Hanna Marta Dusza

Contaminants of emerging concern in the fetal environment

unravelling the exposure and effects of endocrine disrupting compounds and micro(nano)plastics in utero

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Contaminants of emerging concern in the fetal environment

unravelling the exposure and effects of endocrine disrupting compounds and micro(nano)plastics *in utero*

Opkomende stoffen in de foetale omgeving

ontrafelen van de blootstelling en effecten van hormoonverstorende stoffen en micro(nano)plastics *in utero*

(met een samenvatting in het Nederlands)

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“In a woman’s womb
another chance to make the world better”

Ellen Hopkins

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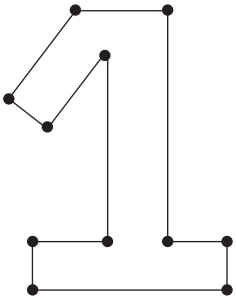

General introduction and thesis outline

Fetal development represents the most vulnerable phase of human life. From the moment when sperm and egg cells join together in a woman's womb, millions of events will take place to shape a human being. It is here *in utero* where a dynamic interplay between genome, epigenome and environmental factors knits us, cell by cell, into existence and prepares us for life outside of the mother's womb. Just like our environment affects our health, the *in utero* environment can have a profound impact on the way an organism programs itself for later life. From conception to birth, the placenta is exposed to a broad range of environmental pollutants women are exposed to during pregnancy and contrary to old beliefs, it does not form an impermeable barrier to these agents. A wide range of exogenous compounds has been shown to pass the placental barrier and reach the direct environment of a developing fetus. These exogenous compounds, collectively called "contaminants of emerging concern", may include new chemicals for which we have little toxicity data, but also known contaminants for which new insights on their biological effects are emerging. Of specific concern is the *in utero* exposure to endocrine disrupting compounds (EDCs), a diverse group of exogenous chemicals that can interfere with the tightly regulated hormonally driven developmental processes. As described below, such exposures have been associated with adverse pregnancy outcomes, as well as, altered fetal programming during critical windows of development, leading to structural and functional changes manifesting themselves as diseases later in life, or even in subsequent generations. Gestational exposure to EDCs is associated with a broad spectrum of adult-onset diseases and is a public health concern worldwide. This has placed an immense responsibility on scientific and regulatory bodies as well as manufacturers to appropriately characterise, assess and prevent harmful *in utero* exposures. Nevertheless, due to the tremendous diversity of chemical contaminants in the environment and the obvious ethical limitations, the characterization of a whole spectrum of EDCs potentially harmful to the fetus remains exceptionally challenging. Moreover, there are new emerging contaminants of concern, such as micro- and nanoplastics (MNPs) and chemicals associated with them, that could reach the placenta and contribute to *in utero* exposures. However, the potential effects of MNPs on placenta functioning and fetal development have received scant attention so far. These are recognised challenges that require urgent scientific scrutiny. The goal of this thesis is to address major knowledge gaps surrounding *in utero* exposure to these contaminants of emerging concern and their potential effects.

Prenatal vulnerability to environmental insults

Developmental Origins of Health and Disease

In 1990 David Barker published a book titled "The fetal and infant origins of adult disease", where he described the idea that environmental factors acting early in life, particularly during intrauterine life, can have profound effects on the developmental programming of adult-inset pathologies. The so-called "Barker's hypothesis" was initially based on observations that undernutrition during gestation increases the risk for metabolic abnormalities and coronary heart disease in adult life [1,2]. Now, 32 years later, this phenomenon is referred to as "Developmental Origins of Health and Disease" (DOHaD) [3–6]. It is now well established that intrauterine exposure to multiple other non-nutritional factors such as infections, drugs or stress increases sensitivity to diseases and dysfunctions in post-developmental life (Figure 1). Although an overwhelming body of evidence, from epidemiological and experimental animal studies, exists in support of this hypothesis, the exact mechanisms underlying the developmental programming of adult disease are still not fully understood. However, an increasing amount of evidence suggests that epigenetic alterations play a critical role in the developmental plasticity of adult pathologies [4,7,8]. In recent years, environmental contaminants, specifically EDCs, are becoming recognised as significant additional drivers for DOHaD, demonstrating the importance of early environmental conditions for adult health (Figure 1) [4,7,9,10].

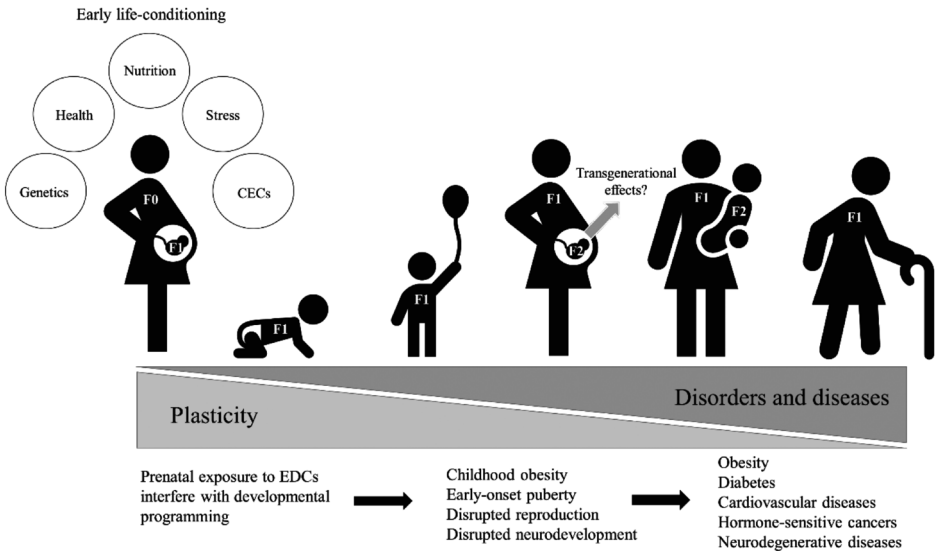

Figure 1. Schematic representation of the Developmental Origins of Health and Disease (DOHaD). Abbreviations: CECs, Contaminants of Emerging Concern; EDCs, Endocrine Disrupting Compounds.

Developmental windows of vulnerability

The timing of exposure to environmental contaminants is extremely important in predicting fetal susceptibility and the risk associated with that exposure. A considerable amount of publications from the field of paediatrics and developmental toxicology has grown up around the term windows of vulnerability, i.e. critical periods in life development where exposures to stressors including environmental pollutants, may disrupt developmental processes and predispose individuals to permanent functional impairments [1,3,4,11,12]. It is now apparent that a fetus in all stages of development is more susceptible to exposure to environmental contaminants than adults. The most critical "windows of vulnerability" exists *in utero* because, during early life programming, different sets of genes are sequentially activated and deactivated, cell reproduction rates are high and biological systems are immature and highly plastic, providing numerous targets for environmental exposures. Other important mechanisms increase both exposure and hazard to the fetus: sensitive periods of development for different organ systems; increased exposures per kilogram of body weight; undeveloped blood-brain barrier; and immature liver and kidney enzyme systems thus not well-developed detoxification processes to metabolize, conjugate, and eliminate toxicants [13–16].

Placenta: the bridge between mother and fetus

In practice, all agents from maternal circulation i.e., nutrients, oxygen, and environmental contaminants, must first pass the placenta to reach the developing fetus. The placenta is the first organ that forms during human embryogenesis. It is a remarkable, multifaceted interface between maternal and fetal circulation that carries out crucial tasks ranging from the physiological adaption of the mother to immunological acceptance, nourishment and support of the developing embryo [17]. The placenta is thus a key organ that enables *in utero* existence and ensures healthy pregnancy outcomes. Trophoblast cells, specifically syncytiotrophoblast are specialized placental cells with diverse biological roles including exchange, endocrine, immune and metabolic functions [18–20]. By the end of the first trimester, the syncytiotrophoblast cells covering the chorionic villi (functional units of the placenta) are fully bathed in oxygenated maternal blood (Figure 2). At term, the extensive branching of chorionic villi creates a large surface area (approximately 13 m^2), which provides ample space for nutritional supply, as well as, for the interaction of environmental contaminants present in maternal blood with the placental syncytiotrophoblast [20,21]. Direct developmental toxicity may arise from contaminants in maternal blood that cross the placental barrier and directly affect fetal development or from exposure to the placenta tissue itself, which may affect placental development and functioning with consequences for both, fetal and maternal health during pregnancy [22].

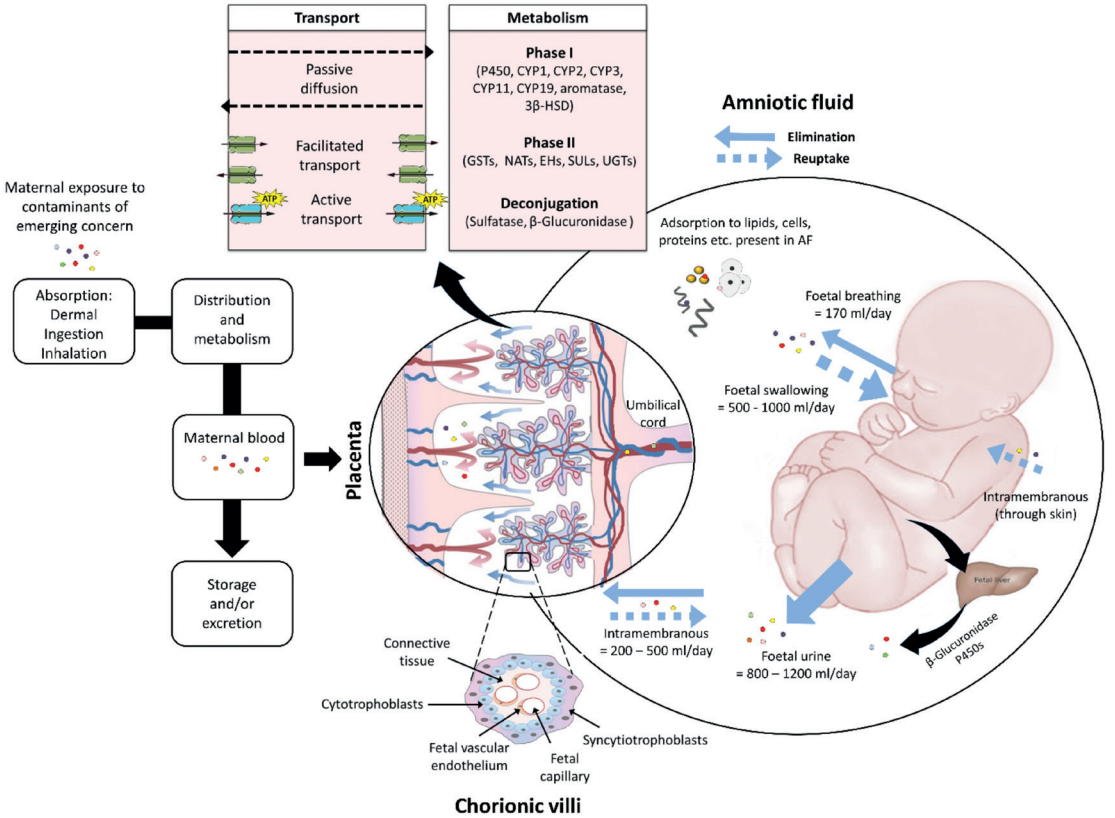

Figure 2. Schematic representation of the complex maternal-fetal toxicokinetics of contaminants of emerging concern including maternal exposure and metabolism; placental metabolism and transport; fetal exposure through the umbilical cord; fetal metabolism and elimination; and reuptake from amniotic fluid. Placental trophoblasts, specifically syncytiotrophoblasts, cover chorionic villi and are in direct contact with maternal blood where they regulate key placental functions i.e., transplacental transport, metabolism, endocrine and immune functions.

Contrary to the old belief, the placenta does not form a barrier for exogenous contaminants but is highly permeable to a large variety of compounds with diverse molecular structures (e.g., phthalates, bisphenols, pesticides, flame retardants, metals) that can (to various degree) cross it from the maternal blood and reach the fetus [21,23,24]. Passive diffusion as well as an array of plasma membrane carriers and export pumps (located on both the fetal and maternal side of the trophoblasts) largely regulate the transplacental transport [25]. However, the placental/fetal toxicokinetics are complex and dependent on multiple other factors: chemical properties (molecular weight, lipophilicity, protein binding); placental characteristics (blood flow, protein and pH gradient; metabolism; placental surface area and thickness that changes throughout pregnancy); and maternal and fetal factors (foetal growth, development, metabolism and tissue binding, maternal metabolism and maternal health) [25]. Moreover, the placenta is highly metabolically active and contains a wide variety of phase I and phase II enzymes [21]. For example, multiple CYPs present in the mitochondria and endoplasmic reticulum of trophoblast cells, participate in the metabolism of endogenous (e.g., hormones) and exogenous (e.g., EDCs) molecules [26]. Moreover, β-glucuronidase, an enzyme responsible for the deconjugation of many xenobiotics, has particularly high activity in placental and fetal tissue hence, there is a potential for β-glucuronidase–mediated deconjugation of xenobiotics to their free form *in utero* (Figure 2). Also, other various breakdown pathways (oxidation, hydrolysis, reduction and conjugation) can be promoted or retarded by placental enzymes, making the placenta the chief regulator of the *in utero* (chemical) environment. Nevertheless, the complex placental toxicokinetics of environmental contaminants are still poorly understood.

Contaminants of emerging concern

Endocrine disrupting compounds

The first important discussion about EDCs came to light in the 1940s, when naturalist Charles Broley linked the use of the insecticide dichlorodiphenyltrichloroethane (DDT) with a declining population of American bald eagles [27]. Later research confirmed that DDT exhibits endocrine-mediated toxicity causing feminization of male embryos weakened eggshells and altered reproductive behaviour of nesting birds. This was one of the first examples to demonstrate that xenobiotic chemicals could spread far beyond their intended use and induce unexpected adverse effects in non-targeted species. In 1962, this and many other examples of EDCs have been described in Rachel Carson's now critically acclaimed book "Silent Spring". But it wasn't till the mid-90's that the concerns about this issue received a great deal of attention, following a series of publications suggesting that pesticides (e.g., DDT, atrazine) and other synthetic chemicals including phthalates (di(2-ethylhexyl) phthalate, DEHP), bisphenols (bisphenol A, BPA), flame retardants

(polybrominated diphenyl ethers, PBDEs), industrial chemicals (polychlorinated biphenyls, PCBs) and by-products could exhibit endocrine-mediated toxicity [28]. Today, the term "endocrine disruptor" refers to an extremely wide variety of exogenous, natural or synthetic chemicals that can interfere with virtually any aspect of hormonal action. Endocrine disruptors are defined by the WHO/IPCS as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations"[28]. EDCs have been shown to interfere with a range of endocrine-related receptors such as estrogen (ER), androgen (AR), glucocorticoid (GR), thyroid (TR) and the peroxisome proliferative-activated receptor (PPAR) family. EDCs are of specific concern because like hormones they can act at extremely low concentrations, exhibit complex (nonmonotonic) dose-response curves and in complex mixtures dose additivity and synergism can occur [29–32].

Nowadays, daily human exposure to a complex mixture of EDCs including persistent organic pollutants (POPs) is inevitable. Biomonitoring studies detected a wide variety of EDCs in the majority of the studied subject and various body tissues and fluids e.g., blood, urine, serum and breast milk, indicating chronic low-level exposure in the human population, including pregnant women [31,33–36]. In addition, a few published studies measured various EDCs in follicular fluid, cord blood, amniotic fluid, fetal serum and meconium demonstrating placental transfer and fetal exposure [37–42]. Considering that the developing fetus is highly sensitive to hormonal perturbation, EDCs are increasingly recognized as important risk factors for the programming of adult-onset diseases as they may interfere with tightly regulated hormonally driven developmental processes [9,12,43–48]. Research shows that the effects of EDCs exposures often do not manifest themselves at birth, but result in subtle functional changes that increase the risk of disease/dysfunction long after transient *in utero* exposure. Such effects include early-onset puberty [49,50], increased male genital abnormalities [51], declined sperm quantity and quality [52,53], increased occurrence of hormone-sensitive cancers (e.g., breast, uterine and testicular) [31], asthma [54], cardiovascular diseases [55], childhood obesity [56–58], diabetes [59], as well as, altered behaviour and neurodevelopment in children [44,60,61]. Moreover, some effects can be transmitted via the germ line to the offspring resulting in multigenerational or even transgenerational effects [10,46,62,63].

Today, more than 350 000 chemicals and mixtures of chemicals have been registered for production and use [64]. More than 1000 compounds with peer-reviewed data have been suggested to have endocrine-disrupting properties, including chemicals used across a range of applications and in a multitude of industrial and consumer products [65]. Although efforts have been made to identify potential novel EDCs, in its statement from 2012, the World Health Organisation expressed that: "identifying chemicals with endocrine disrupting potential among all of the chemicals used and released worldwide is a major challenge, and, likely, we are currently assessing only the tip of the iceberg". This statement

holds true even now, a decade later when only a small fraction of the hundreds of thousands of synthetic chemicals in existence have been assessed for endocrine disrupting activity. A recent statement by European Commission (2020) further highlights this issue: "The exposure of humans and the environment to endocrine disrupting chemicals requires specific attention. These substances are increasingly linked to diseases acting via the hormonal system. Their use is on the rise, representing a serious risk to human health and wildlife as well as creating an economic cost for society" [66]. Considering the complex maternal and *in utero* toxicokinetics and the ethical challenges in characterizing prenatal exposures, identifying EDCs of potential concern for fetal development remains a major challenge.

Challenges in the characterization of *in utero* exposure to EDCs

There are obvious ethical limitations for the detection of environmental contaminants in the fetal environment during gestation. For this reason, the assessment of prenatal exposure is based mainly on maternal biomonitoring studies, mostly using maternal urine, blood, serum or breast milk. An example of this is shown in Figure 3, for Bisphenol A, arguably the most studied EDC. However, the use of maternal matrices as surrogates for fetal exposures is not without limitations. The important shortcomings of the current approaches for *in utero* exposure assessment are summarized below:

1) Use of maternal matrices as surrogates for *in utero* exposure
    a. There is a lack of data on placental toxicokinetics i.e., maternal urine, blood, serum or breast milk are most commonly used for prenatal exposure assessment, however, it is often unknown to what extent, if at all, chemicals present in these matrices will reach the fetus
    b. The complex placental/fetal metabolism is often not accounted for i.e., there are various breakdown pathways (hydrolysis, reduction, conjugation and oxidation) that can be promoted or retarded by placental and/or fetal enzymes, possibly resulting in a different chemical environment *in utero* than in the maternal compartment
    c. The single sampling of the maternal matrices represents only a snapshot of gestational exposure i.e., provides an imprecise estimate of the long-term *in utero* exposure throughout pregnancy
    d. Due to reduced placental/fetal clearance and poorly developed glucuronidation processes, there might be accumulation and continuous re-exposure of the fetus to the circulating levels of parent compounds and their metabolites *in utero*, as compared to maternal matrices

2) The low, presumed "safe" dose of EDCs in adults may have a potent and irreversible effect during early life development

3) Usually, only a limited number of well-known environmental pollutants are investigated, however, a fetus might be exposed to a vast number of compounds throughout gestation, including new previously unidentified EDCs

4) The cumulative effect of co-occurring chemicals, i.e. "cocktail effect" is of great concern, nevertheless, mixture toxicity is still rarely addressed in the studies on early life exposures

Considering the thousands of chemicals in commercial use that pregnant women might be exposed to and the complex maternal/fetal toxicokinetics, characterization of the whole spectrum of EDCs and their metabolites present *in utero* is still a major challenge. Consequently, the disease risk due to *in utero* exposure to a mixture of EDCs may be significantly underestimated. It is, therefore, crucial to move from the quantification of a limited number of known EDCs to the characterization of complex mixtures, including possible novel and biologically active compounds, preferably in *in utero* specific matrices.

