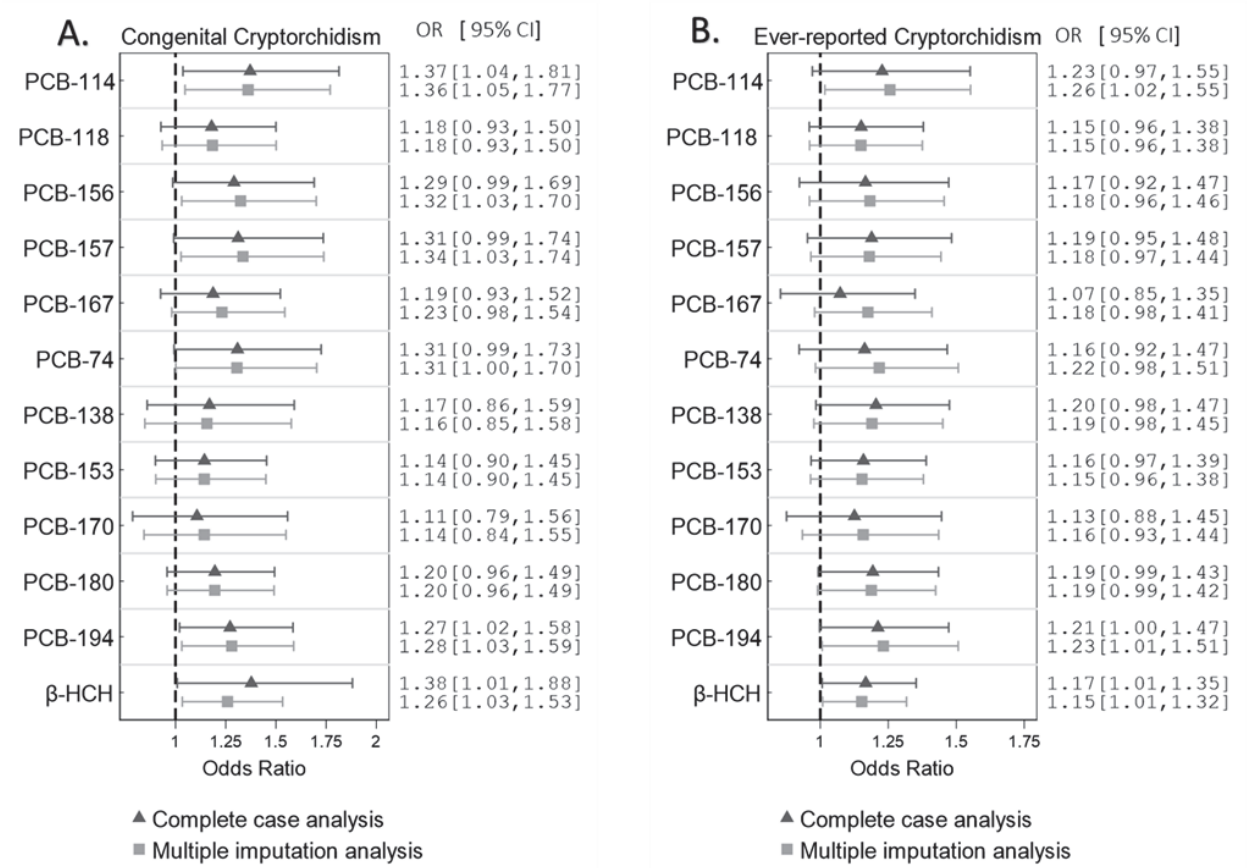

Figure S4. Forest plots comparing complete case analyses and multiple imputation analyses for (A) congenital cryptorchidism, and (B) ever-reported cryptorchidism in the case-cohort study in the HUMIS cohort (n=641, 2002-2009, Norway). Odds ratios were obtained from single pollutant logistic regression model based on either complete case or the multiply imputed dataset (n=100 imputed datasets). The coefficients represent the odds ratio and 95% confidence interval per interquartile range increase in the measured concentration of EDCs in breast milk. The model was adjusted for maternal age, education, pre-pregnancy BMI, smoking and parity (total effect).

Paper II

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Anti-androgenic compounds in breast milk and cryptorchidism among Norwegian boys in the HUMIS birth cohort

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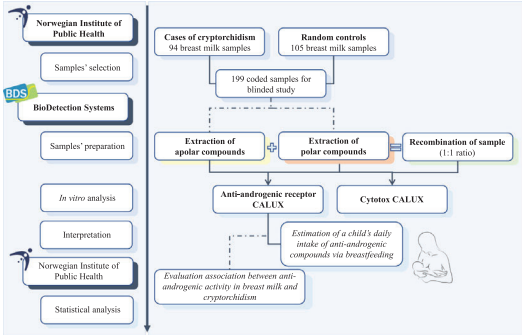
HIGHLIGHTS

- 199 human milk samples were analyzed using the AR CALUX reporter gene bioassay.
- Antagonistic effects towards the androgen receptor were found in human milk samples.
- No association between anti-androgenicity in human milk and cryptorchidism prevalence was found.
- A child's daily intake of milk contaminants was estimated to 78 μg flutamide eq./kg body weigh/day.

GRAPHICAL ABSTRACT



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ABSTRACT

The prevalence of cryptorchidism has increased over the past decades, yet its origins remain poorly understood. Testis descent is dependent on androgens and likely affected by endocrine disrupting compounds (EDCs), targeting the androgen receptor (AR). We investigated the association between anti-androgenic activity, not derived from natural hormones, in maternal breast milk and impaired testis descent among boys. We performed a case-control study based on 199 breast milk samples from 94 mothers of cryptorchid boys and 105 random non-cryptorchid boys participating in the Norwegian HUMIS (Human Milk Study) cohort. For each participant, apolar, and polar fractions were extracted, and combined to reconstitute a mixture. Anti-androgenic activity was measured in all three fractions using the human cell-based in vitro anti-AR CALUX® assay and expressed in μg of flutamide equivalent, a well-known antiandrogen. Results from fraction analyses were compared among boys with cryptorchidism and controls using multiple logistic regression, controlling for appropriate confounders identified using a directed acyclic graph. Children's daily exposure to anti-androgenic EDCs through breastfeeding was estimated to 78 μg flutamide eq./kg of body weigh/day. The activity was higher in the polar fraction (1.48 ± 1.37 μg flutamide eq./g of milk) mainly representing non-persistent chemicals, in contrast to other fractions. However, the activity in the polar extracts was decreased when in mixtures with the apolar fraction, indicating synergistic interactions. No significant difference in the activity was observed according to cryptorchid status for polar, apolar or mixed breast milk fractions. The study showed anti-androgenic activity in nearly

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all human milk samples, and at levels higher than the advisory threshold. However, no significant association was observed between cryptorchidism and antiandrogenic activity measured in either polar, apolar, or mixture fractions derived from breast milk.

1. Introduction

During the last few decades, an increase in the prevalence of undescended testis in male newborns has been reported (Acerini et al., 2009; Boisen et al., 2004; Gaspari et al., 2011; Virtanen and Toppari, 2008). Cryptorchidism is a risk factor for developing testicular germ cell cancer and impaired fertility, but its etiology is to this date still not fully understood (Huyghe et al., 2007; Mieusset et al., 1995; Yiee and Baskin, 2010). Over the years, it has been hypothesized that exposure to environmental factors such as endocrine disrupting chemicals (EDCs) could potentially add up to cause incomplete masculinization in boys exposed during pregnancy (Sharpe and Skakkebaek, 1993, 2003; Toppari et al., 2010).

Many consumer products contain EDCs with either weak estrogenic (bisphenol A) or anti-androgenic (phthalate) properties even in relatively low dosages (Sohoni and Sumpter, 1998; Bergman et al., 2012). Exposure to such EDCs has been associated with various adverse health effects including disruption of the proper masculinization of the gonads (Bergman et al., 2012; Toppari et al., 1996). In experimental animals, impact of EDCs is dependent on the level and sensitive period of exposure, identifying fetal and early life as a particularly vulnerable window (Bergman et al., 2012). In humans this window of androgen dependence of testicular decent occurs between 8 and 10 gestational weeks and much later around 25 to 35 gestational weeks (Hutson et al., 2013; Welsh et al., 2008). Some studies have reported associations between prenatal exposure to EDCs e.g. xenoestrogens, polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DTT), and cryptorchidism at birth (Brucker-Davis et al., 2008; Fernandez et al., 2007). However, subsequent studies on cryptorchidism reported that PCBs and DDT placenta levels, as well as perfluorinated compounds cord blood concentrations were not associated with cryptorchidism (Jensen et al., 2014; Virtanen et al., 2012).