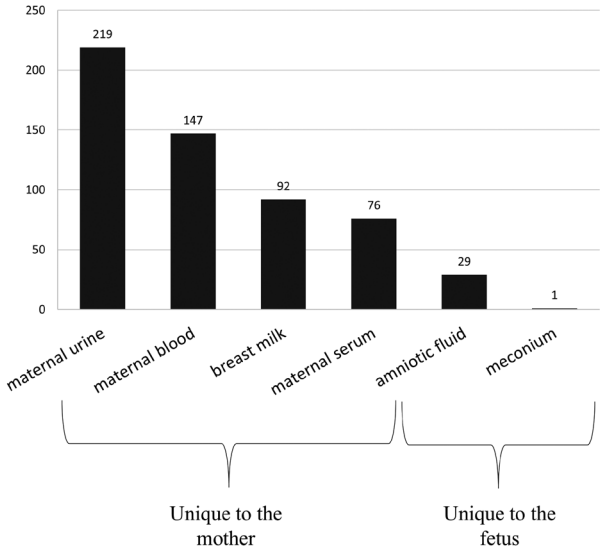

Figure 3. A number of published studies that investigated bisphenol A (BPA) in maternal and fetal matrices, as retrieved from PubMed (June 2022) using a combination of keywords: matrix of choice (and) bisphenol A (and) human.

Micro- and nanoplastics

Since 1950, global plastic production has increased significantly with approximately 367 million metric tons (Mt) produced in 2020 alone. Only approximately 10% of all plastic produced and used is recycled, the rest is incarcerated and ends up in landfills or the environment leading to an increasing plastic pollution crisis [67]. The larger pieces of plastic present a well-documented ecological problem, however, the smaller plastic particles cause a wider, largely unexplored ecological and health concern. Microplastics (MPs, <5 mm) and nanoplastics (NPs, <1 μm) collectively called micro- and nanoplastics (MNPs) can be derived from primary or secondary sources [68]. Primary MNPs are industrially manufactured as microbeads of different sizes and are used in personal care products amongst other. Secondary MNPs are typically formed by weathering, mechanical abrasion, and photodegradation of plastic waste and products [69]. Today, MNPs are abundant and widespread throughout outdoor and indoor environments, contaminate a large variety of foods, beverages and consumer products, and are an emerging global concern for environmental and human health.

Involuntary human exposure to MNPs by ingestion and inhalation is inevitable. It is estimated that each person ingests between 39,000 and 52,000 plastic particles annually and up to 100,000 through inhalation [70,71], however, these estimates do not account for the smaller nanosized particles. Recently, MNPs have been detected in human lung tissue [72,73] and blood [74] suggesting that they can translocate through the gut and lung barrier and end up in systemic circulation. There is convincing evidence from experimental *in vitro* and animal studies that MNPs can accumulate intracellularly, in internal organs and the brain where they can activate the innate immune system, induce inflammatory responses and mediate oxidative stress [75–77]. The toxicity of MNPs may depend on the material, size and shape of the particles, as well as on various environmental contaminants they may carry [78,79]. MNPs can leach plastic-associated compounds such as plasticizers, brominated flame retardants, antioxidants, UV stabilizers and synthetic dyes many of which are known EDCs [78,80,81]. MNPs can also absorb hazardous environmental chemicals (e.g., PCBs, DDT, metals and polycyclic aromatic hydrocarbons (PAHs) which can increase their overall toxic potential. Considering the above, human exposure to MNPs has raised serious health concerns [82].

Recently, (ultra)fire particles from air pollution have been detected in placenta tissue [83,84]. Epidemiological research indicates that exposure to (ultra)fine particles during pregnancy can adversely affect both maternal and fetal health. Various pregnancy complications have been reported such as pre-eclampsia [85,86], pre-term birth [87], low birth weight [88] and stillbirths [89], as well as, increased incidence of postnatal congenital heart defects [90] and respiratory diseases [91]. Recently, MNPs have been detected for the first time in human placenta samples on both, the maternal and fetal sides [92]. This clearly demonstrates that environmental nanoparticles including MNPs may accumulate in placental

cells and that a human fetus may be exposed *in utero*. Considering the current and projected increase in MNP pollution, the potential adverse effects of MNPs on reproductive health and fetal development are of specific concern however largely unexplored [93]. Since the placenta plays a central role in fetal and maternal wellbeing during pregnancy, there is an urgent need to understand the potential placental toxicokinetics and toxicity of these ubiquitous contaminants of emerging concern.

Moving the field forward

Amniotic fluid

Amniotic fluid (AF) is a complex nutritious milieu that surrounds and protects the fetus in the womb. It is a product of fetal urination, tracheal secretions, and intra-/transmembranous pathways and a repository matrix that accumulates a wide range of environmental xenobiotics that the fetus is exposed to throughout pregnancy [94]. AF represents an important route of fetal exposure. It continuously circulates through fetal membranes (through fetal swallowing and excretion, Figure 2) and the ongoing cycle of fetal ingestion and excretion results in a continuous, prolonged *in utero* exposure [94–96]. Moreover, it includes not only maternal/placental but also fetal toxicokinetics, and therefore possibly contains a mixture of metabolites and biomarkers unique to the *in utero* environment. Despite the aqueous nature of AF, the spectrum of hydrophilic and lipophilic compounds detected in this matrix is broad, and their presence in AF indisputably demonstrates placental transfer and fetal exposure [42,97,98]. Consequently, this repository matrix may be more useful for the comprehensive measurement of a whole spectrum of EDCs and their metabolites present *in utero*, than other extensively researched acute maternal matrices, such as urine or serum. The concentrations detected are usually lower (pg to ng/mL range) than those detected in maternal urine, serum, umbilical cord blood or meconium; however recent advances in analytical chemistry allow chemicals to be detected both qualitatively and quantitatively at these low concentrations in complex matrices. AF can be collected non-invasively during delivery or as a surplus sample from amniocentesis, which represents the earlier period of gestation. AF is uniquely suited to study the *in utero* chemical environment however, so far, its use has been limited. To date, only targeted analysis of a limited number of known environmental contaminants has been performed in this unique matrix [97].

Effect-directed analysis

The success of modern methods in analytical chemistry and bioactivity-based assays gives new opportunities to tackle complex chemical mixtures and toxicological problems. Specifically, effect-directed analysis (EDA) can be used as a powerful tool for identifying biologically active compounds in complex mixtures [99]. EDA is an effect-based approach that interconnects advanced analytical techniques with bioassays to isolate substances

of biological relevance [100]. EDA uses chromatographic separation techniques to separate compounds into multiple fractions and consequently reduce the chemical complexity of the sample [101]. The biologically active fractions are then identified with relevant bioassays that exhibit a specific mode of action (Figure 4). EDA has been successfully used to analyze chemical mixtures in environmental samples (e.g. surface waters, sediments, soil and biota) and for the identification of (unknown) compounds with endocrine-disrupting potencies, such as estrogenic, androgenic, anti-androgenic and dioxin-like chemicals [102–106]. Two studies by Indiveri et al. (2014) and Lopez-Espinosa et al. (2009) applied the EDA approach for comprehensive profiling of a mixture of EDCs in placenta samples, with the former identifying several anti-androgenic compounds, including antimicrobials, insecticides and components of plastic, surfactants and cosmetics, as well as, novel EDCs. These are examples of the broad potential of the effect-directed analysis for the prenatal screening of EDCs. Nevertheless, this approach has rarely been applied to human samples and has never been used to identify EDCs in human AF.

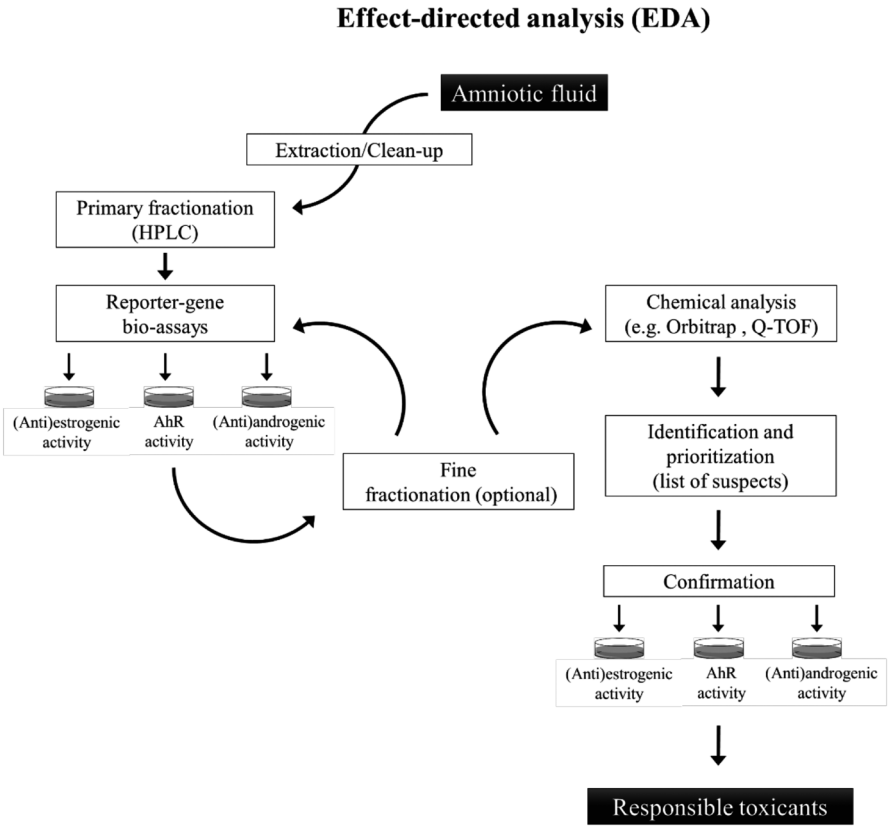

Figure 4. Schematic representation of the workflow for the effect-directed analysis of endocrine disrupting compounds in human amniotic fluid.

Human placental models

There are obvious practical and ethical considerations that limit research efforts in humans, therefore rat and mouse models are generally used to study particle transport and developmental toxicity of MNPs [84,109,110]. However, the placenta is one of the most species-specific organs. The anatomical differences between species including the number of trophoblast cell layers, often reflect the differences in exchange and transport mechanisms and are important factors determining the placental permeability to nutrients, chemicals and particles, making extrapolation from rodent models to humans challenging [111,112]. The difficulty with data extrapolation is not restricted to evolutionary distant species like rodents, but profound differences in placental biology exist even between humans and nonhuman primates [111,113–116]. Consequently, in order to advance our understanding of human placental MNP uptake, transport and toxicity, we need to use predictive *in vitro* and *ex vivo* placenta models using human cells or tissue. Diverse human placental models exist (e.g., plasma membrane vesicles, mono-culture and co-culture, placenta on the chip, villous tissue explants, and placental perfusions), and novel *in vitro* models are being developed to improve the *in vivo* relevance. These models represent different levels of the placental organization and can provide invaluable information on the diverse aspects of placental functioning, accelerating our understanding and providing urgently needed knowledge on the *in utero* health hazards associated with exposure to these contaminants of emerging concern.

Thesis aims and outline

From conception to birth, the placenta and human fetus are exposed to a broad range of contaminants of emerging concern which reach the *in utero* environment with maternal blood. These contaminants may influence maternal and fetal health during pregnancy and contribute to adult-onset diseases. Considering the thousands of chemicals in commercial use that pregnant women might be exposed to and the complex maternal/fetal toxicokinetics, characterization of the whole spectrum of EDCs and their metabolites present *in utero* is still a major challenge. Moreover, there are new emerging contaminants, such as MNPs and chemicals associated with them, that could reach the placenta and contribute to *in utero* exposure. Nevertheless, the effects of MNPs on placenta functioning and fetal development are still largely understudied. The comprehensive characterization of the extent of *in utero* exposure to these contaminants is undeniably crucial to our understanding of the relationship between exposure and health effects. Given the paucity of data on fetal exposure to EDCs and the limited understanding of the transplacental transport and toxicity of MNPs, the goal of our research was to address these major knowledge gaps. The **aim** of our research was two-fold: to develop a method for effect-directed analysis of a wide range of EDCs including novel compounds in human amniotic

fluid; and to investigate possible uptake, transport and toxicity of MNPs and associated compounds in human placenta cells *in vitro*. To this end, this thesis addressed four main **objectives**:

1. Develop a novel method to characterize a broad spectrum of EDCs present *in utero*
2. Identify known and novel, previously unidentified EDCs in the direct fetal environment
3. Determine the appropriate methods and models for studying MNPs in the *in utero* environment
4. Investigate the uptake, transport and toxicity of MNPs in human-relevant placental cell models

This thesis consists of 3 parts**. Chapter 1,** introduces the concept of fetal windows of vulnerability and DOHaD, provides background information on EDCs and discusses the existing challenges and novel approaches to characterize *in utero* exposure to these compounds. Moreover, it discusses MNPs as contaminants of emerging concern for early life health and introduces the placenta as a relevant organ to investigate. In the end, it provides the general aims, objectives and outline of this thesis.

Part I is dedicated to the characterization of fetal exposure to EDCs in AF as a matrix representative of the direct fetal environment. **Chapter 2** describes the development of a nondiscriminating method to extract and fractionate a wide range of lipophilic and hydrophilic EDCs from AF. Here, a chromatographic separation technique and a battery of cell-based reporter gene bioassays were used to identify the (anti)estrogen, (anti) androgen, and dioxin-like activity in the fractions, and, targeted liquid chromatography/ mass spectrometry (LC/MS) analysis was used to measure a range of known xenobiotic and natural compounds responsible for the observed activity in the AF fractions. In **Chapter 3** known and possibly novel EDCs responsible for the observed endocrine activity in nonpolar AF fractions were investigated using targeted and non-targeted gas chromatography high-resolution mass spectrometry (GC-HRMS). The contribution of the known EDCs detected in AF to the activity observed in the fraction was determined using relative potency (REP) values. The compounds responsible for the remaining unexplained activity were investigated further using an innovative, weight-of-evidence approach that takes advantage of curated and freely available databases, predictive QSARs and endpoint-specific profiling models to mine data and predict biological properties of novel EDCs identified through the non-targeted analysis.

Part II focuses on the placenta and MNPs as contaminants of emerging concern and addresses objectives 3 and 4. **Chapter 4** summarizes the physiology and morphology of the human placenta, explores available human-relevant *in vitro* and *ex vivo* placental models, reviews the current state of the science on placental MNP research, and, high-

lights the most important areas that require further scrutiny. **Chapter 5** addresses some of the defined research gaps by investigating the uptake, transport and toxicity of pristine and experimentally weathered MNPs in human placental trophoblasts cells *in vitro*. Here the compositional differences following the weathering were investigated, as well as, the toxicity of MNPs on several endpoints including cell viability, damage to the plasma membrane and effects on placental biosynthesis of steroid hormones.

Part III summarises and discusses the data obtained in **parts I and II** and places this research in a broader perspective.

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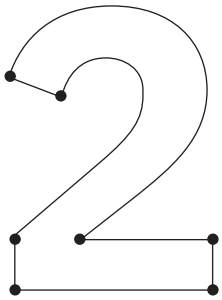
PART I

IDENTIFYING ENDOCRINE DISRUPTING COMPOUNDS IN UTERO:

A NEW APPROACH TO A RECOGNIZED CHALLENGE



Method development for effect-directed analysis of endocrine disrupting compounds in human amniotic fluid

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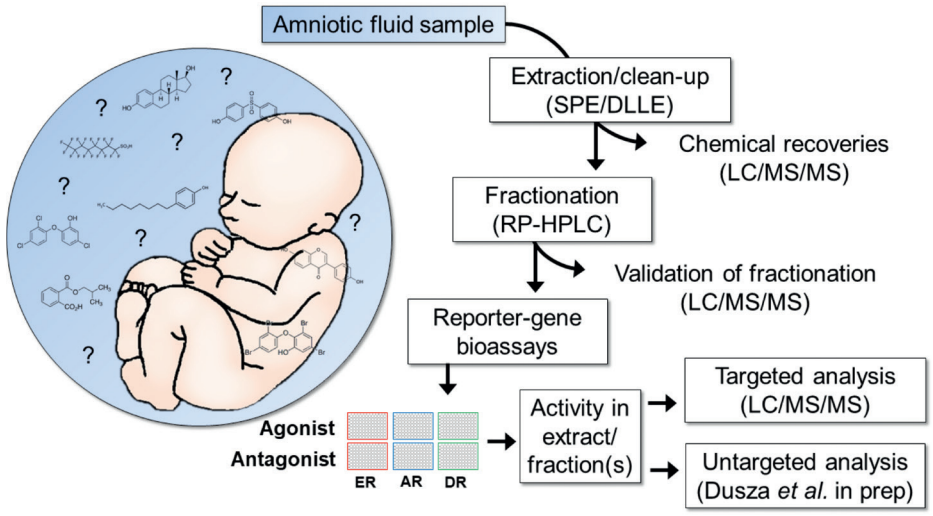
Abstract

The developing foetus represents a highly sensitive period of exposure to endocrine disrupting compounds (EDCs). However, risk assessment of EDCs is hampered by the lack of data on direct in utero exposure. In this study, we developed a robust analytical methodology for the identification of a wide range of known and unknown EDCs in full-term amniotic fluid (AF). First, a method for extraction and fractionation of a broad range of polar and nonpolar EDCs was developed and validated. Maximal recoveries of reference compounds and minimal interference from the matrix were achieved with a combination of solid phase extraction and dispersive liquid/liquid extraction. Bioassay analysis using cell-based reporter gene assays revealed estrogenic, androgenic and dioxin-like activity in AF extract corresponding to 1.4 nmol EEQ/L, 76.6 pmol DHT-EQ/L and 10.1 pmol TEQ/L, respectively. Targeted analysis revealed 13 xenobiotics, phytoestrogens and endogenous hormones in the AF extract that partly contributed to the bioassay activity. Separation of the complex mixture of chemicals in the AF extract with reversed-phase chromatographic fractionation and subsequent bioassay analysis revealed activity in fractions over a wide range of polarity, indicating diverse (unidentified) substances with potential ED activity. The method developed here represents the first methodological step in an effect-directed analysis approach to identify unknown biologically active compounds in the foetal environment.



Graphical abstract

Introduction

The developing foetus is highly susceptible to exposure to endocrine disrupting compounds (EDCs)[1–3], a diverse group of exogenous chemicals that can interfere with the endocrine system and hormone action.[4] A growing body of literature indicates associations between prenatal exposure to EDCs and developmental, reproductive and neurobehavioral disorders, endocrine-related cancers and metabolic diseases.[5–10] Despite the increasing concern for the developmental origins of adult-onset diseases, the extent of exposure to both known and unknown EDCs *in utero* is still largely unknown. This significant data gap hampers comprehensive risk assessment of developmental exposures to EDCs.

Maternal matrices, such as blood, urine and breast milk, are the most commonly used surrogate matrices for prenatal exposure assessment, although it is usually not known to what extent chemicals present in these matrices can reach the foetus.[11] Some compounds (e.g. organochlorines) may pass from mother to foetus by active transplacental transfer rather than passive diffusion and may accumulate selectively in the foetal compartment.[12,13] Moreover, both the placenta and foetal liver express a wide variety of cytochrome P450 enzymes and are metabolically active, resulting in an *in utero* specific chemical environment.[5,14–16] Considering the thousands of chemicals in commercial use that pregnant women might be exposed to and the complex maternal/foetal toxicokinetics, the characterisation of the whole spectrum of EDCs and their metabolites present *in utero* is still a major challenge.[17] Consequently, new methods to measure foetal "exposome", including any potential EDCs, are needed.

Amniotic fluid (AF), a product of foetal urination, tracheal secretions, and intra-/transmembranous pathways, accumulates a wide range of environmental xenobiotics throughout pregnancy.[18] This foetal repository matrix is unique as it can be collected non-invasively during delivery, it includes maternal/foetal toxicokinetics and provides information on the relevant *in utero* exposures occurring prenatally.[19,20] Although the lipid content of full-term AF is low (approx. 0.15 g/L)[21,22], a wide spectrum of hydrophilic and lipophilic xenobiotics has been detected in AF.[18,23–28]

Effect-directed analysis (EDA) is an effect-based approach used to isolate and identify known and unknown biologically active compounds from a complex mixture.[29,30] During EDA, the complexity of the sample is reduced by extraction and fractionation and active fractions are identified with relevant bioassays. Cell-based reporter gene bioassays have been successfully used in EDA studies for the detection of a wide range of EDCs including new, and emerging hormone-like contaminants in environmental samples.[31–33] However, so far this approach has rarely been applied to human samples and, to date, no EDA methodology has been reported for identifying endocrine disrupting activity in AF.

The application of EDA to AF presents a novel opportunity, however, this approach is challenging considering the diversity of EDCs and the complexity of the matrix. i.e., the presence of natural hormones, proteins and lipids that can interfere with bioassay analysis and chemical identification. Therefore, as an important first step in an EDA approach, the goal of this study was to develop a non-discriminating method to extract and fractionate a wide range of lipophilic and hydrophilic EDCs from AF with low interference from the matrix. Xenobiotics and natural hormones were separated through chromatographic separation and the activity profile of collected fractions was measured using a battery of cell-based reporter gene bioassays that measured reporter gene expression under the control of important steroid hormone receptors and transcription factors, including the oestrogen (ER), androgen (AR) and aryl hydrocarbon (AhR) receptors. Targeted LC/MS analysis was used to measure a range of known xenobiotic and natural EDCs that could contribute to the bioassay activity.

Material and methods

All chemicals and reagents were purchased at the highest commercially available purity and are given in Supporting Information S1.

Amniotic fluid samples

Samples of amniotic fluid (AF) were kindly provided by Prof. Andrés López Bernal (University of Bristol). The samples were obtained from healthy pregnancies only, with ethical approval and informed consent (U.K. National Health Service, reference E5431, 2008). Approximately 50 mL of AF was collected during amniotomy at full-term vaginal delivery from each of the four anonymous donors and immediately frozen at -80°C. The samples were pooled, homogenised, and aliquoted in volumes of 5 mL in 15 mL polypropylene falcon tubes and stored at -80°C.

Method development

Sample extraction

A schematic representation of the experimental design for the extraction, fractionation and final analysis of amniotic fluid (AF) samples is presented in Figure 1. Two extraction techniques i.e., solid phase extraction (SPE) and dispersive liquid/liquid extractions (DLLE), were compared and tested under different conditions in order to determine the optimal sample extraction method capable of extracting EDCs with a wide range of chemical and physical properties. For this purpose, a mix of reference compounds containing eighteen EDCs from ten different chemical classes reported previously in AF and covering a wide range of hydrophobicity was used (Table 1, Table S1).

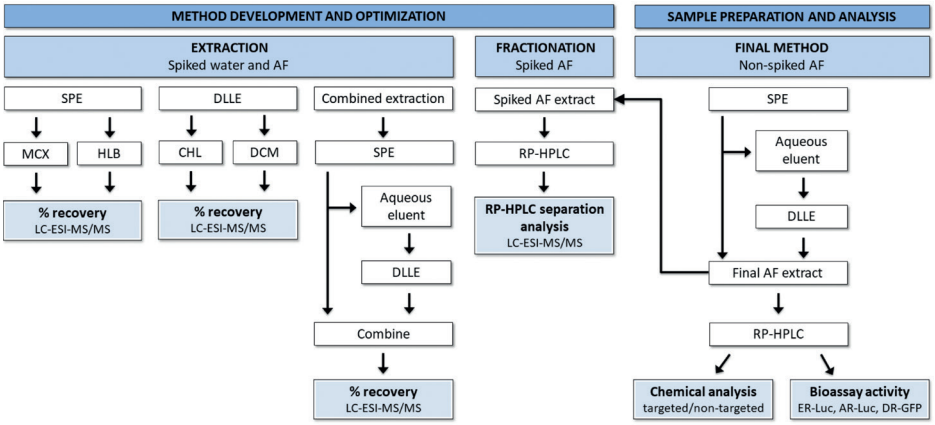


Figure 1. Schematic representation of the experimental design for the extraction, fractionation, and final EDA analysis of AF samples. The extraction efficiency was compared between two SPE sorbents (MCX and HLB), two combinations of DLLE solvents (DCM/acetone and CHL/ acetone), and a combination of SPE and DLLE. The nonspiked and spiked final AF extract was fractionated with RP high-performance LC (RP-HPLC). Chemical recoveries in the nonspiked AF extract and in the spiked fractions were analyzed with LC–ESI–MS/MS, whereas nonspiked fractions were tested for their estrogenic (ER-Luc), androgenic (AR-Luc), and dioxin-like (DR-GFP) activity in reporter gene bioassays.

These included natural hormones, phytoestrogens, exogenous polar and nonpolar EDCs and their hydroxyl metabolites. Two different SPE chemistries, Oasis MCX (mixed-mode, reversed-phase and cation exchange sorbent) and Oasis HLB (multi-purpose reversed-phase sorbent) and two different DLLE extraction solvent combination, dichloromethane (DCM)/acetone and chloroform (CHL)/acetone were tested. Detailed descriptions of the extraction procedures are given in Supporting Information S2. Different extraction methods were performed with spiked (100 µL of reference mix, Table 1, Table S1) LC-MS grade ultra-pure water (W) or/and spiked AF aliquots. The spiked samples were evaporated and reconstituted in 10% MeOH and the analyte recoveries were determined by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The most effective extraction procedure i.e., a combination of DLLE and SPE was used during the final AF extraction, to maximise analyte recovery.

HPLC fractionation

To elucidate the elution profile of the reference compounds, spiked AF extract was fractionated on a reversed-phase Phenomenex Kinetex C18 (100 mm x 2.1 mm, 5 µm pore size) column using an Agilent 1260 HPLC system equipped with binary pump, fraction collector and diode array detector (DAD). Data analysis and processing were performed with Agilent OpenLAB 1.1 Software. Spiked AF extract was injected (50 µL) at a flow rate of 500 µL/min in 95% HPLC grade water and 5% MeOH. The solvent gradient was held as such for 2 min and then increased linearly reaching 100% MeOH at 48 minutes. It was held at 100% MeOH for 2 min and then returned to starting conditions over

a period of 10 min to equilibrate the column before the next injection. The column temperature was set at 35°C. Two-minute fractions were collected throughout the analytical period, excluding the column equilibrium period, resulting in 25 fractions. Each fraction was evaporated to dryness with a gentle stream of nitrogen (at max 40°C), and the remaining residues were reconstituted in 300 µL of 5% MeOH and analysed by LC-ESI-MS/MS to determine the analyte recoveries.

Final sample preparation

SPE columns (Oasis HLB, 6 cc, 150 mg, 30 µm particle size, Waters Corp., Milford, MA) were conditioned with 3 mL of MeOH and 4 mL of deionised water. Aliquotes of AF (5 mL) were diluted with 3 mL of deionised water. The non-spiked samples were loaded on the cartridges at a rate of 1 mL/min. The aqueous eluent was collected for further extraction with DLLE. The cartridges were rinsed with 3 mL of 5% MeOH and the analytes were eluted with 4 x 2 mL of MeOH. The extraction solvent was evaporated (Turbovap, Zymark Corp.) at 40°C and under a gentle stream of nitrogen to a final volume of 1 mL. During DLLE, 0.1 M sodium acetate/acetic acid buffer was added to the aqueous eluent to lower the pH (~ 4.5). The DLLE was performed twice by the addition of 2 mL of acetone (dispersive solvent) followed by the addition of 500 µL of DCM (extraction solvent). Samples were vortexed and then centrifuged at 4081 x g for 15 min at 25°C, organic phases combined and evaporated to dryness under a gentle stream of nitrogen. The 1 mL of extract remaining after SPE was then transferred to the residue in the DLLE tube and vortexed. The extracts were combined and further concentrated to 1 mL, transferred to Eppendorf tube and centrifuged at 20800 × g to separate the precipitate formed after sample concentration. To avoid column overload, 50 µL of the final concentrated extract, representing 100 mL AF, was injected 3 times on the RP-HPLC column, fractionated as above, fractions combined with their complementary fractions, evaporated to dryness and reconstituted in 300 µL of MeOH. 200 µL of each fraction was transferred, evaporated to dryness and reconstituted in 100 µL of DMSO for bioassay analysis. The remaining 100 µL in MeOH was stored for further chemical analysis. All samples were capped and stored at -20°C in amber glass vials.

LC-ESI-MS/MS analysis

Chemical analysis was carried out LC-ESI-MS/MS using an Agilent 1100 Series LC system coupled with an AB SCIEX API ® 5000 triple quadrupole MS operating in MRM mode. 20 µL of the extract was injected on a reversed-phase Phenomenex Kinetex C18 column (100 mm x 2.1 mm, 5 µm pore size). The chromatographic parameters (composition and flow rate of the mobile phase) were optimised. Mobile phases included HPLC-grade water and MeOH. The ionisation was supported via adjustment of pH to 10, through the addition of 0.1% ammonia to both mobile phases. The gradient elution program con-

sisted of 10% MeOH (0-2 min), 70% MeOH (2.5-6.5 min), 100% MeOH (7-9 min), 10% MeOH (9.5-11.5 min) and a flow rate of 0.5 mL/min. After elution, the analytes were ionised using negative ion electrospray ionisation mode (ESI, TurboIonSpray® probe). The optimum operating parameters for ESI were as follows: capillary voltage -4500 V, source temperature 500°C, curtain gas 15, CAD gas 4, GS1 40 and GS2 30. The column temperature was set at 25°C. The collision cell parameters and precursor/product ion transitions (one for quantification and one for confirmation) were optimised for each analyte (Table S2).

Reporter gene bioassays

The estrogen receptor-mediated luciferase reporter gene (ER-Luc) human breast carcinoma (VM7Luc4E2) cells[34], the androgen/glucocorticoid receptor-mediated luciferase reporter gene (AR-Luc) human breast carcinoma (MDA-kb2) cells[35], and the aryl hydrocarbon receptor enhanced green fluorescent protein reporter gene (DR-GFP) mouse hepatoma (H1G1.1c3) cells[36] were maintained and used to test the endocrine activity of extracts and fractions as described in the Supporting Information S3. The AF extract, 25 fractions, solvent (DMSO) control, extraction blank and positive control concentration-response curves were tested in three independent experiments (n=3). 17β-estradiol (E2), dihydrotestosterone (DHT) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were used as positive controls for ER and AR-Luc and DR-GFP, respectively. In the anti-ER/AR/DR assays, cells were co-exposed to the fraction and positive controls at 4 pM, 150 pM and 30 pM of E2, DHT and TCDD, respectively. Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Supporting information S3).

Data analysis

Six concentrations of reference compounds encompassing the entire linear range of the calibration curve were used during LC-ESI-MS/MS analysis. For the quantitative analysis of the reference compounds in the AF extract, LC/MS grade water was injected as calibration point 0. The remaining calibration points were prepared in the matrix to compensate for any matrix-induced ion suppression during analysis and the regression lines of each reference compound were forced through zero. The detection limits (LODs) of measured compounds were calculated as three times the standard deviation (SD) of the extraction blanks i.e., a full extraction procedure performed with a non-spiked HPLC grade water (W). Agilent Analyst® 1.6.2 software was used for data acquisition and analysis. Analyte relative recovery (%) was calculated in AF spiked with reference compounds (Table 2) as measured concentration/spiked concentration x 100.

Table 1. Reference compounds used during method development, including their chemical characteristics, endocrine disrupting mechanism of action (MoA), EC50 or IC50, concentrations in full-term amniotic fluid reported in the literature and measured in this study.

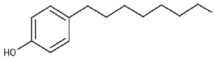
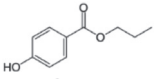
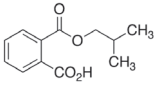
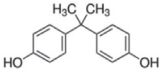
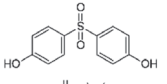
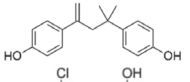
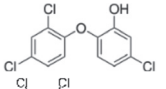
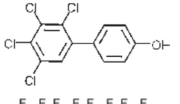
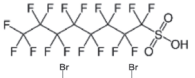
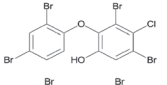
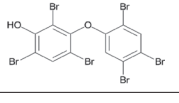
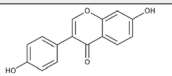
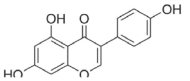
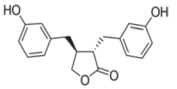
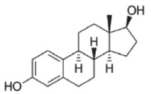
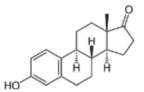
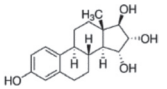
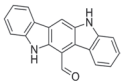
Chemical class	Compound	CAS	Structure	Log*P*	MoA	EC50/IC50 (M)	Reported conc.^ (M)	Measured conc. (M)
Alkyl-phenols	4-OP	71902-25-5		5.50	ER	2.1×10^{-8} (38)	2.77×10^{-8} (39)	<LOD
Parabens	PP	94-13-3		3.04	ER Anti-AR	1.9×10^{-6} (40) 8.6×10^{-5} (41)	1.04×10^{-7} (39)	1.2×10^{-09}
Phthalates	MnBP	30833-53-5		2.84	AR Anti-AR	1.13×10^{-5} (42) 1.22×10^{-7} (42)	3.83×10^{-7} (43)	6.8×10^{-09}
Bisphenols	BPA	80-05-7		3.43	ER Anti-AR	4.0×10^{-7} (38) 3.3×10^{-6} (44)	3.39×10^{-8} (39)	7.3×10^{-09}
	BPS	80-09-1		1.65	ER	1.2×10^{-6} (45)	-	5.6×10^{-09}
	MBP	13464-24-9		5.90	ER	1.1×10^{-9} (46)	-	<LOD
Organo-chlorides	TCS	9012-63-9		4.76	Anti-AR	1.6×10^{-6} (44)	2.43×10^{-8} (38)	1.5×10^{-10}
	OH-PCB-61	67651-34-7		6.10	Anti-AR	1.6×10^{-5} (47)	-	<LOD
PFAS	PFOS	1763-23-1		4.49	ER	-	2.20×10^{-9}* (23)	1.5×10^{-09}
PBDEs	6-OH-BDE-47	79755-43-4		7.19	ER	1.1×10^{-5} (48)	-	2.7×10^{-11}
	3-OH-BDE-153	-		-	-	-	-	1.5×10^{-11}

Table 1. *[continued]*

Chemical class	Compound	CAS	Structure	Log*P*	MoA	EC50/IC50 (M)	Reported conc.^ (M)	Measured conc. (M)
Phyto-estrogens	Daidzein	486-66-8		2.78	ER	6.8×10^{-7} (38)	5.66×10^{-9}*(27)	8.6×10^{-10}
	Genistein	446-72-0		3.04	ER	3.0×10^{-7} (38)	6.25×10^{-9}* (27)	<LOD
	Enterolactone	78473-71-9		3.30	ER Anti-AR,	1.6×10^{-5} (49) 1.3×10^{-5} (50)	3.21×10^{-7}* (51)	1.7×10^{-10}
Estrogens	E2	50-28-2		4.01	ER	3.4×10^{-12} (38)	2.2×10^{-9} (52)	5.1×10^{-10}
	E1	53-16-7		3.13	ER	2.2×10^{-10} (38)	8.65×10^{-10} (53)	2.8×10^{-09}
	E4	15183-37-6		2.21	ER	1.0×10^{-8} (54)	-	4.2×10^{-09}
Other	FICZ	172922-91-7		4.64	AhR	1.0×10^{-8} (55)	-	<LOD

Abbrev: AR, androgenic; ER, estrogenic; AhR, dioxin-like mode of action; PFASs, perfluorinated compounds; PBDEs, polybrominated diphenyl ethers; EC50, half maximal effective concentration; IC50, half maximal inhibitory concentrations; 4-OP, 4-octylphenol; PP, propylparaben; MnBP, mono-n-butyl phthalate; BPA, bisphenol A; BPS, bisphenol S; TCS, triclosan; MBP, 4-Methyl-2,4-bis-(p-hydroxyphenyl)pent-1-ene; OH-PCB-61, 2,3,4,5-tetrachloro-4'-biphenylol; PFOS, perfluorooctanesulfonic acid; 6-OH-BDE-47, 6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether; 3-OH-BDE-153, 3-hydroxy-2,2',4,4',5,5'-hexabromodiphenyl ether; E2, 17β-estradiol; E1, estrone; E4, estetrol; FICZ, 6-formylindolo[3,2-b]carbazole; EC50 and IC50, values based on *in vitro* reporter gene assays; Log*P*, partition coefficient based on octanol-water partitioning, values reported from PubChem or Chemspider database; Symbols: () references from the literature; ^ mean concentrations of unconjugated compounds measured at full-term; * concentrations reported in midgestation; - not reported

Matrix factor (MF) was used as a quantitative measurement of matrix effects and was defined by the compound recoveries in the presence of matrix ions (AF)/compound recoveries in water only (W), where MF≥1 indicates no matrix effect, MF<1 indicates interference from the matrix.[37]

Reporter gene activity was corrected for background luminescence/fluorescence activity and expressed either as % relative luminescence units (% RLUs) for the ER and AR-Luc or % relative fluorescence units (% RFUs) for the DR-GFP. For DR-GFP only, fluorescence in the AF extract was additionally corrected for the background fluorescence in the extraction blank. In each assay, the activity in the extracts/fractions was expressed as the relative % of luminescence/fluorescence of the highest activity observed in the concentration-response curve of the positive control. In each co-exposure assay, the activity in the positive control (n=4 per plate) was set at 100% and the anti-ER/AR/DR activity in the samples (i.e., decrease in luminescence/fluorescence) was expressed relative to the positive control. ER/AR/DR activity in the AF extract was transformed into estrogen equivalent units (EEQ), dihydrotestosterone equivalent units (DHT-EQ) and dioxin equivalent units (TEQs) by interpolating the luminescence/fluorescence response elicited by a diluted extract from the linear region of the four-parameter sigmoidal Hill concentration-response curve prepared with E2, DHT and TCDD, respectively. All equivalent units were expressed in picomoles or nanomoles per litre of AF (pmol/L or nmol/L). Concentration-response curves and statistical significance of the linear correlation between compound retention time and log*P* were analysed/fitted with (GraphPad Software Inc., San Diego, CA).