To date, most case-control studies associating exposure to EDCs and cryptorchidism are based on analysis of a limited number of chemicals. This is an important shortcoming since it has been shown that the effects caused by EDCs can add up in mixtures (Kortenkamp, 2014; Rajapakse et al., 2002). Because most EDCs at realistic exposure levels only show weak binding to nuclear hormone receptors, a more comprehensive analysis of their combined effects is needed to elucidate their possible role in the increased incidence of cryptorchidism. Androgen receptor (AR) is one of the main nuclear receptors involved in male sexual development by binding to androgens. Androgens regulate testicular descent, its suppression by anti-androgenic EDCs (DDE, phthalate, and vinclozolin) or estrogenic EDCs (bisphenol A, DDT, some PCBs, phytoestrogens, and phenols) were directly linked to cryptorchidism development in experimental animal studies (Skakkebaek, 2002, Virtanen and Adamsson, 2012). In addition to disturbance in androgen to estrogen ratio, interference with secretion of insulin-like factor 3 (INSL3), a regulator of the first phase of testicular descent, was also correlated with some EDCs (BPA, phthalates, PFOS) (Chevalier et al., 2015, Toft et al., 2016). Breast milk is a suitable bio-fluid for assessing maternal body burden of persistent chemicals accumulated over life, and thus a good indicator of fetal exposure to persistent chemicals during the inguinoscrotal phase, the critical androgen-driven event of testicular descent (Skaare et al., 1988).

In our previous studies we performed non-targeted chemical analysis on breast milk extracts by UHPLC-Q-TOF-MS/MS and developed a method to distinguish endogenous hormones from EDCs (Collet et al., 2020), while targeted chemical analyses on the human milk samples were performed and reported before (Eggesbø et al., 2004, 2009, Thomsen et al., 2010) Furthermore, we found anti-androgenic activity in most samples and demonstrated that this activity could not be explained by endogenous hormones present in the milk. The present study therefore investigates the possible association between the disruption of androgen action, through mixtures of chemicals antagonizing normal AR functioning, and cryptorchidism. We extend the previous analyses using the anti-AR CALUX® (Chemically Activated LUciferase gene eXpression) bioassay to evaluate the effects of mixtures of EDCs on AR activity (Sonneveld et al., 2006).

2. Material and methods

2.1. Study population

The study population is a subset of participants from the multicenter birth cohort Norwegian Human Milk Study (HUMIS) previously described in detail (Eggesbø et al., 2009, 2011) (Table A.1). In brief, HUMIS is a prospective mother-infant pair study performed at the Norwegian Institute of Public Health. Between 2002 and 2009, 2606 mothers were recruited by public health nurses in seven counties across Norway, except for Østfold county where they were recruited by a pediatrician in Østfold hospital. In the present study, 199 mother-child pairs were selected based on the availability of adequate volume of banked breast milk samples for extraction and analysis for live-born singleton baby boys. For each case, about one potential control was selected by restricting the sex to boys-mother pairs with adequate milk sample for extraction and analysis. Details of the selection procedure are shown in Fig. 1.

2.2. Cryptorchidism: definition and mapping

Mothers were asked to fill in questionnaires at 1, 6, 12 and 24 months after delivery where they gave detailed information about presence or absence of one or both testicles, descent or ascent of the testicles, timing for testicular descent, and whether operation was performed or scheduled for cryptorchidism. The prevalence of congenital cryptorchidism was 6.1%, and more than half descended within the first six months. The prevalence ranged between 2.2 and 2.4% at 6, 12 and 24 months after delivery. Ever-reported cryptorchidism was defined as cryptorchidism reported at any time point (1, 6, 12 or 24 months) was 12.2% (n = 154/1262). Unilateral cryptorchidism was reported twice more than bilateral cryptorchidism, and orchiopexy was performed or planned in only three boys within two years after birth. Persistent cryptorchidism which was defined based on double reporting at one year and two year was 1.6% while recurrent cryptorchidism was 8%.

For the present case-control study, ever-reported cryptorchidism was used as the unique inclusion criteria. A total of 94 out of 154 eligible cases fulfilled the inclusion criteria having enough milk samples for analysis, and 105 controls were selected randomly from the eligible dataset (n = 1262) following the same inclusion criteria. Sensitivity analysis comparing ever-reported cryptorchidism and persistent cryptorchidism definitions have been performed (Fig. A.1).

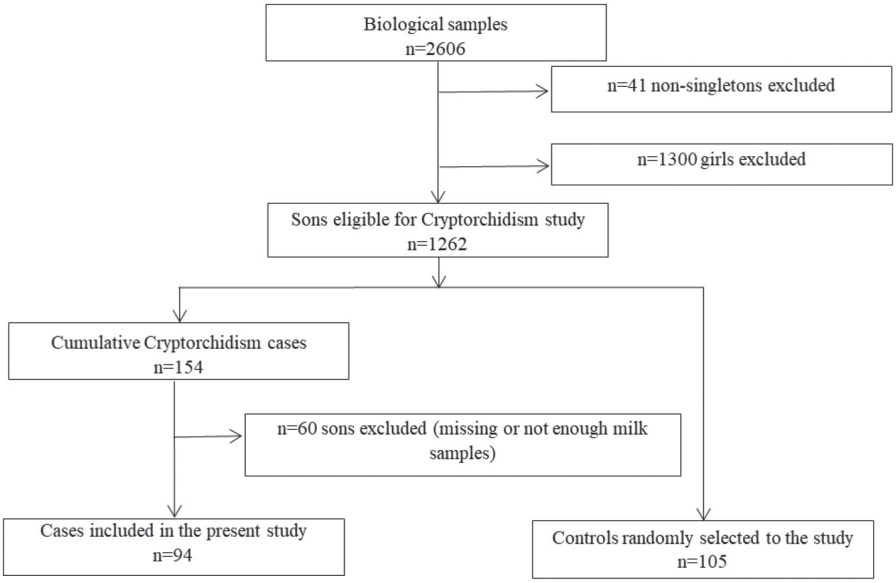

Fig. 1. Selection procedure for samples analyzed for anti-androgenic compounds using the AR CALUX reporter gene assay.

2.3. Mother milk collection

Women were asked to collect 25 mL of milk every morning for eight consecutive days between the first two weeks and two months after giving birth. The median age of milk sampling was 33 days (10th–90th percentile: 18–57) and more than 90% were sampled between two weeks old and before the child reached 2 months of age. In Norway, the prevalence of full breastfeeding during this period is more than 80% while the median duration of breastfeeding is 11–12 months (Häggkvist et al., 2010). Mothers were encouraged to avoid electrical pumping equipment and were asked to register details on how and when the milk was sampled (Eggesbø et al., 2011). Milk samples were collected in 250 mL natural HDPE Packaging Bottles (Cat. No.: 967-21244, Thermo Scientific Nalgene®) made from food-grade high-purity resins, previously tested for potential migration of antiandrogenic chemicals (Collet et al., 2020). The milk bottles were posted by the mothers and stored at −20 °C in a Biobank of the Norwegian Institute of Public Health upon arrival. In this case-control study we selected milk samples from a subset of mothers to 94 boys with cryptorchidism (unilateral or bilateral) and to 105 boys with no cryptorchidism.

2.4. Ethical approval

The study was approved by the Norwegian Data Inspectorate (ref. 2002/1398) and Regional Ethics Committee for Medical Research (ref. S-02122). Mothers were included after oral and written informed consent had been obtained.

2.5. Covariates

Questionnaires filled in by the mothers were used to obtain information on potential confounders. Information on the child's sex, birth weight, preterm, gestational age and maternal smoking during pregnancy was obtained by linkage to the Medical Birth Registry of Norway (Skjærven et al., 2000). Maternal age (˂25, 25–35, >35 years old), pre-pregnancy body mass index (BMI) (under-weight, normal, overweight, obese) and birth weight (˂2500 g, 2500 to 4000 g, >4000 g) were entered as continuous variables. Gestational diabetes, preeclampsia, parity, preterm (child born before 259 days or 37 completed weeks), small for gestational age (<10th percentile), smoking habits (no smoking, daily smoker ˂10 cigarettes, daily smoker >10 cigarettes), as well as maternal education (low, medium, high) were set as categorical variables.

2.6. Sample preparation

Analytes derived from each breast milk sample were extracted following a two-step extraction method previously described in Collet et al. (2020). To extract apolar compounds, 5 mL of homogenized breast milk per sample was transferred to a clean 60 mL glass tube with 5 mL of 2-propanol (CAS. no.: 67-63-0, BioSolve) and shaken for 10 min on a shaker at 200 ± 20 strokes per minute. 14 mL of n-hexane (CAS no.: 110-54-3, BioSolve) was added and the tubes were shaken for an extra hour at 200 ± 20 strokes per minute. The upper layer was collected into a clean collecting tube and the procedure was repeated twice with a shorter shaking time (30 min). Collected fractions were evaporated to dryness and reconstituted in 1 mL of n-hexane. Glass columns were prepared with 5 g of 2% deactivated silica and conditioned with 12 mL of n-hexane. Samples were loaded and eluted with 30 mL of a combination of n-hexane and dichloromethane (CAS no.: 75-09-2, BioSolve) to a ratio of 3:1. The final extract was evaporated until dryness, reconstituted in 30 μL of DMSO and stored at −20 °C.