Results and discussion

Extraction of EDCs in amniotic fluid: method optimization

EDCs belong to an extremely diverse group of compounds. They include pesticides, phthalates, alkylphenols, flame retardants, polychlorinated biphenyls (PCBs), dioxins, parabens, bisphenols, and synthetic hormones.[56] An appropriate extraction technique, capable of extracting chemically and structurally diverse chemicals, is essential for the comprehensive characterisation of a broad spectrum of known and novel EDCs that might be present in AF. In this study, a reference mixture consisting of eighteen EDCs from ten different chemical classes was used during method development to account for this chemical diversity (Table 1, Table S1). Although AF is less complex in its composition than other foetal matrices such as umbilical cord blood or meconium, it contains a mixture of biological components and developmental products, such as cells, nutrients, growth factors, lipids and proteins, that may interfere with chemical and bioassay analysis. Two extraction methods, SPE and DLLE were tested for their extraction efficiency using a mixture of reference compounds (Table 1) and a combination of different SPE sorbents and

DLLE solvents. External calibration standards were used for the quantification of compound recoveries with a correlation coefficient exceeding 0.99. For DLLE, compound recoveries with DCM and acetone as extraction solvent and dispersive solvent respectively were higher than with chloroform and acetone (Table 2), and higher than with DCM alone (data not shown). The mixture of DCM/acetone as a stronger nonpolar solvent performed better in extracting more hydrophobic compounds (e.g., triclosan, hydroxy-PCB and PBDEs) than the mixture of chloroform/acetone. However, in both cases, recoveries below 40% were found for E4, genistein and FICZ. FICZ is stable as a solid but decomposes quickly in solution and the presence of light and air (half-life of ~3h).[55] It is thus likely that FICZ was lost due to light and air-induced decomposition or hydrolysis during solvation, extraction and/or evaporation. In general, weak acids with a high number of hydroxyl groups, such as genistein and E4, have a low affinity to partition to nonpolar organic solvents with low dielectric constants such as DCM or chloroform. To aid the partition of polar compounds into the organic phase, DLLE was additionally tested with salting-out. However, the addition of sodium chloride resulted in poor organic/aqueous phase separation and the presence of salt residue in the final sample extracts (data not shown).

Next to DLLE, two different SPE columns, MCX and HLB, were tested. Both sorbents performed better in extracting hydrophilic compounds than the nonpolar compounds (Table 2). Nevertheless, extraction with HLB resulted in overall higher recoveries, with recoveries above 40 % for all compounds except for 4-OP (6 %). The two most promising extraction conditions, DLLE (DCM/acetone) and SPE (HLB) were evaluated further for their matrix effect (ME). Lipids, specifically phospholipids contribute significantly to ME. They interfere with reversed-phase chromatographic methods, as they are strongly retained on hydrophobic columns and can cause significant ionization suppression during mass spectrometric detection. [57] The total amount of lipids in the full-term AF is generally low (~0.15 g/L)[21,22], nevertheless significant preconcentration of the AF during EDA may cause accumulation of lipids and other small organic constituents that may interfere with the bio-/chemical analysis. Considerable interference from the matrix was observed for the strongly hydrophilic hydroxy-PCB (MF 0.4) and hydroxy-PBDEs (MF 0.2) after DLLE extraction (Table 2). Matrix effects for these compounds after HLB-SPE extraction were much less pronounced (MF 0.5-0.6). Considering that lipids (phospholipids) are highly soluble in methanol[58], SPE clean-up with 5% MeOH might have removed part of the lipids adsorbed on the SPE cartridge, resulting in less interference from the matrix. In general, the overall average recoveries and MF for the two extraction methods were comparable, however, SPE-HLB showed lower variability in the recoveries than DLLE (Table 2). Since HLB-SPE recovered more polar compounds with higher efficiency than DLLE, whereas DLLE (DCM/acetone) recovered mid-polar to nonpolar compounds with higher efficiency than HLB-SPE, the two methods were combined to maximise the extraction efficiency.

Table 2. Analyte Recoveries (%) of the Reference Compounds Extracted with DLLE Using Two Different Extraction Solvent Combinations (DCM/Acetone and CHL/Acetone) and SPE Using Two Different SPE Sorbents (HLB and MCX); Chemical Recoveries (%) ± Relative Standard Deviation (RSD) and MF Measured in the Spiked LC/MS Grade Water (W) vs Spiked AF Extracted with DCM/A, HLB, and a Combination of the Two Extraction Techniques (Final Extraction) as Described in the Method Section[a]

	Spike conc. (ng/mL)	DLLE		SPE		DLLE - DCM/A			SPE - HLB			final extraction
		Recovery (%) (n =1)		Recovery (%) (n =1)		Recovery ± RSD (%) (n = 3)			Recovery ± RSD (%) (n = 3)			Recovery ± RSD (%) (n = 3)
		DCM/A	CHL/A	HLB	MCX	W	AF	MF	W	AF	MF	AF
4-OP	27.6	56	9	6	9	45 ± 3	66 ± 27	1.5	26 ± 5	16 ± 5	0.6	64 ± 8
PP	109.2	87	109	81	59	102 ± 9	106 ± 18	1.0	80 ± 3	83 ± 3	1.0	84 ± 15
MnBP	107.4	48	74	78	62	71 ± 4	75 ± 13	1.1	109 ± 14	98 ± 12	0.9	86 ± 15
BPA	50.7	60	54	70	51	69 ± 9	86 ± 17	1.2	64 ± 7	58 ± 20	0.9	81 ± 30
BPS	116.0	47	35	105	99	54 ± 3	44 ± 6	0.8	77 ± 4	50 ± 5	0.7	115 ± 13
MBP	102.1	65	54	62	76	73 ± 4	74 ± 27	1.0	61 ± 13	46 ± 8	0.8	79 ± 32
triclosan	58.0	84	26	60	32	72 ± 22	59 ± 14	0.8	40 ± 5	33 ± 2	0.8	104 ± 9
OH-PCB-61	100.0	62	14	72	55	71 ± 32	32 ± 8	0.4	51 ± 4	28 ± 7	0.5	109 ± 3
PFOS	62.4	109	85	73	17	78 ± 10	90 ± 27	1.1	65 ± 3	52 ± 9	0.8	98 ± 10
6-OH-BDE-47	49.5	81	25	65	28	68 ± 34	14 ± 6	0.2	51 ± 7	22 ± 6	0.6	103 ± 10
3-OH-BDE-157	117.4	82	29	47	8	38 ± 18	6 ± 2	0.2	42 ± 1	21 ± 3	0.5	86 ± 13
daidzein	104.2	82	93	88	55	107 ± 7	108 ± 11	1.0	95 ± 3	103 ± 6	1.1	102 ± 5
genistein	103.8	6	2	65	64	7 ± 3	4 ± 2	0.5	113 ± 7	97 ±8	0.9	54 ± 10
enterolactone	38.7	102	97	83	76	81 ± 5	103 ± 19	1.3	94 ± 3	72 ± 7	0.8	98 ± 11
E2	43.0	67	63	59	19	67 ± 10	87 ± 17	1.3	80 ± 4	70 ± 9	0.9	87 ± 15
E1	55.7	68	59	66	15	67 ± 5	102 ± 29	1.5	76 ± 2	77 ± 8	1.0	95 ± 9
E4	84.0	19	16	98	76	21 ± 2	23 ± 7	1.1	103 ± 5	90 ± 19	0.9	121 ± 7
FICZ	45.4	32	20	43	43	44 ± 25	6 ± 9	0.1	1 ± 1	0 ± 0	-	6 ± 2
average	**76**	**64**	**48**	**68**	**47**	**63 ± 11**	**60 ± 14**	**0.9**	**68 ± 5**	**57 ± 8**	**0.8**	**87 ± 12**

[a]*n* = number of extractions

During the final sample preparation, dilution of AF with HPLC-grade water decreased the sample viscosity and improved extraction efficiency (data not shown). Disruption of protein binding was omitted in the pre-treatment stage as the amount of total protein in AF is low (~0.4%) and lower than in maternal blood (~7%) or umbilical cord blood (~5%).[59–61] The nonpolar compounds that did not adsorb to SPE during loading were recovered with DLLE (DCM/acetone). Here, acidification of the sample (to pH=4.5) increased the extraction efficiency of the weak acids (data not shown). Prior extraction with SPE resulted in cleaner aqueous eluent for DLLE and better recoveries of nonpolar compounds. During the final extraction, the combination of the off-line SPE and DLLE was successful in recovering reference compounds with high efficiency (Table 2). The relative recoveries ranged from ~54 to 121%, except for the chemically unstable FICZ (<10%). In general, the average relative recovery in AF was higher for the combined method (87% ± 12%), than for SPE (57% ± 12%) or DLLE (60% ± 14%) alone (Table 2).

HPLC fractionation

AF extracted with SPE and DLLE was spiked, and fractionated on an RP-HPLC column and each fraction was analysed with LC-ESI-MS/MS. Considering the overall low concentrations of EDCs in AF reported in the literature, lower resolution fractionation (25 fractions) was applied to avoid loss in sensitivity during bioassay analysis. The concentration of the reference compounds in the fractions was quantified with an external calibration curve. Increased column temperature (35°C) during chromatographic separation resulted in better peak separation (data not shown). In general, a good separation of all reference compounds was achieved (Figure 2). A statistically significant correlation was observed between retention time and the compound log*P* ($r=0.644$, $p<0.001$, Figure S2). Natural hormones, E2 (log*P* 4.01) and E1 (log*P* 3.69) and the synthetic estrogen BPA (log*P* 3.43), eluted in fractions 13 to 15 at mid-retention times (26-30 min) (Figure 2). More polar E4 (log*P* 2.21), a weaker steroid hormone produced exclusively by the foetal liver during gestation, eluted in the earlier fractions (8-9). As expected, the nonpolar reference compounds (Figure 2, Table 1) eluted in the late fractions (17-20) with more than 75% of organic solvent in the chromatographic gradient. Although MBP extraction efficiency was high (79%), it was the only compound not detected in any of the fractions. Just like FICZ, MBP can decompose when dissolved in an aqueous solution and exposed to sunlight.[62] The decomposition during the fractionation and evaporation process might, at least partially, contribute to its absence in the fractions. For photosensitive compounds, sample preparation under low UV exposure should be therefore considered.

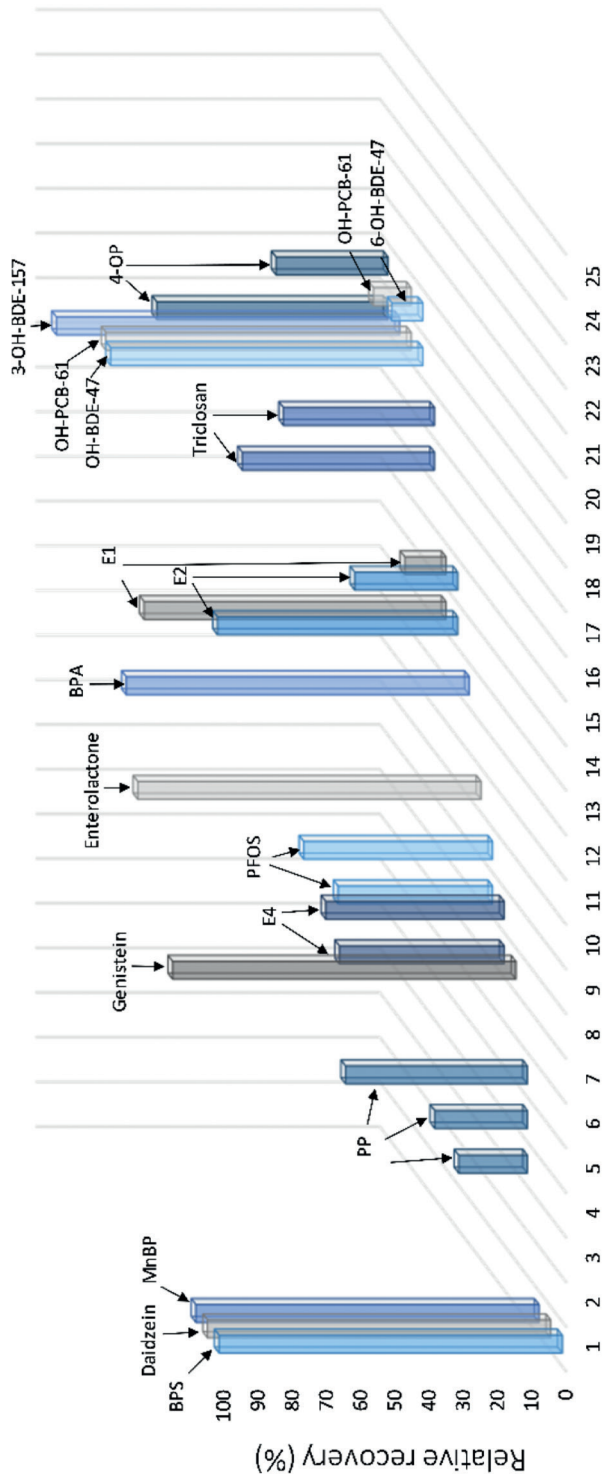


Figure 2. Elution profile of reference compounds during RP-HPLC fractionation of spiked AF extract. The total concentration of reference compound eluted during the whole chromatographic run was set at 100% and the recovery in each fraction was reported as relative to it.

Bioassay analysis

Non-spiked AF was fractionated on an RP-HPLC column (Figure S3, Figure 1). Serial dilutions of the AF extract and the 25 fractions were tested on six different endpoints from three different reporter gene assays i.e., the AR-Luc, ER-Luc and DR-GFP in agonistic (Figure 3B and 4A) and antagonistic mode (Figure 3C and 4B). The reporter gene bioassays used are considered highly sensitive with responses to the respective positive controls i.e., E2, DHT and TCDD, comparable to the responses reported in the literature, with EC50 values of 3, 52 and 11 x 10^{-12} M, respectively (Figure 3A). The diluted AF extract was active in all three bioassays tested (Figure 3B). Estrogenic potency was high with estrogenic activity observed even in the extract diluted 500 times, corresponding to 1.4 nmol (374.5 ng) EEQ/L. The AR and DR activity of the AF extract was found at lower dilutions, corresponding to 76.6 pmol (22.2 ng) DHT-EQ/L and 10.1 pmol (3.3 ng) TEQ/L, respectively (Figure 3B, n=3). The co-exposure experiments showed no significant antagonistic effect of the AF extract in any of the assays tested (Figure 3C).

In order to determine if known hormones and xenobiotics contributed to the biological activity measured in vitro, the 18 reference compounds in the non-spiked AF extract were analysed. From 18 reference compounds, 13 were detected above LODs (Table 1, Table S2). The concentration of natural estrogens, E2 (0.5 nM), E1 (2.8 nM) and E4 (4.2 nM), were detected at levels comparable to the concentrations reported in the literature (Table 1). Most xenobiotics and phytoestrogens were detected at a low nM range. The two hydroxy-PBDEs were detected close to LODs (pM range). Considering that the EC50 values for most of the exogenous reference compounds fall within the high nM to µM range (Table 1), their contribution to the observed activity is minor.

To our knowledge, estrogenic, androgenic and dioxin-like activity has only been reported once before in a recent study by Long et al. (2019) in samples from second-trimester AF. The median concentration of the total estrogenic activity was determined at 545.8 ng EEQ/L (range 149.0-1308, n=75).[28] The authors reported a high variation in the levels of E2, ranging from 55 to 1005 E2 ng/L. After E2 subtraction, estrogenic activity induced only by xenoestrogens was estimated at 214.4 ng EEQ/L (n=68).[28] In this study, the combined levels of E2, E1 and E4 measured in AF (Table 1) were determined at 0.56 nmol (152 ng) EEQ/L and contributed to 48% of the activity observed in the ER-Luc bioassay. A combination of xenoestrogens, phytoestrogens and other minor and weaker steroid estrogens (e.g., estriol (E3),17α-estradiol, hydroxylated estrogen metabolites) are most likely contributors to the remaining activity.

The combined effect on AR of endogenous androgens and xenoandrogens reported by Long et al. (2019) was in a range of 16-290 ng DHT-EQ/L, and, the combined effect on DR of dioxin-like compounds was in a range of 0-2.10 ng TEQ/L. The values obtained in this study fall in the range obtained by Long et al. (2019), except for dioxin-like activity.

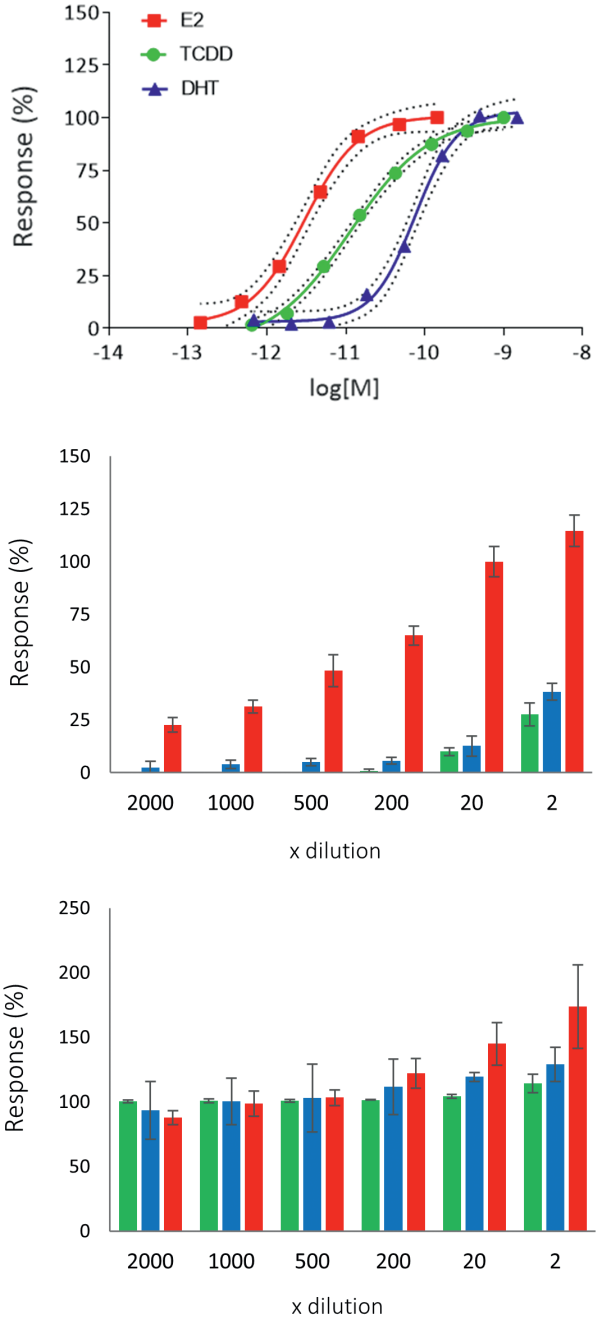


Figure 3. Concentration–response curves (A) with 95% confidence intervals of ER, AR, and DR responsive cell lines exposed to E2 (red), DHT (blue), and TCDD (green), respectively (n = 3). Agonistic (B) and antagonistic (C) activity of the AF extract represented as luminescence response relative to the highest response of the respective positive control (average % ± SD, n = 3). For antagonism experiments (C), extract dilutions were co-exposed with 4, 150, and 30 pM of E2, DHT, and TCDD, respectively. "x dilution" represents the final dilution factor of the AF sample.

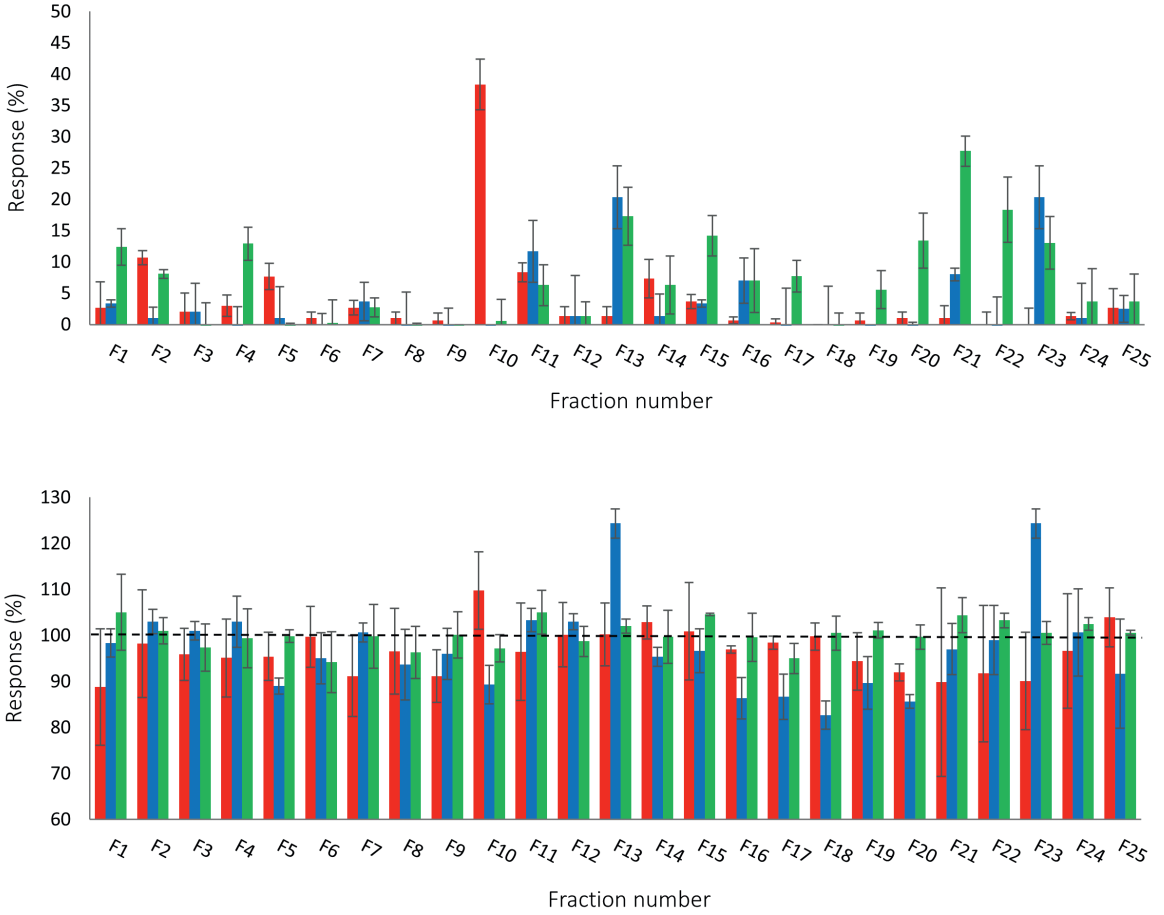


Figure 4. Agonistic (A) and antagonistic (B) activity of AF fractions in ER (200× diluted, red), AR (2× diluted, blue), and DR (2× diluted, green) responsive cell lines. Antagonistic activity is measured as a decrease in luminescence after co-exposure with 4, 150, and 30 pM of E2, DHT, and TCDD, respectively. Results presented as average % of RLUs of positive control ± SD between experiments (n = 3).

Here, the observed activity was approximately one and a half times higher than the highest value reported by Long et al. (2019). However, direct comparison is somewhat limited. Considering that both hormone levels and the volume of AF change during pregnancy, full-term AF may significantly differ from second-trimester AF in chemical composition and concentration.[63] Moreover, the more lipophilic dioxin-like compounds may gradually accumulate in AF throughout pregnancy, resulting in the higher activity observed in this study, i.e. samples collected at term compared to samples from mid-gestation.

To further characterize the nature of the chemicals contributing to the biological activity, the AF extract was fractionated and the ER, AR and DR activity in each fraction was determined. Considering that the ER activity of the 2 x diluted extract was above the maximum response of the positive control (~120% activity, Figure 3B), the fractions tested for estrogenic activity were diluted 200 x in order for their response in the bioassay to be interpolated in the linear range of the concentration-response curve of the positive control. In the ER-Luc assay, elevated activity was observed mainly in the early polar fractions (Figure 4A, F2 and F5) and fractions of intermediate polarity (Figure 4A, F10-11, F14-15). Fraction 10 showed the highest estrogenic activity, however, none of the potent natural estrogens (E1, E2 or E4) eluted in this fraction (Figure 2). The reference compound PFOS, a weak estrogen receptor ligand [64], eluted in fraction 10 and was detected in AF at 1.5 nM (Table 1). However, at 200 x dilution, the concentration would be too low to significantly contribute to the observed activity. Nevertheless, it is possible that similar perfluorinated compounds and weak ER ligands (e.g. perfluorooctanoic (PFOA), perfluorononanoic (PFNA), perfluorodecanoic (PFDA), acids)[64,65] could elute in this fraction and contribute to the observed activity. Moreover, these compounds have been previously detected in maternal blood and cord blood.[66] Estrogenic activity in fractions 2 and 5 could not be explained by any of the reference compounds measured in this study. The retention time at which another agonistic ER activity was observed corresponded well with the retention times of reference estrogenic compounds i.e., natural hormones (E2 and E1, F14-15) and phytoestrogens (enterolactone, F11) (Figure 2, Figure 4A). Although E4 was detected in AF (4.25 nM, Table 1) and was shown to elute in fractions 8 and 9 (Figure 2), we did not observe activity in these fractions at 200 x dilution (Figure 4A). However, these two fractions showed weak ER activity when tested at lower dilutions (Figure S4). The anti-estrogenic activity of environmental chemicals is much less commonly reported than the estrogenic activity. This corroborated our findings i.e., in the anti-estrogenic assay, only few fractions showed a small decrease in the luminescence induced by the positive control E2 (Figure 4B).

In this study, three mid-polar (F11, F13, F16) and two nonpolar fractions (F21 and F23) showed elevated androgenic activity (Figure 4A). Although we did not analyse the levels of DHT (log*P* 3.55) and testosterone (log*P* 3.32), these hormones have similar log*P* values to BPA and enterolactone (log*P* 3.3) which eluted in fractions 11 and 13. Both tes-

tosterone and DHT are commonly detected in AF.[28,67] It is thus possible that the activity in these fractions is, at least to some extent, caused by endogenous androgens. The androgenic activity found in fractions 20 and 21 is noteworthy and could be of xenobiotic origin, as natural androgens are not expected to elute in these highly nonpolar fractions. Antiandrogenic activity, commonly reported in the literature, was observed for multiple fractions (F5, F10 and F16-F20, Figure 4B). MTT assay showed no decrease in cell viability in any of the fractions tested. Interestingly, anti-androgenic activity was not observed in the unfractionated extract, which may be explained by the co-elution of androgenic compounds that, when mixed, could mask the anti-androgenic effects.[68] A similar result was observed by Indeveri et al. (2014), who reported lower total antiandrogenic activity in a placental extract than in the summed activities in the fractions.[69] The anti-androgenic reference compounds propylparaben and triclosan eluted in the fractions that showed anti-androgenic effect i.e., F5 and F17/18, respectively (Figure 4B) and were detected in the non-spiked AF extract (Table 1). Moreover, both compounds were previously reported in maternal blood and cord blood.[39] However, the IC50 for their anti-androgenic effects are reported at levels 3 to 4 orders of magnitude higher than the measured concentrations (Table 1). Therefore, their contribution to the observed activity is likely minimal.

Most dioxin-like compounds are highly lipophilic and include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated/polybrominated biphenyls (PCBs/PBBs), polychlorinated dibenzofurans (PCDFs) or polycyclic aromatic hydrocarbons (PAHs).[70,71] Reports on the quantification of these compounds *in utero* are surprisingly scarce, with only a few publications reporting trace levels of dioxin or dioxin-like compounds in AF.[27,72] Despite their lipophilic nature, the high activity observed in this study shows that the extraction method presented here could be successfully used for the detection of dioxin-like activity in AF extract. Consistent with the general chemical characteristics of these compounds, most dioxin-like activity was found in the nonpolar fraction (F19 to F23) (Figure 4A). The observed activity in these late nonpolar fractions indicates that the extraction method presented here extracted compounds with higher log*P* values than the log*P* value reported for most nonpolar reference compounds used in this study (6-OH-BDE-47, log*P* 7.19). Interestingly, weaker dioxin-like activity was also found in earlier fractions (F13 – F17), possibly indicating the presence of dioxin-like compounds with intermediate polarity or the presence of hydroxylated metabolites. Similar to anti-androgenic activity, the summed dioxin-like activity in the fractions was significantly higher than the activity observed in the unfractionated extract. None of the fractions showed anti-DR activity in the cells co-exposed to TCDD (Figure 4B). This is consistent with the literature, where the majority of reported AhR ligands are agonists.

This study shows for the first time that a combination of off-line SPE and DLLE can be used to extract a broad spectrum of EDCs from AF with low interference from the matrix.

Although it is generally assumed that the chemical concentrations in AF are lower than those detected in maternal urine, serum, or blood, we show here that the sample pre-concentration, fractionation and the use of cell-based reporter gene assays provide a sensitive method for successful detection of a range of endocrine disrupting activities in AF extract. The biological activity found in fractions of differing polarity gives a first indication of the diversity of endogenous and exogenous ER, AR and AhR ligands present in AF. The method presented here sets the foundation for further qualitative and quantitative identification of known and unknown EDCs with biological activity, a necessary step forward in hazard characterisation and risk assessment of EDC exposure *in utero*.

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Supporting Information

Additional information on MBP synthesis, extraction procedures, cell culture, parameters used during LC-ESI-MS/MS analysis and RP-HPLC fractionation.

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Supplementary Materials

S1. Chemicals

α-Minimal Essential Medium (MEM) with GlutaMax (32561-029), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (DMEM/F12) with GlutaMax (31331-028), DMEM/F12 medium without phenol red (11880-028), Leibovitz's L-15 medium (L-15, 11415-049), L-15 medium without phenol red (21083-027), Hank's Balanced Salt Solution (HBSS, 14175-053), penicillin/streptomycin (P/S, 15140-122), trypsin (25300-054) and foetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany); G418 (ant-gn-1, ant-gn-5, 10832-42-2) was obtained from InvivoGen (Toulouse, France); Steady-Glo® Luciferase Assay system (E2550) was purchased from Promega (Leiden, The Netherlands). Methanol (MeOH) and dichloromethane (DCM) were purchased from Thermo Fisher Scientific (Loughborough, UK); acetone was obtained from BDH Laboratory (Poole, Dorset, UK); dimethyl sulfoxide (DMSO, 99.9+%) was obtained from Alfa Aestar (MA, USA). All solvents used were of HPLC grade. Ammonium hydroxide solution (ACS reagent, 28.0-30.0% NH3 basis) was purchased from Sigma Aldrich (Poole, Dorset, UK); LC-MS grade water (LiChrosolv®) was obtained from Merck KGaA (Darmstadt, Germany); chloroform (ACS reagent, 99.8+%) was purchased from Acros Organics (Geel, Belgium). Two hydroxylated metabolites of brominated flame retardants, namely 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47) and 3-hydroxy-2,2',4,4',5,5'-tetrabromodiphenyl ether (3-OH-BDE-153) were generously provided by Dr. Lillemor Asplund (Stockholm University). All other reference compounds were purchased from Sigma Aldrich (Poole, Dorset, UK) at the highest commercially available purity. 4-methyl-2,4-bis(4-hydroxyl- phenyl) pent-1-ene (MBP), a potent metabolite of bisphenol A, was synthesized and its identity and purity was confirmed spectrometrically (NMR, IR and HR-MS) as described below.

S1.1. Synthesis of 4,4'-(4-methylpent-1-ene-2,4-diyl)diphenol (MBP)

Materials and Methods: NMR spectra were recorded on a Bruker Avance 500 (125.78 MHz for ^{13}C) using the residual solvent as internal standard (^{1}H: δ 2.05 ppm, $^{13}C\{^{1}H\}$: δ 206.26 ppm for acetone-d_6. Chemical shifts (δ) are given in ppm and coupling constants (*J*) are given in Hertz (Hz). Melting points were recorded on a Büchi M-565 melting point apparatus. Electrospray Ionization (ESI) high-resolution mass spectroscopy was carried out using a Bruker micrOTOF-Q instrument in negative ion mode. Infrared spectra were recorded neat using a Shimadzu FTIR-8400s spectrophotometer and wavelengths are reported in cm^{-1}. Flash chromatography was performed on Silicycle Silia-P Flash Silica Gel (particle size 40-63 μm, pore diameter 60 Å) using the indicated eluent. Thin layer chromatographic (TLC) analysis was performed with Merck F254 silica gel-60 and visualized by UV light.

4-Methyl-2,4-bis-(p-hydroxyphenyl)pent-1-ene (MBP): Bisphenol A (2.00 g, 8.76 mmol) was dissolved in concentrated sulfuric acid (6.86 mL) and the orange solution was poured over ice water (116 mL) under vigorous stirring. The resulting solids were collected by filtration. The filtrate was washed with MTBE (3 x 20 mL) and the previously obtained solids were dissolved in the combined organic layers. Traces of sulfuric acid in the organic layer were neutralized with saturated aqueous $NaHCO_3$, after which the organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash silica gel chromatography (gradient, CH_2Cl_2:EtOAc 97.5:2.5 → 90:10) to give MBP (235 mg, 20 %) as a white solid.

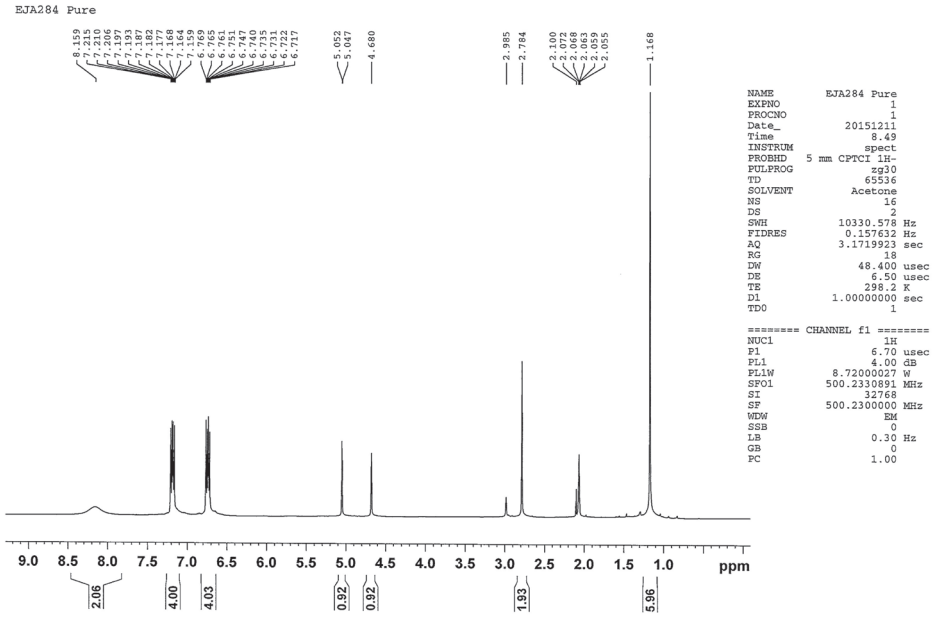

Figure S1.1. Proton NMR spectrum of synthesized MBP
^{1}H NMR: (500 MHz, Acetone-d_6) δ 8.16 (br s, 2H), 7.19 (ddd, 4H, *J* = 11.0 Hz, 9.0 Hz, 2.5 Hz), 6.74 (ddd, 4H, *J* = 11.0 Hz, 9.0 Hz, 2.0 Hz), 5.05 (d, 1H, *J* = 2.5 Hz), 4.68 (s, 1H), 2.78 (s, 2H), 1.17 (s, 6H);

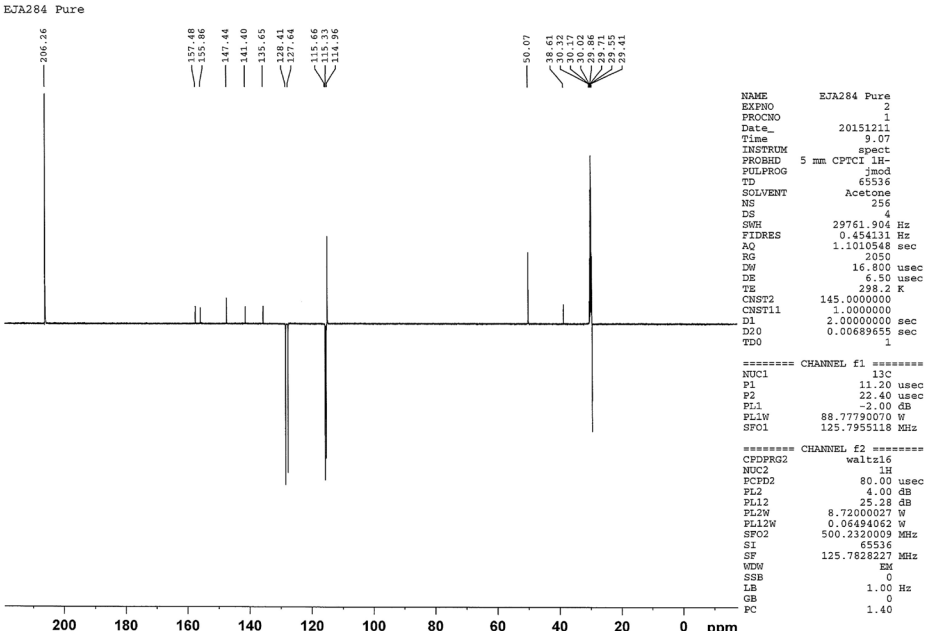

Figure S1.2. ^{13}C NMR spectrum of synthesized MBP
^{13}C NMR: (125 MHz, Acetone-d_6) δ 157.5, 155.9, 147.4, 141.4, 135.7, 128.4 (2C), 127.6 (2C), 115.7 (2C), 115.3 (2C), 115.0, 50.1, 38.6, 29.4 (2C);