One control consisting of 5 mL of breast milk primarily spiked with 50 μL of a solution of 13C-labeled internal standard containing PCB153L and PCB180L (200 ng/mL) (MBP-D7, Wellington Laboratories) was added to every ten-sample batch. Controls were processed as described earlier (Collet et al., 2020). In brief, controls were extracted following the same procedure as the samples with the exception that the final fraction was reconstituted in isooctane (CAS no.: 540-84-1, BioSolve). Internal standard recoveries were assessed on a gas chromatograph/tandem mass spectrometer (GC–MS/MS) system using gas chromatograph GC-2010 Plus (Shimadzu) and gas chromatograph mass detector GCMS-TQ8050 (Shimadzu) controlled by the program GCMS Real Time Analysis (Shimadzu) and a CTC CombiPal autosampler controlled by the software Cycle Composer (CTC Analytics AG). Parameters and settings used for the analysis were similar to those described by Collet et al. (2020). Overall, recovery values were 96 ± 13 and 115 ± 21% for PCB153 and PCB180, respectively.

Polar compounds from breast milk samples were extracted according to the method detailed by Collet et al. (2020). In brief, we adapted and optimized a QuEChERS (Quick Easy Cheap Effective Rugged and Safe) solid phase extraction and cleaning method for isolating polar

EACs in the breast milk samples (Anastassiades et al., 2003). The 199 samples were homogenized prior to the procedure. In a clean 50 mL tube (Grenier Bio-One), 5 mL of sample was transferred and completed with 15 mL of acetonitrile (ACN) (CAS no.: 75-05-8, BioSolve). After 30 s of vigorous shaking, one QuEChERS EN 15662 extraction packet (Cat. no.: 5982-5650, Agilent) was added. Tubes were shaken for 15 min using a circular shaker and then centrifuged for 5 min at 4000 rpm at 4 °C. The upper phase was collected in a clean 60 mL glass tube and the solid lower phase mainly constituted of salts was re-dissolved by adding 15 mL of ACN. The complete procedure was repeated once using the same Grenier tube but without adding sample. The upper layers were combined and transferred to a 15 mL QuEChERS d-SPE (Cat. no.: 5982-5158, Agilent) clean-up tube. After 1 min vortex for homogenization, the d-SPE tubes were centrifuged for 5 min at 4000 rpm at 4 °C. Subsequently, the upper layer was collected and evaporated until dryness. All extracts were reconstituted in 30 μL of DMSO and stored at −20 °C until analysis.

Samples were extracted by sets of 10. A procedure control consisting of 5 mL of spiked breast milk was included to each batch to assess the efficiency of the extraction. 100 μL of a mixture of internal standards (100 μg/mL) of bisphenol A (CAS no.: 80-05-7, Sigma-Aldrich), 17β-estradiol (E2) (CAS no.: 50-28-2, Sigma-Aldrich) and testosterone (CAS no.: 58-22-0, Sigma-Aldrich) was used, similar to the pilot study (Collet et al., 2020). Controls were extracted following the same procedure as the samples with the exception that the final extract was reconstituted in pure ACN. Controls were analyzed by liquid chromatography using a Kinetex Biphenyl column (150 × 4.6 mm 2.6 μ particle size) (Cat. no.: 00F-4622-E0, Phenomenex). The system setup was the same as detailed earlier (Collet et al., 2020). After analysis, recovery values were calculated by comparing the peak height in the control samples with the initial internal standard spiking solution. BPA, E2 and testosterone were recovered to a rate of 35 ± 13, 50 ± 6.8 and 59 ± 5.9%, respectively.

Recovery calculations using GC- and LC-based analysis showed that 105% of apolar compounds were extracted while only 48% of polar compounds could be found back after the procedure. Therefore, to compensate for that loss and produce a realistic sample, twice as much of the polar fraction were added to the final reconstituted mixture (ratio 2:1 polar/apolar).

Previous work showed that endogenous hormones including 17β-estradiol and testosterone could be extracted by our optimized QuEChERS method to an average yield of about 50% (Collet et al., 2020). Chemicals with a weak log K_{ow} value (i.e. coefficient of the relationship between lipophilicity and hydrophilicity of a substance) such as phthalates and phytoestrogens are expected to be extracted along those natural compounds in the polar fraction. On the contrary, hydrophilic compounds (log K_{ow} value >5) including PCBs, PAHs and brominated flame retardants, are predominantly extracted by n-hexane and will therefore be found in the apolar fraction.2.7 Anti-AR CALUX bioassay.

Apolar, polar and mixed fractions derived from each sample were individually analyzed on the anti-AR CALUX reporter gene assay (Sonneveld et al., 2005). The anti-AR CALUX assay is based on human osteoblastic osteosarcoma U2-OS cell-line (American Type Culture Collection), cultured as described previously (Sonneveld et al., 2005). The AR cell line is stably transfected with a full-length human AR expression vector and a minimal promoter element coupled to a luciferase reporter construct containing three androgen responsive elements (Sonneveld et al., 2005).

Blinded analysis was chosen to reduce bias. Anti-AR CALUX analysis was performed using a Hamilton Starlet liquid handling robot coupled to a Cytomat incubator. The procedure was adapted from the automated version of the CALUX bioassay described earlier in Van der Burg et al. (2014). For this, cultured AR CALUX cells were re-suspended in assay medium (1×10^5 cells/mL) consisting of DMEM/F12 medium without phenol red indicator (Cat no.: VX1041025, Fisher) supplemented with 10 U/mL penicillin and 10 μg/mL streptomycin (P/S), non-essential amino acids (NEAA) (Cat no.: 11140-03, Gibco) and 5% charcoal-stripped fetal calf serum (DCC). 100 μL were transferred in 96 well plates and incubated for 20 ± 4 h at 37 °C and 5% CO_2. After incubation, samples were placed in the handling robot along with a nine-point calibration line of the reference compound flutamide ranging from 0 M, consisting of pure DMSO, to $1.0E^{-05}$ M (stock solution). The robot was programmed to dilute each extract (dilution series 1-3-10-30-100x) using assay medium supplemented with $3.0E^{-10}$M of 5α-dihydrotestosterone i.e. DHT (CAS: 521-18-6, Sigma Aldrich). The final DMSO concentration was set to 0.2%. The robot was programmed to fill the 96-plate with 100 μL of exposure medium. All dilution points including the calibration line were tested in triplicate on the same 96-well plate. A solvent control (DMSO) was added to each plate to evaluate background activity. After 22 ± 2 h of incubation the medium was discarded and replaced by 30 μL of a Triton-lysis buffer. Plates were shaken for 10 min and the luciferase signal in cellular lysates was measured using a Tristar luminometer (Berthold).

2.7. Controls

All anti-androgenic measurements were performed along with the Cytotox CALUX® assay. Used as a control, the assay identifies non-specific luciferase activity repression e.g. caused by cellular death. The Cytotox CALUX bioassay consists of U2-OS cells constitutively expressing the luciferase gene (Van der Linden et al., 2014) and was essentially performed as described above with the exception that the analysis was done by hand with undiluted samples. A full dose response curve of the reference compound tributyltin acetate (CAS: 56-36-0, Merck Chemicals B.V.) was added to each Cytotox CALUX plate. Samples inducing a decrease in luminescence of more than 20% (i.e. cytotoxicity ≥20%) were further diluted and reanalysed. For further evaluation of non-specific signals, ten breast milk samples randomly selected from the sample set were incubated with an excess amount of DHT (i.e. 1000-times the EC50 concentration). DHT saturating concentration is set to maintain receptor activation through competing with anti-androgenic compounds. In this way, a remaining decrease in luminescence demonstrates a non-specific repression, independent from AR inhibition. As none of the tested samples showed such repression of the signal, further measurements were considered as true antagonistic activity.