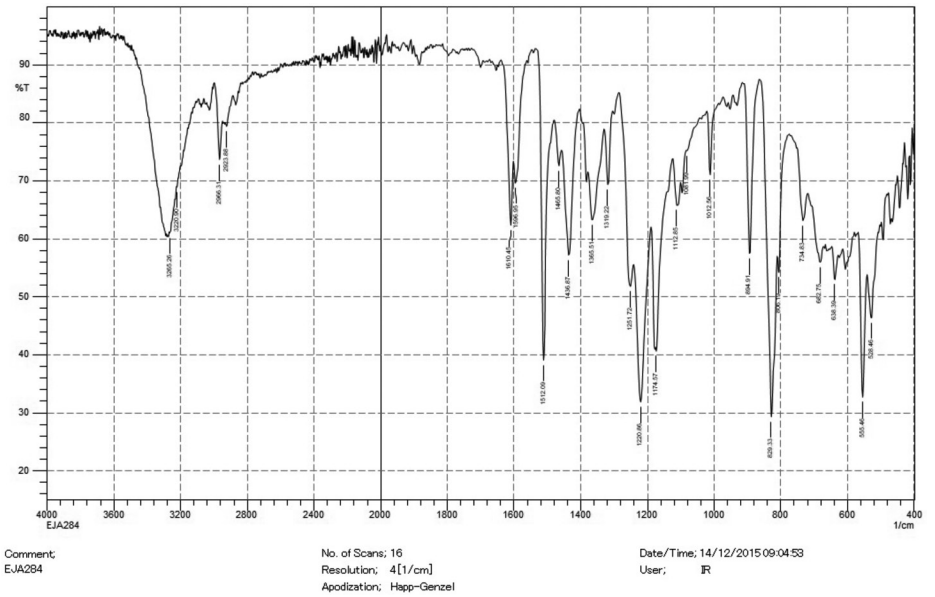

Figure S1.3. Infra-Red (IR) spectrum of synthesized MBP
IR (neat) νmax (cm^{-1}): 3265 (s), 1610 (m), 1512 (s), 1437 (m), 1366 (m), 1221 (s), 1175 (s), 895 (m), 829 (s), 555 (s);

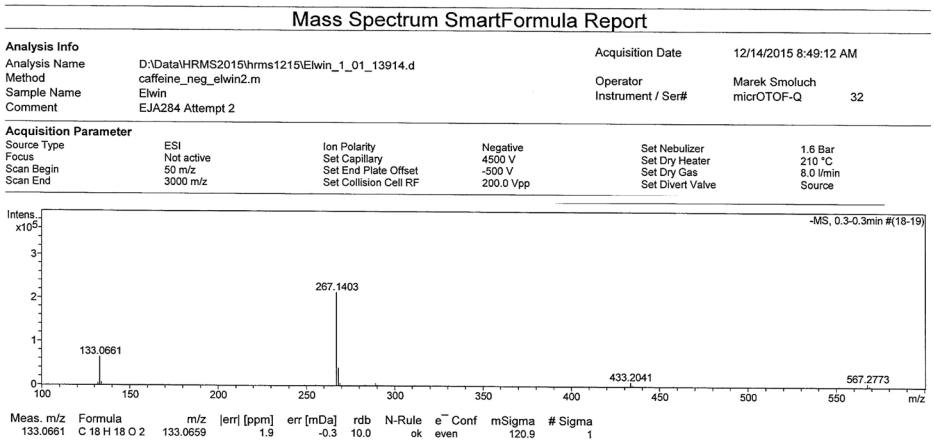

Figure S1.4. HRMS (ESI) spectrum of synthesized MBP
HRMS (ESI): *m/z* calculated for $C_{18}H_{19}O_2$ $[M-H]^-$ 267.1391, found 267.1403, $C_{18}H_{18}O_2$ $[M-2H]^{2-}$ 133.0659, found 133.0661; melting point 127.3-129.5 °C.

Table S1. The concentration of the reference compounds in the spike mix used during the method development of extraction and fractionation of amniotic fluid.

Compound	Concentration in the spike mix (µg/mL)
4-OP	0.788
PP	0.394
MnBP	0.708
BPA	0.570
BPS	0.679
MBP	0.336
TCS	0.423
OH-PCB-61	0.729
PFOS	0.263
6OH-BDE-47	0.378
3OH-BDE-157	0.741
daidzein	0.705
genistein	0.188
enterolactone	0.308
FICZ	0.345
E2	0.693
E1	0.292
E4	0.797

S2. Extraction procedures

Solid phase extraction (SPE): MCX columns (Oasis, 6 cc, 150 mg/3 mL, 30 µm particle size, Waters Corp., Milford, MA) and HLB columns (Oasis, 6 cc, 150 mg, 30 µm particle size, Waters Corp., Milford, MA) were conditioned with 3 mL of methanol and 3 mL of deionised water. Matrix (5ml of LC-MS grade water or AF) was spiked with the reference mix (100 µL), pH was adjusted to 2 with 98% formic acid (for MCX extraction only), and then samples were loaded on the cartridges at a rate of 1 mL/min. The MCX and HLB cartridges were rinsed with 3 mL of 2% formic acid (in deionized water) and with 5% methanol respectively, and then air dried for 1 min. The analytes were eluted from the MCX cartridges with 2 x 2 mL of 100% methanol and 2 x 2 ml of 5% of ammonium hydroxide, and from the HLB cartridges with 4 x 2ml of 100% methanol. Deionised water (0.2 mL) was added to the extracts and the extracts were put in a water bath (max. temperature 40°C) where the solvent was evaporated under a gentle stream of nitrogen to a final volume of 0.2 mL. The samples were then reconstituted with 0.3 mL of 10% methanol in deionised water, transferred to glass vials for analysis and analysed on LC-ESI-MS/MS for chemical recoveries.

Dispersive liquid/liquid extraction (DLLE): 5 ml of LC-MS grade water or amniotic fluid was spiked with the reference mix (100 µL) and pH adjusted to ~4.5 with sodium acetate/acetic acid buffer (0.1 M). DLLE was performed by addition of DCM (0.5 mL) or chloroform (0.5 mL) only, or by addition of a mixture of acetone (1ml) (dispersive solvent) and DCM (0.5 mL) or chloroform (0.5 mL) (extraction solvent). Samples were then vortexed 2 x for 15 seconds and centrifuged at 4081 x g for 15 min at 25°C. The supernatant was removed, 50 µL of deionised water was added, the organic layer evaporated under a gentle stream of nitrogen and the residue was reconstituted with 10% MeOH to a final volume of 0.5 mL. Samples were transferred to glass vials and analysed on LC-ESI-MS/MS for chemical recoveries.

Table S2. Parameters used during LC-ESI-MS/MS Multiple Reaction Monitoring (MRM) detection of the analytes including collision cell parameters and monitored precursor/product ions. The limit of detection (LOD) for each analyte was calculated as described in the method section.

Compound	Q1	Q3a	Q3b	DT	EP	DP (Q/C)	CE (Q/C)	CXP (Q/C)	LOD (M)
4-OP	205.18	106.0	189.0	25	-10	-55	-28	-15	4.4×10^{-10}
PP	179.13	91.9	136.0	25	-10	-55	-32	-13	6.0×10^{-11}
MnBP	221.05	121.1	70.9	25	-12	-80	-20	-13	$2.0. \times 10^{-10}$
BPA	227.20	133.1	212.0	25	-11	-90/-90	-40/-26	-13/-13	3.7×10^{-09}
BPS	249.10	107.9	156.0	25	-10	-70	-38	-17	3.7×10^{-11}
MBP	267.20	133.1	117.2	25	-11	-100/-150	-25/-50	-13/-15	9.4×10^{-12}
TCS	287.09	34.9	241.1	25	-10	-40	-48	-7	5.4×10^{-11}
OH-PCB-61	306.95	270.8	34.9	25	-10	-55/-55	-30/-82	-19/-17	9.9×10^{-11}
PFOS	498.95	79.9	98.9	25	-10	-170	-84	-17	9.9×10^{-12}
6OH-BDE-47	500.75	78.8	80.8	25	-10	-75	-40	-11	2.7×10^{-11}
3OH-BDE-157	658.55	80.8	576.6	25	-10	-105/-105	-100/-38	-13/-41	5.8×10^{-12}
daidzein	253.14	132.0	223.0	25	-10	-90/-90	-54/-36	-7/-13	1.7×10^{-11}
genistein	269.10	133.1	63.1	25	-10	-135/-135	-46/-50	-11/-11	2.9×10^{-11}
enterolactone	297.16	253.0	107.0	25	-10	-110/-150	-28/-50	-17/-15	2.7×10^{-11}
FICZ	283.30	254.2	255.3	25	-10	-90	-40	-13	2.1×10^{-11}
E2	271.15	183.0	145.0	25	-10	-80/-50	-56/-56	-11/-17	4.1×10^{-11}
E1	269.12	145.0	142.9	25	-10	-105	-52	-23	1.6×10^{-11}
E4	303.12	241.0	273.0	25	-14	-90	-28	-15	3.8×10^{-11}

Q1, precursor ion (*m/z*); Q3a, quantification product ion (*m/z*); Q3b, confirmation product ion (*m/z*); DT, dwell time (ms); DP, declustering potential (V); EP, entrance potential (V); CE, collision energy (V); CXP, collision cell exit potential (V)

S3. Cell culture

The estrogen receptor-mediated luciferase reporter gene (ER-Luc) human breast carcinoma (VM7Luc4E2) cells[1], the androgen/glucocorticoid receptor-mediated luciferase reporter gene (AR-Luc) human breast carcinoma (MDA-kb2) cells[2] and the aryl hydrocarbon receptor enhanced green fluorescent protein reporter gene (DR-GFP) mouse hepatoma (H1G1.1c3) cells[3] were routinely maintained in 75 cm^2 canted neck tissue culture flasks (Greiner Bio-One Ltd.). ER-Luc and DR-GFP cells were maintained in DMEM/F12 and α-MEM medium respectively, supplemented with 10% FCS and 1% P/S (culture media) and incubated in a humidified incubator at 37 °C and 5% CO_2. DR-GFP cells were additionally supplemented with 1% G418. AR-Luc cells were maintained in Leibowitz-15 (L-15) medium supplemented with 10% FCS (culture media) and incubated in a humidified incubator at 37 °C without additional CO_2. Cells were subcultured when 70-80% confluent.

ER, AR and DR reporter gene assays

ER-Luc: Prior to experiments, ER-Luc cells were maintained in DMEM/F12 medium without phenol red (assay media) supplemented with 10% charcoal dextran stripped FCS (cdFCS) and 1% P/S for two media changes (4 days). Cells were seeded at a density of $2x10^4$ cells per well in 100 µL of assay media in clear-bottomed 96-well tissue culture dishes (Greiner Bio-One Ltd.) and allowed to attach. After 24 h media was removed and 100 µL of assay media containing test chemicals or DMSO control (0.5%) was added to the wells. After 24h of exposure, media was removed, cells were washed with PBS (200 µL), PBS was removed and lysis buffer was added (20 µL). After cell lysis (15 min with shaking) the luciferase activity was determined in a plate reader (LUMIstar Optima, BMG Labtech GmbH) with 100 µL of luciferase reagent media injected directly into each well during measurement.

AR-Luc: Prior to experiments, AR-Luc cells were maintained in L-15 medium supplemented with 10% cdFCS (pre-assay media) for two media changes (4 days). Cells were then seeded at a density of $2x10^4$ cells per well in 100 µL of phenol red-free L-15 medium supplemented with 10% cdFCS (assay media) into white, clear-bottomed 96-well tissue culture dishes and allowed to attach. After 24 h, 100 µL assay medium was added to the wells with twice the intended final concentration of test chemicals or DMSO control (0.5%), to obtain the desired test concentrations. After 24 h of exposure, 100 µL of media was removed and SteadyGlo assay reagent (Promega UK Ltd.) (100 µL) was added to the wells and the plate was left shaking for 10 min. The luciferase activity was determined as described above.

DR-GFP: DR-Luc cells were maintained in culture media and when confluent seeded in black, clear-bottomed 96-well tissue culture dishes (DR-Luc) (Gre- iner Bio-One Ltd.) in MEM Alpha media supplemented with 10% FCS, 1% P/S, but without the addition of

G418 (assay media). Cells were seeded at a density of $3x10^4$ cells per well in 100 µL of assay media and left to attach. After 24 h, media was aspirated and cells were exposed to test chemical or DMSO control (1%) in 100 µL of assay media. After 24 h of exposure, fluorescence was measured in the intact cells (without removal of media) using a fluorescent plate reader (POLARstar Galaxy, BMG Labtech GmbH) with an excitation wavelength and an emission wavelength set at 485 nm and 510 nm, respectively. In order to normalize results the fluorescence gain was adjusted per plate to the highest activity of the positive control. In each assay, the outer wells of the plate were not included in the measurements and were filled with 100 µL assay media only.

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, after 23 h exposure, media was removed from each well, 100 µl of 5 mg/ml freshly made MTT solution was added and plates were incubated at 37 °C. At t= 24 h, MTT solution was removed, lysis buffer (150 uL of isopropanol) was added to the wells and the plate was left shaking for 15 min. The absorbance at (λex = 590 nm) was measured with a POLARstar fluorescent plate reader.

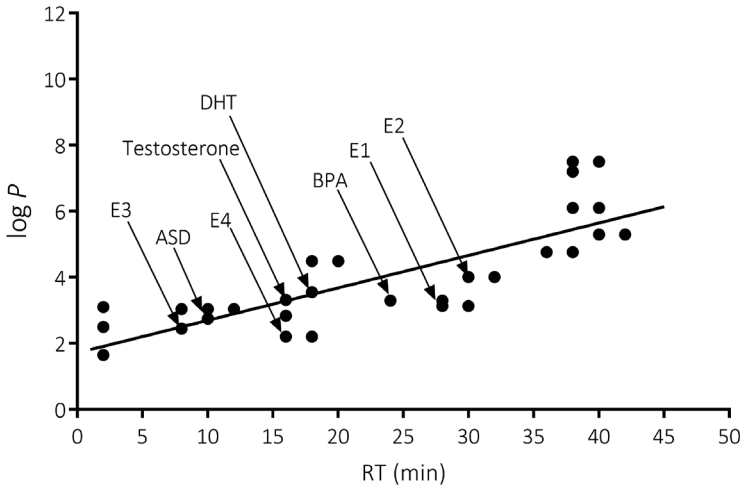

Figure S2. The log*P* of reference compounds retrieved from the PubChem database (www.pubchem.ncbi.nlm.nih.gov) plotted against retention times (RT) during RP-HPLC fractionation of spiked AF extract, including estetrol (E4), 17β-estradiol (E2) estrone (E1) and bisphenol A (BPA). Log*P* of estriol (E3), androstenedione (ASD), dihydrotestosterone (DHT) and testosterone was plotted against their predicted RTs interpolated from linear regression analysis (GraphPad Software Inc., San Diego, CA).

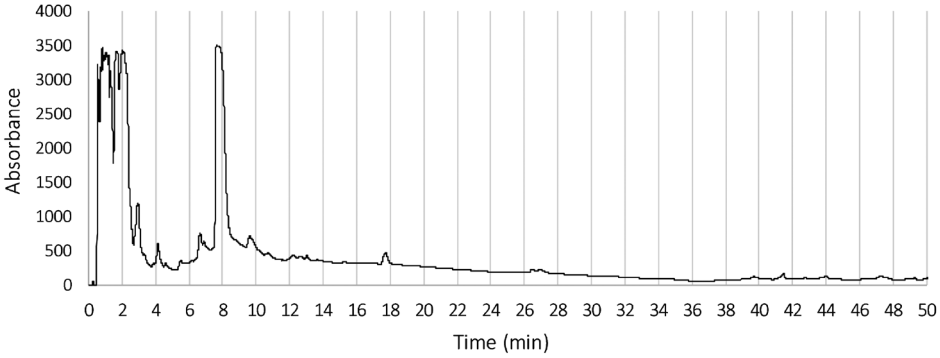

Figure S3. An example of a chromatogram obtained during reversed-phase fractionation of AF extract. The sample was injected onto a reversed-phase Phenomenex Kinetex C18 (100mm x 2.1 mm, 5 µm pore size) column using an Agilent 1260 system equipped with a diode array detector (DAD). Absorbance was monitored with a 253 nm wavelength and plotted against retention time. Eluted fractions were concentrated, resuspended in DMSO and used for bioassay analysis.

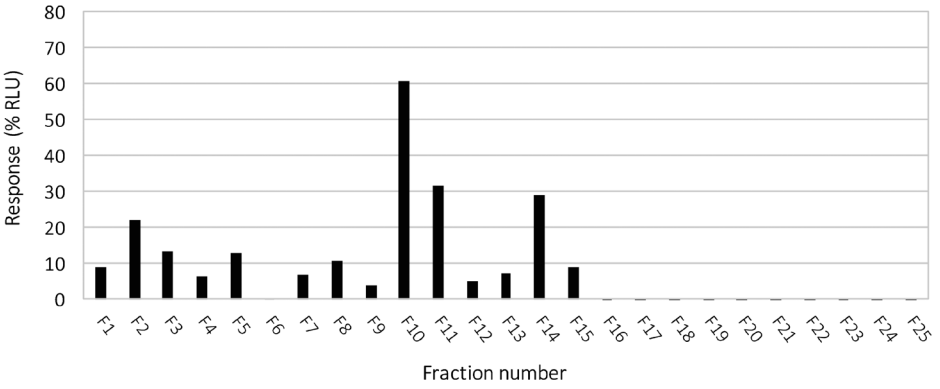

Figure S4. The agonistic activity of AF fractions (50 x diluted) in the ER-Luc assay. ER activity was measured as described in the method section (n=1).