2.8. Data handling

For each plate, the EC50 DHT agonistic signal was set to 100% and the maximum signal response induced by the antagonist flutamide was defined as 0% (Fig. 2). Relative Light Units (RLUs) measurements were

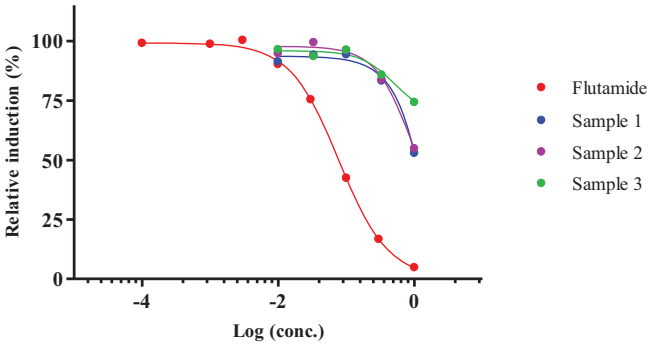

Fig. 2. Typics dose-response curves for anti-androgenic compounds and activities in human milk extracts.

corrected for background using DMSO. The average of each triplicate was plotted to the reference dose-response curve. Subsequently, all results were transformed using the statistical software package GraphPad Prism 5.0 (non-linear regression, variable slope, 4 parameters, robust fit), and expressed as a percentage of the maximum signal (relative induction). The relative induction was used to quantify the anti-androgenic activity in each sample expressed as μg equivalent of flutamide per gram of milk (μg flutamide eq./g of milk). "Quantification of Flutamide equivalents (Flu-EQs) of a sample extract is based on the anti-AR response of the dilution closest to 80% relative induction on the anti-AR dose response curve".

A sample is considered active if its activity surpasses the limit of quantification (LOQ) of the anti-AR CALUX bioassay, set to ≥0.40 μg flutamide eq./g of milk. A non-active sample i.e. activity ≤ LOQ, was replaced by an estimation of the activity equal to half the LOQ value. Determination of the LOQ was based on the standard deviation of the solvent blank DMSO (LOQ = average (solvent blank) + 10 * SD (solvent blank)). Anti-AR CALUX measurements were evaluated according to the following criteria: IC50 of the reference compound between assay-specific predetermined limit values (1.1E−07–1.1E−06 M); R2 of standard curve >0.98; z-factor of standard curve >0.6. The measurements performed for this study fit into the predetermined criteria as the standard curve depicted an overall IC50 range of 5.7E−7 to 1.0E−06 M and mean z-factor of 0.822.10.

2.9. Statistical analysis

Differences in maternal and child characteristics between cryptorchidism cases and controls in our study were tested in a univariate analysis using Pearson's chi-square test for binary or categorical variables, and the Wilcoxon rank-sum test for the continuous variable (gestational age).

2.9.1. Adjustment models

To estimate the association between anti-AR activity in human milk samples and the risk of cryptorchidism, appropriate confounders were first identified using a Direct Acyclic Graph (DAG) (DAGitty v3.0) (Fig. 3). Multiple logistic regression models were used to estimate adjusted Odds Ratio (OR) and 95% Confidence Interval (95% CI). Two sets of potential confounders were identified. Set 1 included maternal education, maternal age, pre-pregnancy BMI, preterm, gestational age and parity, in which the overall percentage of missing values was 7%. Set 2 included small for gestational age, gestational diabetes, preeclampsia, smoking during pregnancy, alcohol consumption during pregnancy, antibiotic use, fat content of the milk, and a selected maternal toxicant. The overall missing when adjusting for both set 1 and 2 was 83% (Table A.2). The high percentage of missing was due to the fact that information on some covariates were obtained through linkage with the Norwegian MoBa study, for the small set of participants who also participated in the MoBa study. However, the high percentage of missing values in the fully adjusted model was problematic, so we decided to use multiple imputation by predictive mean matching in Stata as a sensitivity analysis. As a result, the other set of potential confounders (alcohol use, smoking, antibiotics use, maternal toxicant and fat content) were dropped since they had high missing values, and sensitivity analysis after multiple imputation by predictive mean did not change the result (Fig. A.2). The final model was therefore a complete case analysis of the association between anti-AR activity measured in breast milk fractions (polar, apolar, mixture) and the risk of cryptorchidism adjusted for variables in set 1.

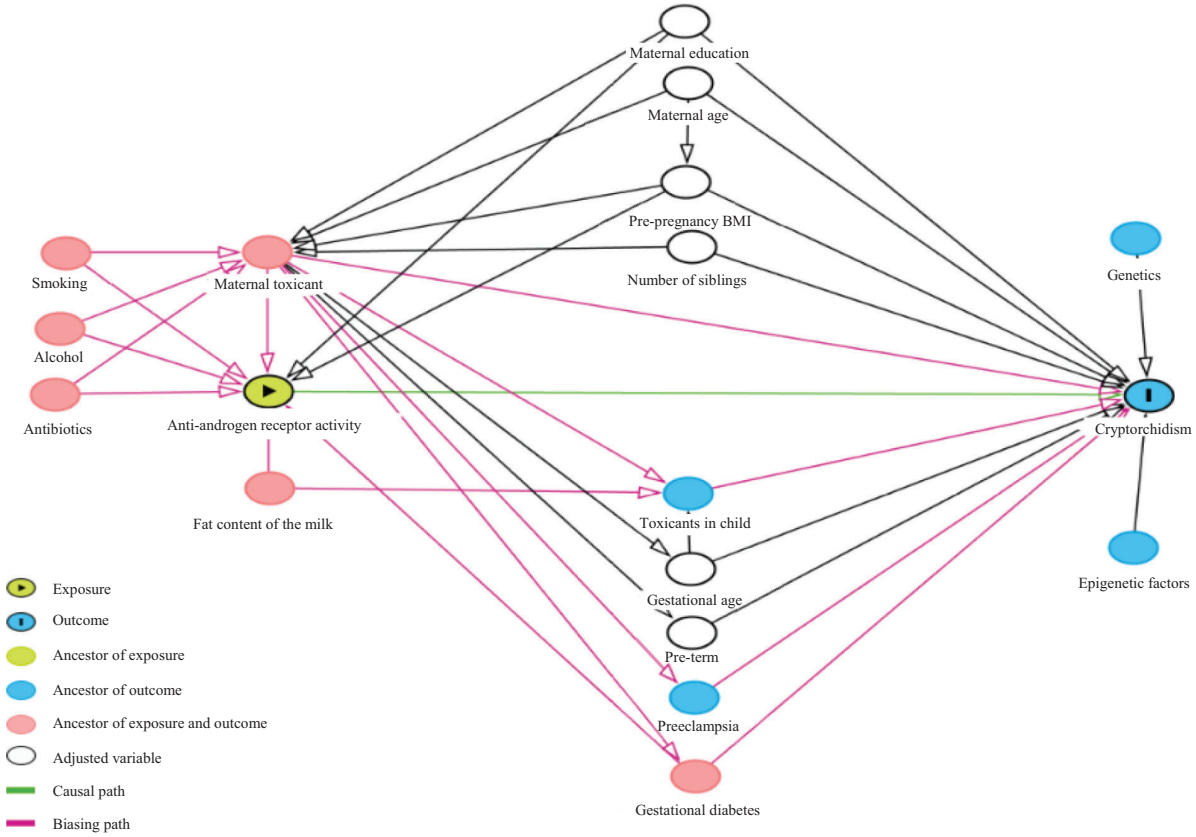

Fig. 3. Representation of appropriate confounders in relation to possible association between anti-AR activity in uman milk and cryptorchidism identified using a Direct Acyclic Graph (DAG).

Table 1
Univariate analysis of the association between maternal and child characteristics of the study participants (n (%) or median (IQR)) and the occurrence of cryptorchidism in a case-control study among 199 mother-child pairs, Norway.

Characteristic	Total n = 199	Controls n = 105	Cases (cryptorchidism) n = 94	p-Value
Maternal age (years)				
<25	30 (15.1%)	15 (14.3%)	15 (16.0%)	*0.94*
25–35	148 (74.4%)	79 (75.2%)	69 (73.4%)	
>35	21 (10.6%)	11 (10.5%)	10 (10.6%)	
Maternal education				
Low	19 (9.5%)	7 (6.7%)	12 (12.8%)	*0.16*
Medium	26 (13.1%)	11 (10.5%)	15 (16.0%)	
High	150 (75.4%)	84 (80.0%)	66 (70.2%)	
Missing	4 (2.0%)	3 (2.9%)	1 (1.1%)	
Birth weight (grams)				
<2500	5 (2.5%)	3 (2.9%)	2 (2.1%)	*0.62*
2500–4000	146 (73.4%)	74 (70.5%)	72 (76.6%)	
>4000	48 (24.1%)	28 (26.7%)	20 (21.3%)	
Nulliparity	78 (40.0%)	43 (41.7%)	35 (38.0%)	*0.60*
Small for gestational age	19 (9.5%)	9 (8.6%)	10 (10.6%)	*0.62*
Gestational age (days)	281.5 (273–288)	284 (272–289)	280 (273–287)	*0.18*
Preterm (before 37 week/259 d)	13 (6.5%)	8 (7.6%)	5 (5.3%)	*0.51*
Pre pregnancy BMI				
Under weight (<18.5)	4 (2.0%)	4 (3.8%)	0 (0.0%)	*0.27*
Normal (18.5–24.9)	125 (62.8%)	65 (61.9%)	60 (63.8%)	
Overweight (25–29.9)	39 (19.6%)	19 (18.1%)	20 (21.3%)	
Obese (≥30)	22 (11.1%)	12 (11.4%)	10 (10.6%)	
Missing	9 (4.5%)	5 (4.8%)	4 (4.3%)	
Smoking at the end of pregnancy				
No smoking	160 (80.4%)	83 (79.0%)	77 (81.9%)	*0.55*
Daily smoker < 10	5 (2.5%)	2 (1.9%)	3 (3.2%)	
Daily smoker > 10	1 (0.5%)	1 (1.0%)	0 (0.0%)	
Missing	33 (16.6%)	19 (18.1%)	14 (14.9%)	
Preeclampsia	7 (3.5%)	2 (1.9%)	5 (5.3%)	*0.19*
Gestational diabetes	1 (0.5%)	1 (1.0%)	0 (0.0%)	*0.34*

Note: BMI: body mass index; IQR: interquartile range.