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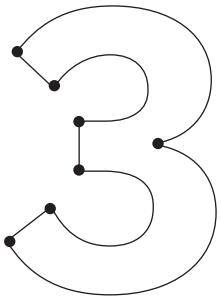

Identification of Known and Novel Nonpolar Endocrine Disruptors in Human Amniotic Fluid

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Abstract

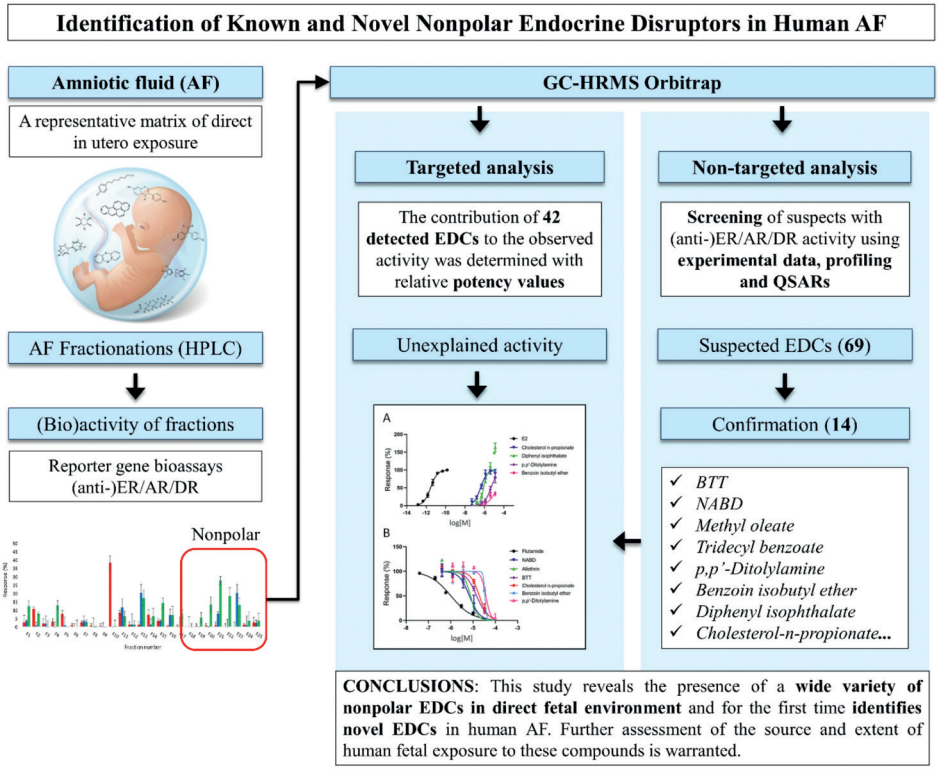
BACKGROUND: Prenatal exposure to endocrine disrupting compounds (EDCs) may contribute to endocrine-related diseases and disorders later in life. Nevertheless, data on *in utero* exposure to these compounds are still scarce.

OBJECTIVES: We investigated a wide range of known and novel nonpolar EDCs in full-term human amniotic fluid (AF), a representative matrix of direct fetal exposure.

METHODS: Gas chromatography high-resolution mass spectrometry (GC-HRMS) was used for the targeted and non-targeted analysis of chemicals present in nonpolar AF fractions with dioxin-like, (anti-)androgenic, and (anti-)estrogenic activity. The contribution of detected EDCs to the observed activity was determined based on their relative potencies. The multitude of features detected by non-targeted analysis was tentatively identified through spectra matching and data filtering and further investigated using curated and freely available sources to predict endocrine activity. Prioritized suspects were purchased and their presence in AF was chemically and biologically confirmed with GC-HRMS and bioassay analysis.

RESULTS: Targeted analysis revealed 42 known EDCs in AF including dioxins and furans, polybrominated diphenyl ethers, pesticides, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. Only 30% of dioxin activity and less than 1% of estrogenic and (anti-)androgenic activity were explained by the detected compounds. Non-targeted analysis revealed 14,110 features of which 3,243 matched with library spectra. Our data filtering strategy tentatively identified 121 compounds. Further data mining and *in silico* predictions revealed in total 69 suspected EDCs. We selected 14 chemicals for confirmation, of which 12 were biologically active and 9 were chemically confirmed in AF, including the plasticizer diphenyl isophthalate and industrial chemical p,p'-ditolylamine.

CONCLUSIONS: This study reveals the presence of a wide variety of nonpolar EDCs in direct fetal environment and for the first time identifies novel EDCs in human AF. Further assessment of the source and extent of human fetal exposure to these compounds is warranted.



Graphical abstract

Introduction

Environmental pollutants are increasingly recognized for their ability to alter fetal programming during critical windows of development [1,2]. Prenatal exposure to endocrine disrupting compounds (EDCs) is of particular concern, as EDCs can interfere with hormonal signalling and affect many developmental processes tightly regulated by hormones (Ho et al. 2016; Vaiserman 2014). Growing scientific evidence suggests that prenatal exposure to EDCs is a contributing factor in the increasing prevalence of endocrine-related disorders such as early onset of puberty (Lee et al. 2019), hormone-sensitive cancers [8], metabolic and neurobehavioral disorders and cardiovascular diseases [9–11] and that these effects might be transgenerational (Heindel and Vandenberg 2015). The EDC-mediated developmental programming of adult-onset disease is now a public health concern that requires careful scrutiny. Nevertheless, the tools for comprehensive evaluation of *in utero* exposure to EDCs, crucial for accurate risk assessment, are still lacking.

EDCs are chemically very diverse and include compounds with a wide range of physicochemical properties, including differing polarity. Nonpolar, lipophilic EDCs are ubiq-

uitously present in the environment with widespread chronic, low-level exposure in the general population [14,15]. Many nonpolar EDCs have long elimination half-lives, tend to accumulate over time in the maternal body lipids, and can be mobilized during gestation when maternal energy expenditure and metabolism change rapidly to support the developing fetus [16]. Pollutants eliminated from the maternal compartment (e.g., via placental transport to the fetus) might, at the same time, be gradually released from maternal lipophilic storage depots back into the blood [17]. The circulating low level of persistent pollutants in the maternal compartment may result in chronic exposure to the fetus. Although many factors influence the transplacental transport of environmental chemicals, in general EDCs with higher lipophilicity are thought to more readily cross the placenta than the more hydrophilic compounds [18,19].

Nowadays, there are thousands of chemicals in commercial use that pregnant women might be exposed to, with the vast majority lacking toxicity data on endocrine endpoints [20,21]. The majority of research on prenatal exposure is focused only on a limited number of well-known environmental pollutants often measured in maternal matrices as a surrogate for the fetal environment [22–26]. However, considering complex placental and *in utero* specific toxicokinetics, extrapolation of exposure from the maternal to the fetal compartment remains challenging [27,28]. It is, therefore, crucial to move from the quantification of a limited number of known EDCs to the characterization of complex mixtures, including possible novel and biologically active compounds, preferably in *in utero* specific matrices.

Amniotic fluid (AF) is a unique complex nutritious milieu and a repository matrix for many environmental xenobiotics that the mother and the fetus are exposed to throughout pregnancy. Compounds in AF continuously circulate through foetal membranes (through foetal swallowing and excretion) and therefore AF represents not only maternal but also fetal toxicokinetics [29]. The ongoing cycle of fetal ingestion and excretion of AF may result in fetal accumulation [30] and/or prolonged fetal exposure [31]. Despite the low lipid content of AF (approx. 0.15 g/L), highly lipophilic compounds, such as polybrominated diphenyl ethers (PBDEs) or organochlorines, have been previously detected in this matrix, and their presence in AF indisputably demonstrates placental transfer and fetal exposure (Barmpas et al. 2020; Miller et al. 2012). Although concentrations of xenobiotics detected in AF are usually lower than in maternal blood and difficult to detect using conventional methods, advances in analytical chemistry provide sensitive methods for qualitative and quantitative detection of these compounds, even at trace levels (pg-ng/mL range). Nevertheless, the use of AF for the assessment of prenatal exposure to nonpolar EDCs is still largely unexplored.

Recently, we developed a robust, non-discriminating method for the extraction of EDCs with a wide range of polarities from full-term AF [35]. We used an effect-directed approach (EDA) in which chromatographic separation of chemicals in AF extract allowed

for a separation of compounds with different polarities into fractions and consequent reduction of the chemical complexity. The endocrine disrupting activity profile of the fractions was determined with a battery of cell-based *in vitro* reporter gene bioassays. This approach revealed significant dioxin-like, (anti-)androgenic, and (anti-)estrogenic activity in the nonpolar AF fractions, that could not be attributed to the presence of endogenous hormones [35]. The goal of the current study was to identify known and possibly novel EDCs responsible for the observed endocrine activity. To this end, we performed targeted and non-targeted analyses of EDCs in nonpolar fractions using gas chromatography high-resolution mass spectrometry (GC-HRMS). The contribution of the known EDCs detected in AF to the activity observed in the fraction was determined using relative potency (REP) values. To investigate the remaining unexplained activity further, we developed an innovative, weight-of-evidence approach to prioritize novel EDCs from the multitude of *m/z* features identified through the non-targeted analysis. This approach takes advantage of curated and freely available resources to mine data and predicts the biological properties of unknown chemicals. We compared the 'hits' from the non-targeted GC-HRMS analysis to a battery of experimental data obtained from high-throughput bioassays (ToxCast and Tox21 databases [36] available through EPA CompTox Chemicals Dashboard [37]), predictive QSARs (OPERA [38] and Danish (Q)SARs Database [39]) and endpoint specific profiling models (OECD QSAR Toolbox [40]). Unknown compounds prioritized based on *in silico* and *in vitro* profiling were purchased and their presence in the active fraction was chemically and biologically confirmed with GC-HRMS and bioassay analysis, respectively.

Materials and methods

AF sampling, extraction and fractionation

Approximately, 50 mL of AF was collected during amniotomy at full-term vaginal delivery, from four healthy pregnancies, with ethical approval and informed consent, and immediately frozen at -80 °C (U.K. National Health Service, reference E5431, 2008). Samples were pooled, homogenised, and 100 mL was extracted with a combination of solid-phase extraction (SPE) and dispersive liquid/liquid extraction (DLLE) as described in Dusza et al. (2019). Briefly, aliquots of AF (5 mL) diluted with 3 mL deionized water were loaded on the SPE columns (Oasis HLB, Waters Corp., Milford, MA), the aqueous eluents were collected for further extraction with DLLE, the cartridges were rinsed and the analytes eluted with MeOH. The solvent was evaporated with nitrogen, to a final volume of 1 mL. DLLE was performed twice, on the aqueous eluents acidified with acetate/acetic acid buffer (pH ~4.5), using acetone as dispersive solvent and DCM as extraction solvent. The samples were vortexed, centrifuged and the organic phases combined and evaporated to dryness with nitrogen. The extracts remaining after SPE were transferred to the residues in the DLLE tubes, vortexed, combined and further concentrated to 1 mL. The final

extract was centrifuged at 20 800g to separate the precipitate formed after sample concentration. The extraction procedure was repeated with an AF sample spiked with a mix of reference compounds containing 18 EDCs from 10 different classes and covering a wide range of hydrophobicity (log*P* 1.65 – 7.19). The average relative recovery of the analytes was 87 ± 12% RSD, n=3 (see Dusza et al. 2019).

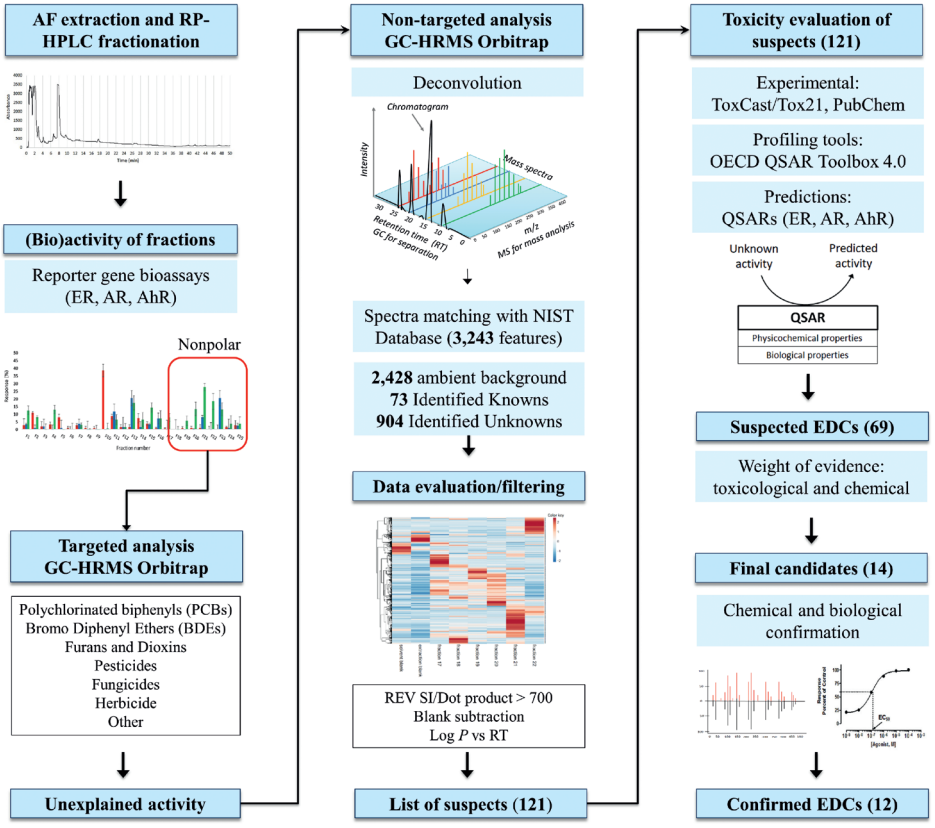


Figure 1. Study design for identification of nonpolar endocrine disruptors (EDCs) in full-term amniotic fluid (AF), using a combination of targeted and non-targeted chemical analysis, reporter gene bioassays, toxicity profiling and prediction tools.

A total of 150 µL of non-spiked AF extract (representing 100 mL AF) was injected on a reversed-phase (RP) Phenomenex Kinetex C18 (100 mm × 2.1 mm, 5 µm pore size) column and separated into 25 fractions using high-performance liquid chromatography (Agilent 1260 HPLC system) and a gradient elution using water and MeOH. Each of the 25 fractions was tested for the (anti-)estrogenic, (anti-)androgenic and dioxin-like activity using reporter gene bioassays [35]. In this study, the nonpolar fractions were further analysed with targeted and non-targeted GC-HRMS analysis to screen for the known and

unknown (novel) nonpolar EDCs present in the active fractions. Moreover, an extraction blank (i.e., a full extraction procedure performed with a non-spiked HPLC grade water) and a solvent blank (i.e., solvents used to prepare AF fractions for GC-HRMS analysis) were also included in the analysis. A schematic representation of the study design is presented in Figure 1. Details of chemicals, reagents and bioassays used are given in Supplementary Material S1.1 and S1.2.

Relative effect potency (REP)

REP values of the individual compounds were determined by dividing the EC50 value obtained for the respective positive control (E2, DHT or TCDD) by the EC50 obtained for the test compound. In the AR-antagonistic assays, the REP values were calculated as the potency of a test compound relative to the EC50 of the positive control, flutamide (Flu). The REP values were calculated based on EC50 data derived in this study or collected from open literature sources or retrieved from ToxCast/Tox21 (Table S1). The ER/anti-AR/DR activity was transformed into estrogenic (EEQs), anti-androgenic (Anti-AR-EQs) and dioxin (TEQs) equivalent units, by multiplying the REP values with the concentrations of compounds measured quantitatively in the AF fraction. The equivalent units were expressed in ng of E2-EQs, Flu-EQs or TCDD-EQs per litre AF (ng/L), respectively.

GC-HRMS Sample Preparation

Prior to GC-HRMS analysis, the methanol fractions were evaporated to 50 µL under nitrogen gas using a Thermo Scientific Reacti-Therm (Waltham, MA 02541). The 50 µL sample and two 30 µL dichloromethane rinses were transferred to an amber GC vial with a 350 µl fused glass insert. Each sample was spiked with 10 µL of internal standard solution to achieve a final concentration of phenanthrene-d10 and chrysene-d12 of 20 ng/mL and a concentration of each compound in the carbon number distribution marker of 93 ng/mL. The final volume of each sample was brought to 150 µL with dichloromethane. The deuterated internal standards were used to verify injection consistency and column performance, while the carbon number distribution marker was used for non-targeted HRMS analysis as discussed below. Overall, the injection consistency during GC-HRMS analysis was high, with the coefficient of variation (CV) for both internal standards of less than 5% (Supplementary Excel File). The two columns used showed a high level of reproducibility with CV for RT drift for both internal standards of less than 0.1%.

GC-HRMS Data Collection and Targeted Analysis

Sample extracts were analyzed using a Thermo GC-Orbitrap mass spectrometer, which was equipped with a Thermo Trace 1300 GC and TriPlus RSH autosampler operated in positive electron ionization (EI) mode. Helium (99.9999% purity) and nitrogen (99.999% purity) were used as the carrier and c-trap gases, respectively. Two GC runs

were performed; the first run was used to quantify dioxins, furans, and polybrominated diphenyl ethers (PBDEs) separated on a 15-m Restek Rtx-5SilMS column (0.25 mm inner diameter x 0.25 µm film thickness), while the second run was used to quantify polychlorinated biphenyls (PCBs), pesticides, and non-brominated flame retardants and for non-targeted HRMS analysis separated on a 30-m Restek Rtx-5Sil MS column (0.25 mm inner diameter x 0.25 µm film thickness). For the full list of compounds see Supplementary Material, Table S2. For targeted analysis of dioxins, furans, and PBDEs, 6 µL of the extract was injected into a programmable temperature vaporizer inlet (PTV) operating in large volume injection (LVI) mode. The PTV-LVI injection occurred in the following stages: (1) injection: split mode for 0.05 minute at 45°C with a split gas flow rate of 100 mL/min, (2) evaporation: split mode for 1 minute with a split flow of 250 mL/min while the temperature increased at 14.5°C /s to 60°C, (3) transfer: splitless mode for 2 min and increased to 330°C at 14.5°C /s, (4) cleaning: split mode for 5 min with a split flow of 100 mL/min and the temperature increased 14.5°C /s to 350°C, and (5) post-cycle: split mode and the temperature was maintained at 350°C for the remainder of the GC run. The carrier gas flow rate was 1.5 mL/min. The oven temperature ramp was: 75°C for 0.4 min, 200°C at 30°C /min, 260°C at 10°C /min with a 3 min hold, 310°C at 5°C /min, and to 360°C at 50°C /min with a 3 min hold. The transfer line and source were maintained at 300°C. Data were collected between 5.3 and 23 minutes (the total run time was 34 minutes) in full-scan mode with 60,000 resolution and a scan range of 220 to 850 *m/z*. The instrument was calibrated before sample analysis to less than 1 ppm mass accuracy.

Sample extracts were injected a second time to evaluate the concentration of PCBs, pesticides, and non-brominated flame retardants and for non-targeted analysis. A 3-µl sample was injected into a 290°C splitless inlet operated in split-less mode. The carrier gas flow rate was 1 mL/min. The transfer line and the source temperature were maintained at 300°C. The oven temperature ramp was as follows: 50°C hold for 0.5 min, 221°C at 7°C /min and held for 2 minutes, 222°C at 0.1°C /min, and 320°C at 7°C /min and hold for 3 minutes (total run time of 49 minutes). Data were collected in full-scan mode between 5 and 45 minutes with a scan range of 50 to 750 *m/z*.