2.9.2. Software

Stata (version 16.1; Stata Corp LP, College Station, Texas, USA) was used for all statistical analyses. Statistical significance was set up at $p < 0.05$.

3. Results

The characteristics of the study population in which anti-androgenic activity was assessed are presented in Table 1. Of 199 mothers, 148 (74.4%) were between 25 and 35 years of age at delivery, comparable between cases and controls. Most of the participants were highly educated (75.4%). The study population shows a minority of overweight or obese women (31%) and more than 80% reported no smoking history. In both cases and controls, most boys weighted within 2500–4000 g at birth. A non-significant tendency of lower education among mothers of cases was observed. Overall, no major difference was found between the mothers giving birth to a cryptorchid boy versus a healthy child. Also, the selection of the case-control study participants is representative of the study population enrolled in the HUMIS cohort with respect to maternal age, maternal education, and small birthweight for gestational age (9.5% vs 10%). Preterm was lower in the present study compared to the study population (6.5% vs 9%). Detail of the profile is presented in the appendix (Table A.1).

Anti-androgenic activity was evaluated in 199 breast milk samples and expressed as μg equivalent of the reference compound flutamide, per gram of milk. For each participant, the apolar and polar fractions including the endogenous hormones, and the combination of both have been analyzed separately using the anti-AR CALUX bioassay (Fig. 4, Table 2).

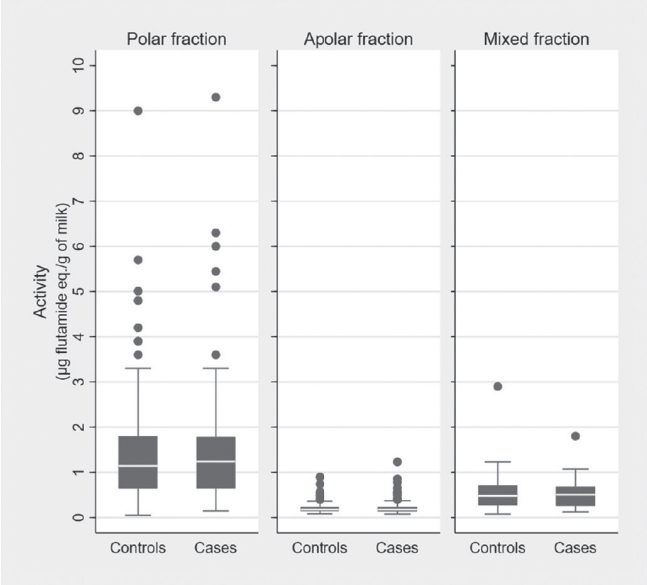

Fig. 4. Boxplots showing the distribution of anti-androgenic activity in polar fraction, apolar fraction, and reconstituted breast milk samples (mixture) measured via the anti-AR CALUX bioassay, and over cryptorchidism cases and controls.

Fig. 4 and Table 2 present the distribution of anti-androgenic activity in the apolar and polar fractions along with the mixtures of both. The mixtures showed antagonistic properties ranging from 0.40 to 2.82 μg flutamide eq./g of milk. In nine milk samples, the mixtures were found to be ≥1.0 μg flutamide eq./g. of milk while in 70 samples, the mixtures were below LOQ (data not shown). Polar analyte-based measurements ranged from 0.41 to 9.25 μg flutamide eq./g of milk, and most samples (124) were above ≥1.0 μg flutamide eq./g of milk. In contrast, apolar fractions showed a smaller range of values (0.40 to 1.16 μg flutamide eq./g of milk) with 23 milk samples eliciting activity above the LOQ cut-off value, and only one sample scoring above 1.0 μg flutamide eq./g of milk. Yet, considering that more than half of breast milk samples showed anti-androgenic activity when the mixture was assessed, we estimated the potential daily exposure to anti-androgenic EDCs of a breastfed child during the first twelve months of life.

According to WHO guidelines on breastfeeding (WHO, 2011), a child should be exclusively breastfed for the first six months to one year of life. During this period, an infant ingests about 150 mL of milk per kg of body weight (bw) per day, on average. Our previous results showed that breast milk from Norwegian mothers of the HUMIS cohort could be contaminated with anti-androgenic toxicants, equivalent to 0.52 μg flutamide eq./g of milk, on average. Considering these data, and the volume of daily intake of an infant, we estimated the average exposure to anti-androgenic EDCs during the first year of life, in μg of flutamide/kg bw/day (Table 3). The anti-androgenic activity was stable during breast feeding duration as can be seen with absence of the correlation with the child age during breast milk collection (Fig. A.3).

The distribution of anti-androgenic activity in control and cryptorchid groups is shown in Table 2. The mean of the mixed fractions was 0.53 ± 0.36 and 0.51 ± 0.28 μg flutamide eq./g of milk in controls and cases, respectively. Most apolar fractions fell below the LOQ value of the anti-AR CALUX bioassay regardless of the studied group ($p75 = 0.23$ μg flutamide eq./g of milk). Finally, the mean activities of the polar fractions were 1.47 ± 1.33 and 1.49 ± 1.42 μg flutamide eq./g of milk. In conclusion, the anti-androgenic activity measured in breast milk samples from controls and cases were similar across all the tested fractions.

The association between anti-AR activity measured in breast milk fractions (polar, apolar, mixed) and the occurrence of cryptorchidism was estimated using logistic regression (Fig. 5, Table A.2). The

Table 2
Distribution of anti-AR activity in polar, apolar, and combined fractions in breast milk from mothers of cryptorchidism 94 cases and 105 controls enrolled in the Norwegian HUMIS study.

	N	Mean	SD	p5	p25	p50	p75	p95	Max	Range	IQR
Mixtures											
All	199	0.52	0.32	0.13	0.27	0.50	0.70	0.98	2.90	2.82	0.43
Cryptorchidism	94	0.51	0.28	0.14	0.26	0.51	0.68	0.98	1.8	1.68	0.42
Controls	105	0.53	0.36	0.13	0.27	0.48	0.71	0.97	2.90	2.82	0.44
Polar fraction											
All	199	1.48	1.37	0.18	0.64	1.24	1.80	4.20	9.30	9.25	1.16
Cryptorchidism	94	1.49	1.42	0.19	0.64	1.24	1.78	5.1	9.3	9.16	1.14
Controls	105	1.47	1.33	0.17	0.64	1.14	1.80	3.90	9.00	8.95	1.16
Apolar fraction											
All	199	0.22	0.16	0.10	0.14	0.17	0.23	0.55	1.23	1.16	0.09
Cryptorchidism	94	0.23	0.18	0.1	0.14	0.17	0.23	0.57	1.23	1.16	0.1
Controls	105	0.22	0.14	0.10	0.14	0.17	0.23	0.50	0.90	0.82	0.09

Note: Concentration are expressed in μg flutamide eq./g of milk. N: number of samples per category; Mean: average activities; SD: standard deviation; p5–95: 5th to the 95th percentile; Max: maximum activity measured for the category; Range: difference between minimum and maximum activity measured for the category; IQR: interquartile range, difference between p75 and p25.

minimally adjusted model contained maternal age, maternal education, pre-pregnancy BMI, gestational age, and number of siblings, while the fully adjusted model also contained gestational diabetes and pre-eclampsia. The other set of variables (alcohol use, smoking, antibiotics use, maternal toxicant and fat content) were dropped since they had high missing, and sensitivity analysis after multiple imputation by predictive mean didn't change the result (Fig. A.2). Sensitivity analysis has also been performed by excluding pre-terms, and to check whether the use of breast pumps affect cryptorchidism risk. No statistically significant association was observed for anti-androgenic activity in the polar fractions, apolar fractions or mixture with ever-reported cryptorchidism. Sensitivity analysis with persistent cryptorchidism as an outcome also did not show any association with the different fractions (Fig. A.1).

4. Discussion

This study shows that nearly all Norwegian babies are exposed to anti-androgenic activity through breast milk and at levels higher than the advisory threshold. Children's daily exposure to anti-androgenic EDCs through breastfeeding was estimated to 78 μg flutamide eq./kg of body weigh/day. However, no significant association between cryptorchidism and anti-androgenicity from polar or apolar contaminants in breast milk, nor from the overall reconstituted samples (mixtures), was found.

Anti-AR CALUX mother milk analysis showed that anti-androgenic activity was higher among polar breast milk fractions in contrast to apolar fractions that gave a lower anti-AR activity signal. This was unexpected, since the latter fraction is supposed to contain various toxicants such as DDT, DDE, brominated flame retardants, and organochlorines, known to exhibit anti-androgenic activity (Kelce et al., 1995; Lemaire et al., 2004; Van der Burg et al., 2010). Yet, the present results match our previous pilot study demonstrating less activity in apolar extracts in comparison with polar measurements (Collet et al., 2020). Due to their resistance to degradation and extended half-life, persistent toxicants are able to bioaccumulate throughout life in diverse fatty tissues, including breasts. Over the past decades, many studies highlighted EDCs presence in mother milk, demonstrating breastfeeding as an important source of exposure for the infant (Norén and Meironyté, 2000; Sonawane, 1995; Thomsen et al., 2010). The present study provides insights regarding child's early life exposure showing an almost ubiquitous anti-androgenicity in breast milk. Although generally less potent, apolar fraction analysis provides a better general insight of perinatal exposure in opposition to the polar fraction, which mainly reflects the action of non-persistent compounds i.e. less stable exposure. Yet, exposure may be stable if mothers have a stable consumption/use of products containing non-persistent chemicals. Nevertheless, reconstituted samples analysis highlighted the importance of studying both fractions, alone and in combination. Anti-AR CALUX analysis showed a decline in anti-androgenic activity from polar compounds (p50 = 1.80 μg flutamide eq./g of milk) when assessed along with apolar chemicals as a mixture (p50 = 0.50 μg flutamide eq./g of milk). These results demonstrate that the presence of apolar compounds influences anti-androgenicity by weakening stronger polar chemical effects. While various classes of lipophilic chemicals were proven to cause anti-androgenic activity, there are likely many other chemicals in this fraction, with unknown effect on the AR which could cause unpredicted

Table 3
Estimation of a child's daily intake of anti-androgenic EDCs through breastfeeding at one month, six months and twelve months.

Average activity (μg flutamide eq./mL)	Age (months)	Daily intake of milk[a] (mL)	Estimated intake per day (μg flutamide eq./day)	Body weight[b] (kg)	Nursing child dose per day (μg flutamide eq./kg bw/day)
0.52	1	525	273	3.5	78
0.52	6	1125	585	7.5	78
0.52	12	1380	718	9.2	78

[a] Assuming that the child is exclusively breastfed.
[b] Average body weight at 1, 6 or 12 months.

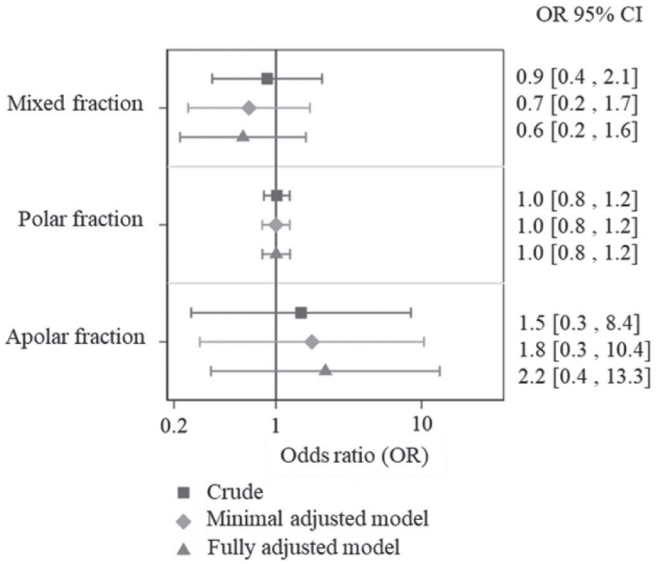

Fig. 5. Association between anti-AR activity measured in breast milk fractions and the occurence of cyptorchidism as estimated using logistic regression.

mixture effects (Bergman et al., 2012; Kortenkamp, 2014). Overall, these anti-AR CALUX measurements provide an overview of the AR activity present in human milk. A nursing child's dose exposure to anti-AR EDCs based on the AR activity levels present in human milk was estimated to 78 μg of flutamide eq./kg bw/day approximately. This level of anti-androgens, expressed as flutamide equivalent, largely exceeds the acceptable daily doses of a flutamide exposure for humans reported in 2017 (no adverse effects level NOAEL of 0.025 mg/kg/day) (Zacharia, 2017). Additional studies involving targeted methods and mixture effect analysis are necessary to identify specific compounds and their origins in order to advice regulatory authorities on necessary further chemical restrictions. Nevertheless, the present extraction and analysis of 199 breast milk samples gave a starting point in the path of exploring anti-androgenicity and mixture effect in human milk. Different potential EDCs were measured in HUMIS cohort, and the potential association with anti-androgenic activity in breast milk will be reported in a separate paper.

Anti-androgenic measurements in cryptorchid and control groups were similar regardless of the assessed fraction. Experimental studies on animals, however, clearly demonstrated that cryptorchidism can be caused by the disruption of hormones involved in regulation of testicular descent (MacLeod et al., 2010; Welsh et al., 2008). The mechanistic studies investigating anti-androgenic activity and cryptorchidism in humans were mainly focused on the relationship between hormone (testosterone/estrogen) levels or non-persistent i.e. polar compounds and cryptorchidism. (Key et al., 1996; Virtanen and Adamsson, 2012). Still, there are a few studies investigating on cryptorchidism risk and apolar chemicals. In 2007, a Danish-Finnish case control study among 280 boys suggested a possible association between flame retardants (polybrominated diphenyl ethers) levels in breast milk and congenital cryptorchidism (Main et al., 2007). Brucker-Davis et al. (2008) showed that cryptorchid boys were more likely to present higher levels of DDE and polychlorinated biphenyls in colostrum in comparison with the control group.

This case-control study has some strengths. First of all, it is the first to investigate the association between anti-androgenic receptor activity in human breast milk fractions and cryptorchidism in sons selected from a prospective cohort. The selection of the study participants in this case-control study is a representative of the entire (HUMIS) cohort with respect to maternal (age, education, parity, pre-pregnancy BMI) and child (gestational age, small for gestational age) characteristics. The HUMIS cohort is a multi-center birth cohort enrolling participants across Norway to represent Norwegian population. The exposure, breast milk extracts were collected after the outcome, however, represents real-life exposure covering both prenatal and postnatal windows especially for lipophilic compounds as it accumulates in lipid-rich tissues and represents mothers body burden. There is also a positive correlation between levels measured in breast milk and levels in the umbilical cord for persistent chemicals making it a suitable proxy for prenatal exposure (Kanja et al., 1992; Verner et al., 2013; Waliszewski et al., 2001). Moreover, using CALUX bioassays and total EDC content, this study takes into account the interactions among “real” chemical mixtures in the breast milk. In addition, we had information on a large set of potential confounders and the outcome, cryptorchidism, was based on repeated questionnaire (maternal reports) at 1, 6, 12, and 24 months which enables to capture recurrent cryptorchidism; normally descended testis at birth that subsequently ascended.

One of the major limitations of this study is that the outcome ascertainment which was based on maternal report. The reliability of cryptorchidism ascertainment could be questionable even for trained observers. To reduce the bias from maternal report, questionnaires were filled separately at 1-,6-,12-, and 24-months with detailed information including performed or planned operation. Even though cryptorchidism was not included, high reliability has been demonstrated for important birth and pregnancy parameters from maternal reports compared to registry data in Norwegian mothers (Skulstad et al., 2017). Another limitation was the sample size (199 samples) that might have underpowered the study, preventing the detection of any significant difference. In addition, fractions of breast milk showing anti-androgenic activity above LOQ was low (n = 23/199) in the apolar fraction. Breast milk was used as a proxy for prenatal chemical exposure which is not suitable for polar chemicals. While it is well-known that apolar compounds bioaccumulate in breast tissues, polar chemicals, i.e. non-persistent compounds, content tends to fluctuate during life. Although polar activity measurements were corrected for loss during the extraction procedure, an underrepresentation of non-persistent EDCs cannot be ruled out. Androgen-dependent phases of testis descent occur during early fetal development (8–10 weeks) and much later around 25–35 gestational weeks (Hutson et al., 2013; Welsh et al., 2008). Whereas breast milk appears as a suitable matrix for monitoring late fetal environmental, earlier steps of cryptorchidism determinism might have been overlooked. Moreover, even if this study was the first to investigate anti-androgenic receptor activity in human breast milk fractions and the risk of cryptorchidism, it was only limited to androgen receptors. Therefore, studies on more androgen pathways such as its synthesis or metabolism may be required in future studies.

5. Conclusion

In conclusion, in this case-control study we found the ubiquitous anti-androgenicity present in breast milk samples from Norwegian mothers. We highlighted the interactions between chemicals of different polarity and the interest in assessing them alone and in combination. No association between anti-androgenic activity in mother milk and the occurrence of cryptorchidism in the offspring was found. Additional studies based on a larger sample size are needed to further explore the relationship between anti-androgenic activity in milk and the risk of cryptorchidism in humans.

CRediT authorship contribution statement

Bérénice Collet: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Writing – original draft. **Anteneh A. Desalegn:** Investigation, Formal analysis, Methodology, Writing – original draft. **Kees Swart:** Methodology, Investigation, Validation, Writing – review & editing. **Matthijs Naderman:** Methodology, Investigation, Validation. **Nina Iszatt:** Methodology, Supervision, Formal analysis, Writing – review & editing. **Hein Stigum:** Methodology, Supervision, Formal analysis, Writing – review & editing. **Tina K. Jensen:** Methodology, Formal analysis, Writing – review & editing. **Abraham Brouwer:** Conceptualization, Methodology, Funding acquisition, Supervision, Resources, Writing – review & editing. **Merete Eggesbø:** Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing, Resources. **Bart van der Burg:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149746.

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Supplementary (Paper II)

Table A.1. Profile of study participants for anti-AR case-control study compared to the eligible and entire cohort.

	Enrolled in HUMIS cohort	**Eligible (mother-son) Cohort**	**Case-control study participants**
	N=2,606	N=1,262	N=199
Maternal age (years)	30 (27-33)	30 (26-33)	30 (26-33)
Maternal Education level			
Low	223 (8.6%)	113 (9.0%)	19 (9.5%)
Middle	319 (12.2%)	158 (12.5%)	26 (13.1%)
High	1,952 (74.9%)	970 (76.9%)	150 (75.4%)
Missing	112 (4.3%)	21 (1.7%)	4 (2.0%)
Birth weight			
<2500	178 (6.8%)	56 (4.4%)	5 (2.5%)
2500-4000	1,853 (71.1%)	876 (69.4%)	146 (73.4%)
> 4000	574 (22.0%)	329 (26.1%)	48 (24.1%)
Missing	1 (0.0%)	1 (0.1%)	
Nulliparity	1,063 (42.5%)	531 (43.6%)	78 (40.0%)
Gestational age (days)	281 (273-288)	282 (274-288)	281.5 (273-288)
Small for gestational age	261 (10.0%)	113 (9.0%)	19 (9.5%)
Preterm	242 (9.3%)	101 (8.0%)	13 (6.5%)
Caesarean section	434 (16.7%)	205 (16.2%)	27 (13.6%)
Pre-pregnancy BMI			
Under weight	80 (3.1%)	39 (3.1%)	4 (2.0%)
Normal	1,536 (58.9%)	750 (59.4%)	125 (62.8%)
Overweight	565 (21.7%)	280 (22.2%)	39 (19.6%)
Obese	260 (10.0%)	127 (10.1%)	22 (11.1%)
Missing	165 (6.3%)	66 (5.2%)	9 (4.5%)
Smoking in pregnancy			
never smoking	1,557 (59.7%)	747 (59.2%)	132 (66.3%)
past smoker	682 (26.2%)	342 (27.1%)	52 (26.1%)
occasional	63 (2.4%)	35 (2.8%)	3 (1.5%)
daily smoker LE 10	208 (8.0%)	97 (7.7%)	8 (4.0%)
daily smoker >10	32 (1.2%)	17 (1.3%)	2 (1.0%)
Missing	64 (2.5%)	24 (1.9%)	2 (1.0%)
Preeclampsia	115 (4.4%)	55 (4.4%)	7 (3.5%)
Gestational diabetes	15 (0.6%)	9 (0.7%)	1 (0.5%)

Table A.2. Missing-value patterns of the data.

	Pattern								
Percent	1	2	3	4	5	6	7	8	9
17	1	1	1	1	1	1	1	1	1
41	1	1	1	1	1	1	1	0	0
23	1	1	1	1	1	1	1	1	0
11	1	1	1	1	1	1	1	0	1
2	1	1	1	1	1	1	0	1	0
1	1	1	1	0	1	1	1	1	0
1	1	1	1	1	1	1	0	0	0
<1	0	1	1	1	1	1	0	1	0
<1	1	0	1	1	0	0	0	0	0
<1	1	0	1	1	1	1	1	0	0
<1	1	1	0	1	1	1	1	0	1
<1	1	1	0	1	1	1	1	1	0
<1	1	1	0	1	1	1	1	1	1
<1	1	1	1	0	0	0	0	0	0
<1	1	1	1	0	1	1	1	0	0
<1	1	1	1	1	0	0	0	0	0
<1	1	1	1	1	0	0	1	0	0
<1	1	1	1	1	1	0	1	0	0
100									

Note: 17% of the data is complete for all variables while 83% are missing for either one or more of the variables.
1. Gestational age 2. Smoking 3. Fat content percent 4. Maternal education 5. Number of siblings 6. Antibiotics use during pregnancy 7. Pre-pregnancy BMI 8. PCB194 9. Alcohol consumption.

However, the 83% missing was reduced to 7% when variables with high missing (alcohol use, smoking, antibiotics, maternal toxicants, and fat content) were removed after Sensitivity analysis showed that including them in the model didn't change the result (See below Supp. Figure 1-5). Therefore, after removing these variables, 93% had complete data for the rest of the variables.

Table A.3. Unadjusted and adjusted estimates from logistic regression for the associations between anti-AR activity from different fractions (mixture, polar, apolar) and the risk of cryptorchidism among cases (n=94) and controls (n=105) in HUMIS cohort.

	Crude				Adjusted[a]				Further adjusted[b]			
	OR	**95% CI**		***p-value***	**OR**	**95% CI**		***p-value***	**OR**	**95% CI**		***p-value***
Ever-reported cryptorchidism												
Mixed fraction	0.87	0.36	2.07	*0.75*	0.65	0.25	1.71	*0.39*	0.59	0.22	1.61	*0.31*
Polar fraction	1.01	0.82	1.24	*0.89*	1.00	0.80	1.24	*0.99*	1.00	0.80	1.25	*0.97*
Apolar fraction	1.49	0.26	8.44	*0.65*	1.77	0.30	10.39	*0.53*	2.18	0.36	2.58	*13.3*

Note: [a]Minimal adjusted model: Maternal age, socio economic status, Pre-pregnancy BMI, gestational age and parity

[b]Fully adjusted model: Additional adjustment including gestational diabetes and preeclampsia.

OR: Odd Ratio; 95% CI: 95% Confidence interval

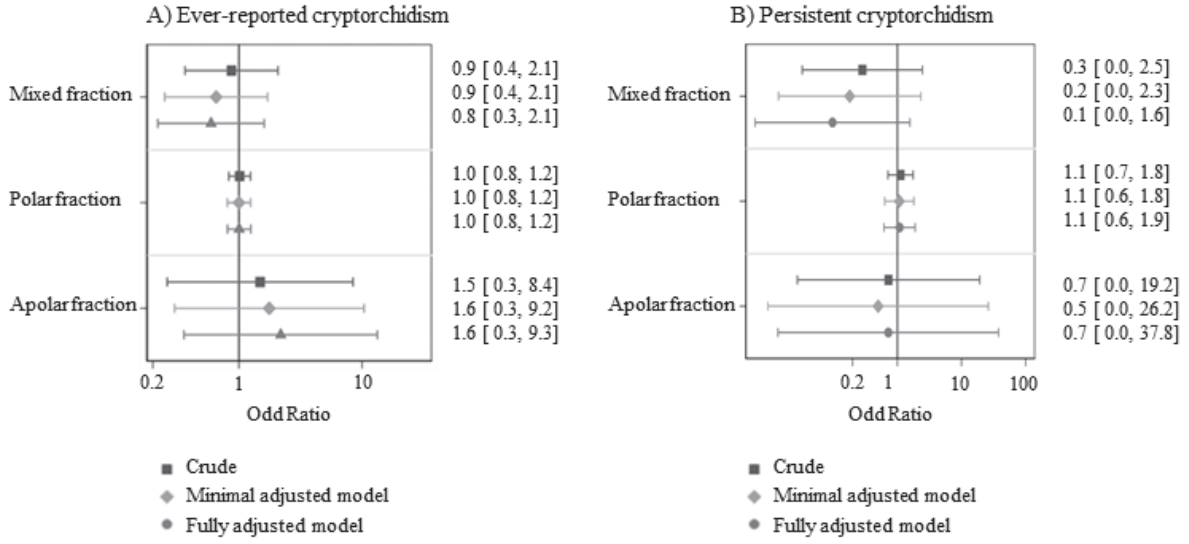

Figure A.1. Comparison of the association between anti-androgenic activity in different breast milk fractions (polar, apolar, mixture) with ever-reported cryptorchidism (A) and persistent cryptorchidism (B).

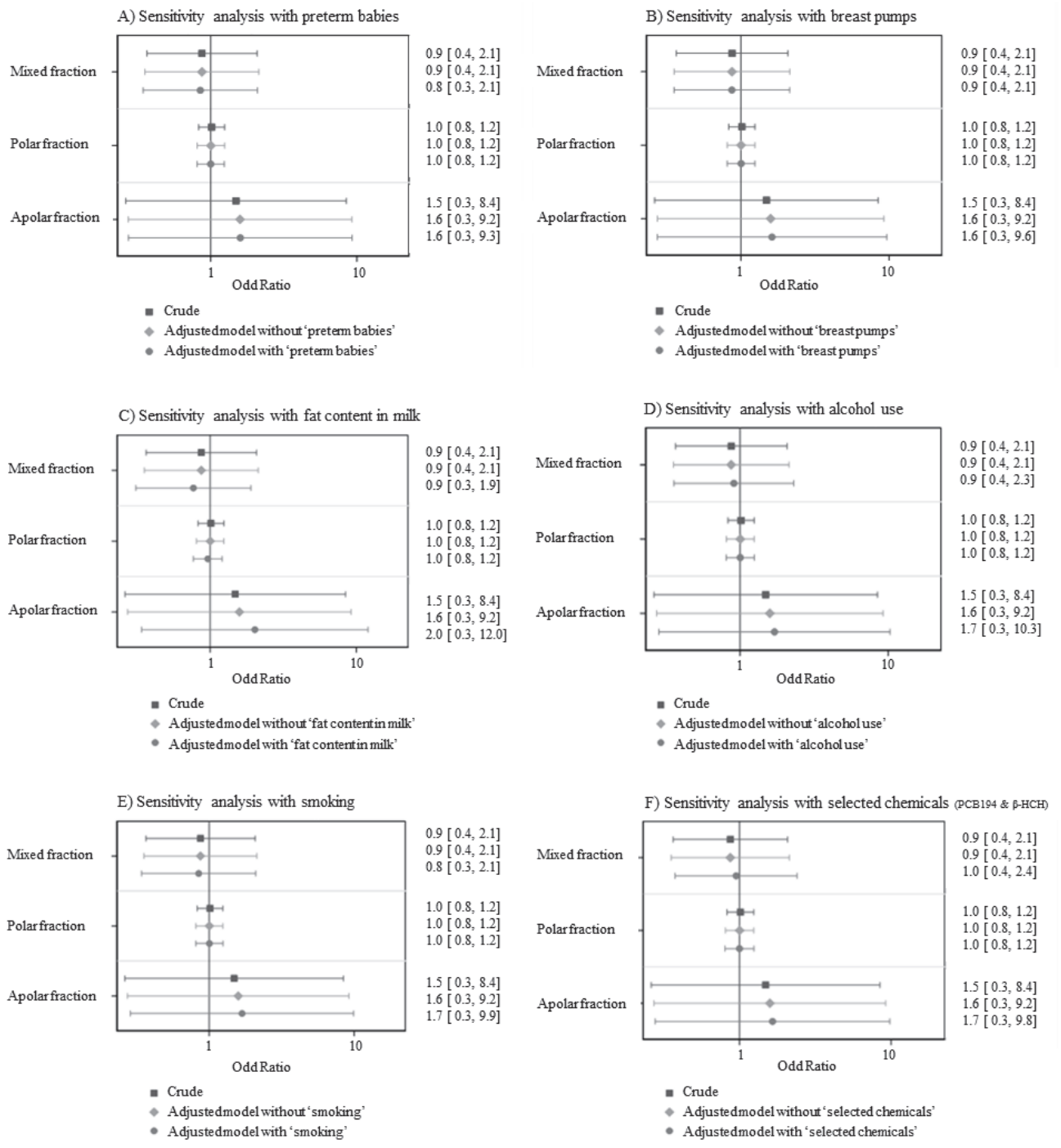

Figure A.2. Sensitivity analysis including potential confounders (A-F; preterm babies, use of breast pumps, fat content of the milk, alcohol use, smoking, and selected chemicals in breast milk maternal toxicants, and fat content).

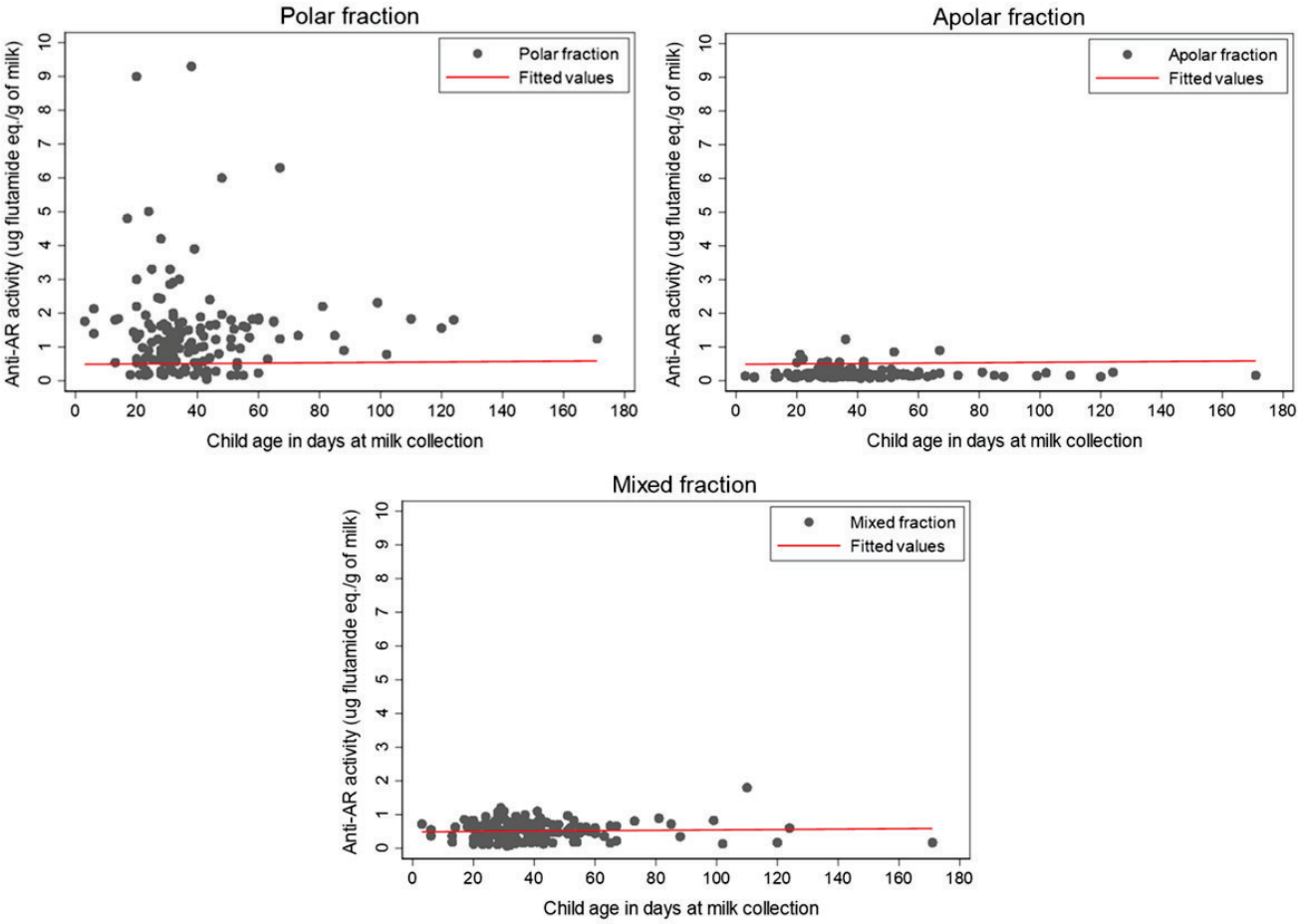

Figure A.3. Association between anti-AR activity and child age (days) at breast milk collection for polar, apolar and mixed fractions.

Paper III

Paper IV