The extracted ion chromatogram (XIC) was used for quantification using the most abundant peak in the mass spectrum for each compound. Compound identity was confirmed using retention time and two confirming ions (Supplementary Material, Table S2). Quantification was performed by an external eight-point calibration curve prepared by serial dilutions of the calibration standards (0.005 to 15 ng/mL). LOD for each compound was determined by injecting 7 low-calibration standards near the detection limits (between 0.05 and 2 ppb, depending on the concentration of the analyte in the standards purchased from Accustandard) and was calculated as t (the student's t value for a 99% confidence level with n-1 degrees freedom, t=3.14) times standard deviation divided by the calibration curve slope (Long and Winefordner 1983). The concentration of compound

detected in AF fractions above LOD was corrected for the preconcentration factor and reported in ng/L AF, ng/g lipids (based on the average AF lipid content at full term (0.15g/L) as reported in the literature [41,42]) and as a non-lipid adjusted molar concentration (M).

Non-targeted GC-HRMS Data Analysis

The data files produced by the GC-HRMS runs on the 30-m column were re-processed for non-targeted analysis using Thermo TraceFinder software (EFS Version 4.1 SP 1) with the Deconvolution Plugin. The Deconvolution Plugin was used to automate forward mass spectra searching to the 2017 NIST Mass Spectra Library (NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library Version 2.3) and a high-resolution library developed using certified standards. The NIST library contains over 250,000 compounds while the in-house library contains 300 compounds. Features were integrated for a signal-to-noise (S/N) ratio of greater than 10, a minimum total ion chromatogram (TIC) intensity of 1,000, and a 99% allowable ion overlap window. The compounds in the carbon number distribution marker were used to align peaks and to calculate the retention index (RI) of each *m/z* feature. The RI of each peak was compared to the library match RI data contained in the spectral libraries. The allowable RI delta was 300. The library search was performed with a reverse match factor (RSI/Rev) score threshold of 500 (a comparison score of the obtained spectrum to the library spectrum). The compound with the highest RSI score was assigned as the identification. A data filtering strategy (Supplementary Material S1.3) based on a calibration standard containing 350 compounds (Supplementary Excel File), was performed using MATLAB (vR2018a). Briefly, the script exported the identified features for all samples to the PubChem database to retrieve International Chemical Identifiers (InChi) and CAS numbers, and compared features to a single standard containing the 350 compounds. When a feature was present in the calibration standard list and the sample, it was flagged as Identified Known, when a feature was present in the sample but not in the calibration standard, it was flagged as Identified Unknown. The unmatched features were not included in this analysis. Features detected only in the solvent blank, extraction blank, and calibration standard (not detected in any of the samples) were also not included in the analysis. The selected Identified Knowns were further quantified using a full calibration curve.

Identification of Candidate EDCs

The peak intensities of the Identified Unknowns found in the AF fractions were visualized with a colour-coded heatmap using the ClustVis web tool [43]. Identified Unknowns that correlated with the active fractions were chosen for further analysis when the following criteria were met: a) not present in the extraction/solvent blank or peak intensity at least 5 x higher than in the extraction/solvent blank; b) RSI/Rev Dot product > 700; c) log *P* value (octanol-water partition coefficient), experimental or predicted > 4.0. Exper-

imental Log *P* values were retrieved from Chemspider (http://www.chemspider.com/), PubChem (https://pubchem.ncbi.nlm.nih.gov/) database or publicly available Episuite Kowwin v1.67, U.S. EPA software (available at www.epa.gov). Predicted values were estimated with Episuite Kowwin v1.67 and OPERA software (OPEn structure-activity/property Relationship App) [38]. Identified Unknowns that met the above criteria were further evaluated for their potential (anti-)estrogenic/androgenic/dioxin-like activity using a combination of publicly available experimental data and predictive models.

Experimental Data

Experimental data were retrieved from ToxCast and Tox21 high-throughput bioassays [36] that measured agonistic and antagonistic activity directly linked to estrogen receptor (ER), androgen receptor (AR) and aryl hydrocarbon receptor (AhR) activation or binding using The U.S. Environmental Protection Agency's (EPA) web-based CompTox Chemicals Dashboard platform (https://comptox.epa.gov/dashboard, Williams et al. 2017). InChi identifiers of the Identified Unknowns were used as an input format. Compounds were screened, using a batch search mode, against a total of 31 bioassays (2 AhR, 12 AR and 17 ER assays), comprised of various reporter gene and binding assays. Where possible, for each antagonistic assay complementary viability assays were included to ensure that the observed antagonistic activity was not caused by cell death. For a detailed list of bioassays used see Table S3. Additionally, Identified Unknowns were checked against the experimental database available through the QSAR Toolbox application (OECD QSAR Toolbox, https://www.qsartoolbox.org), which included data on three receptor-mediated endpoints i.e., androgen binding affinity (ARBA) for 1099 compounds, estrogen relative binding affinity (ERBA) for 3715 compounds, and AhR activity for 142 compounds. Identified Unknowns were also checked against a high throughput screening database for the activation of AhR, developed by The Scripps Research Institute Molecular Screening Center (available online at https://pubchem.ncbi.nlm.nih.gov/bioassay/2796,) and against Human Metabolome Database [45], to screen for known endogenous compounds.

Profiling and QSARs

Quantitative-Structure Activity Relationship (QSAR) models were used as a part of the weight of evidence to fill data gaps where experimental data were scarce and to further predict chemicals with potential (anti-)estrogenic/androgenic/dioxin activity. InChI strings of the Identified Unknowns were converted to a simplified molecular-input line-entry system (SMILES) with OpenBabel software [46], accessed through http://www.chem-info.org/. SMILES structure descriptors, were used as input for QSARs. Two separate QSAR tools were used. First, OPERA (OPEn structure-activity/property Relationship App) a command-line application with two QSAR models i.e., CoMPARA (Collaborative Modelling Project for Androgen Receptor Activity) [47] and CERAPP (Collaborative Estrogen

Receptor Activity Prediction Project) [48]. Second, an online-based Danish QSAR Database with estimates from more than 200 free and commercially available (Q)SAR platforms (Danish (Q)SAR Database, Division of Diet, Disease Prevention and Toxicology and Food Institute, Technical University of Denmark 2015, http://qsar.food.dtu.dk/). Here, a battery of modelling systems was used to predict ER activation and binding, AR antagonism and AhR activation (Klimenko et al. 2019). Moreover, ER binding profiler available through QSAR Toolbox was additionally used (https://www.qsartoolbox.org). The profiler predicts chemical binding to ER based on molecular weight (MW) and structural characteristics, classifying chemicals as very strong, strong, moderate, weak or non-binders. A detailed description of each QSAR and profiling tool is given in Table S4 and Figure S1. Identified Unknowns were ranked based on the weight of evidence for their possible endocrine disrupting potency (the experimental, profiling and QSAR data was given the same weight) and the correlation between the elution profile and the activity observed in the fractions (checked manually for each suspected EDC).

Chemical and biological confirmation

Where possible, reference standards for the selected candidate EDCs were purchased at the highest commercially available purity (see Supplementary Material S1.1), and their presence in the AF fraction(s) was further confirmed with GC-HRMS by comparing their exact masses (5 ppm mass accuracy tolerance), mass spectra and retention times (RT difference of ±0.05 min) to the data obtained during the non-targeted analysis. The (anti-) estrogenic/androgenic/dioxin-like activity of the selected candidates was further confirmed in the respective bioassays. A concentration range of the candidate EDCs (0.01 to 100 µM) was prepared by serial dilution in DMSO and, where needed, was adjusted to include more points in the linear part of the concentration-response curve. Candidate EDCs were tested with the same media composition and final DMSO concentration as used to test AF fractions.

Results

Endocrine activity in nonpolar AF fractions

In the previous study by Dusza *et al.* (2019), extraction and fractionation of full-term AF into 25 fractions resulted in elevated (anti)-AR and DR activity in nonpolar fractions F17-F25. In this study, ER activity was tested at the same fraction dilution in which we observed (anti)-AR and DR activity, i.e. 2 times dilution. ER activity was observed in all except two (F19 and F22) nonpolar fractions tested (Figure 2A). The highest ER activity was observed in F23 and reached 28% of the activity of positive control E2, the most potent ER agonist. As shown in our previous study [35], elevated DR activation was observed in 6 (F17 and F19-23) out of the 9 nonpolar fractions tested, with the highest observed

activity (F21) reaching 30% of the activity of TCDD, the most potent AhR agonist (Figure 2A). AR activity was present only in F21 and F23, with 8% and 20% induction of luciferase activity as compared to the highest activity observed for the positive control DHT, respectively (Figure 2A). None of the nonpolar fractions showed anti-ER or anti-DR activity. However, anti-AR activity was observed, with 4 fractions (F17-F20, Figure 2B) showing up to 18% decrease of DHT-induced luminescence. Based on their activity profile, seven nonpolar fractions (F17-F23) were chosen for further targeted and non-targeted chemical analysis.

GC-HRMS Targeted Analysis

A total of 48 known environmental pollutants were measured with targeted GC-HRMS analysis of the active nonpolar AF fractions (Table S2). Fractions were additionally screened for the presence of environmental pollutants by comparing features present in the sample to a single standard point in the calibration standard containing 350 environmental chemicals (Supplementary Excel File), using the data filtering strategy (Supplementary Material S1.3). Here, 73 compounds were detected as Identified Knowns. From these, 62 were present in ToxCast/Tox21database, but only 14 showed endocrine activity in one or more high-throughput *in vitro* screening (HTS) bioassays (Table S3) and therefore were additionally quantified with a full calibration curve. Compounds from the quantitative, targeted analysis of nonpolar AF fractions are presented in Table 1. Moreover, 5 additional nonpolar compounds previously detected in the nonpolar AF fractions reported by Dusza et al. (2019), were also included in Table 1. In total, 42 compounds were detected above LODs (Table 1 and S2), including 4 dioxins and furans, 10 PBDEs, 12 pesticides, fungicides and herbicides, 6 PCBs, and 10 other compounds, such as plasticizers and polycyclic aromatic hydrocarbons (PAHs). The concentrations of most compounds were in the ng/L range, with the highest concentration detected for o,p'-DDD, δ-HCH and p,p'-DDD at 537.2, 498.1 and 274.8 ng/L AF, respectively. The lipid-adjusted concentrations, based on the AF lipid content of 0.15 g/L were, as follows, 6.6 times higher than the non-adjusted values (Table 1). Eight compounds were detected in the extraction blank above the LODs, namely phenol, 2,4,5-trichloro-, PCB 114, TPP, EDP, B[a]P, B[b]F, mesitylene and azobenzene (Table 1 and Table S2). For all, except phenol, 2,4,5-trichloro- and azobenzene, the concentrations found were close to LOD levels (Table S2).

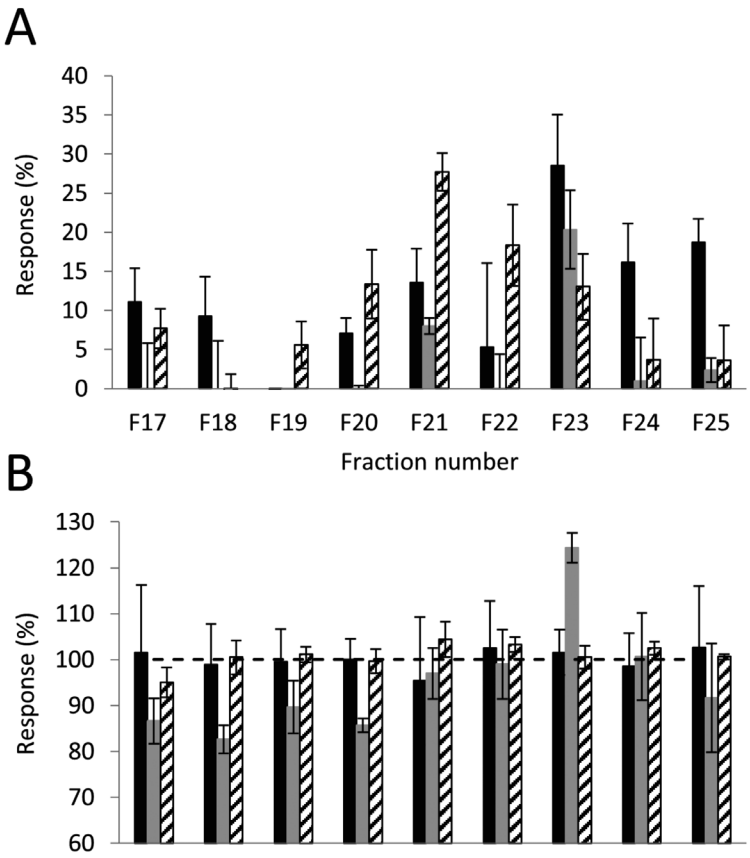


Figure 2. Agonistic (A) and antagonistic (B) activity of nonpolar fractions of amniotic fluid (AF) in ER (black), AR (grey), and DR (striped) responsive cell lines. Agonistic response is expressed relative to the maximum induction observed in the concentration-response curve of the positive control E2 (50 pM), DHT (0.5 nM) and TCDD (1 nM). Antagonistic activity is measured as a decrease in luminescence after co-exposure with 4, 150, and 30 pM of E2, DHT, and TCDD, respectively. Results presented as average % of response of respective positive control ± SD (n = 3).

Table 1. Environmental pollutants in nonpolar fractions of full-term amniotic fluid (AF) measured with targeted GC-HRMS analysis, including molecular weight (MW), partition coefficient (log*P*), total concentration measured in fractions (expressed in ng/L AF, ng/g lipids and non-lipid adjusted molar (M) concentration), and fraction(s) in which compound was detected above the limits of detection (LOD).

Chemical group	Compound name	MW	Log*P*^	Conc. (ng/L AF)	Conc. (ng/g lipid)	Conc. (M)	Fraction nr.
Dioxins and Furans							
	2,3,7,8-TCDF	306.0	6.53	<LOD	<LOD	<LOD	-
	2,3,7,8-TCDD	322.0	6.80	1.4	9.3	4.6×10^{-12}	23
	1,2,3,7,8-PCDF	340.4	6.79	<LOD	<LOD	<LOD	-
	1,2,3,7,8-PCDD	356.4	6.64	0.9	6.0	2.5×10^{-12}	19-20
	1,2,3,4,7,8-HCDF	374.9	NA	4.5	30.0	1.2×10^{-11}	21-22
	1,2,3,4,7,8-HCDD	390.9	7.80	<LOD	<LOD	<LOD	-
	1,2,3,4,6,7,8-HCDF	409.3	7.92	<LOD	<LOD	<LOD	-
	1,2,3,4,6,7,8-HCDD	425.3	8.20	<LOD	<LOD	<LOD	-
	OCDF	443.7	8.60	1.9	12.7	4.3×10^{-12}	17
	OCDD	459.7	8.20	<LOD	<LOD	<LOD	-
Polybrominated Diphenyl Ethers (PBDEs)							
	BDE 3*	249.1	NA	129.6	864.0	5.2×10^{-10}	21-22
	BDE 28	406.9	5.94[a]	32.4	216.0	8.0×10^{-11}	17-20
	BDE 47	485.8	6.81[a]	1.0	6.7	2.0×10^{-12}	21-22
	BDE 99	564.7	6.84	1.1	7.3	1.9×10^{-12}	22
	BDE 100	564.7	7.24[a]	15.1	100.7	2.7×10^{-11}	20
	BDE 153	643.6	7.90[a]	7.0	46.7	1.1×10^{-11}	17-18
	BDE 154	643.6	7.82[a]	<LOD	<LOD	<LOD	-
	BDE 180	722.5	NA	<LOD	<LOD	<LOD	-
	BDE 183	722.5	8.27[a]	1.8	12.0	2.5×10^{-12}	20
	BDE 209	959.2	9.97[b]	34.3	228.7	4.7×10^{-11}	22-23
	6-OH-BDE 47[e]	501.8	NA	13.7	91.3	2.7×10^{-11}	19-20
	3-OH-BDE 157[e]	659.6	NA	9.5	63.3	1.4×10^{-11}	19-20
Pesticides, Fungicides, and Herbicides							
	p,p'-DDT	354.5	6.91	0.8	5.3	2.2×10^{-12}	17
	o,p'-DDT	354.5	6.79[c]	7.8	52.0	2.2×10^{-11}	19-22
	p,p'-DDE	318.0	6.51	<LOD	<LOD	<LOD	-
	o,p'-DDE	318.0	6.00[d]	<LOD	<LOD	<LOD	-
	p,p'-DDD	320.0	6.02	274.8	1832.0	6.6×10^{-10}	17-22
	o,p'-DDD	320.0	5.87[d]	537.2	3581.3	1.7×10^{-9}	17-22
	Mirex	545.5	6.89	<LOD	<LOD	<LOD	-
	Endrin	380.9	5.20	51.3	342.0	1.3×10^{-10}	21-22
	Fonofos*	246.3	3.94	1.5	10.0	6.0×10^{-12}	17
	Heptachlor	373.3	6.10	1.0	6.7	2.8×10^{-12}	20
	γ-HCH	290.8	3.72	2.5	16.7	8.6×10^{-12}	22
	α-HCH	290.8	3.80	<LOD	<LOD	<LOD	-

Table 1. *[continued]*

Chemical group	Compound name	MW	Log*P*^	Conc. (ng/L AF)	Conc. (ng/g lipid)	Conc. (M)	Fraction nr.
	β-HCH	290.8	3.78	8.6	57.3	2.9×10^{-11}	17-22
	δ-HCH	290.8	4.14	498.1	3320.7	1.7×10^{-9}	17-20
	Triclosan[e]	289.5	4.76	45.7	304.7	1.5×10^{-10}	17-18
	cis-Chlordane	409.8	6.10	<LOD	<LOD	<LOD	-
	trans-Chlordane	409.8	6.22	<LOD	<LOD	<LOD	-
	trans-Nonachlor	444.2	6.35	<LOD	<LOD	<LOD	-
	Hexachlorobenzene	284.8	5.73	<LOD	<LOD	<LOD	-
	Phenol, 2,4,5-trichloro-*	197.4	3.72	27.3	182.0	1.4×10^{-10}	17-23
Polychlorinated biphenyls (PCBs)							
	PCB 18*	257.5	5.48	0.7	4.7	2.7×10^{-12}	22
	PCB 65	292.0	NA	<LOD	<LOD	<LOD	-
	PCB 81*	292.0	NA	<LOD	<LOD	<LOD	-
	PCB 105*	326.4	6.79	0.1	0.7	3.1×10^{-13}	21-22
	PCB 114*	326.4	NA	3.5	23.3	1.1×10^{-11}	22-23
	PCB 118	326.4	7.12	<LOD	<LOD	<LOD	-
	PCB 126	326.4	NA	<LOD	<LOD	<LOD	-
	PCB 138*	360.9	7.44	1.4	9.3	3.9×10^{-12}	20-22
	PCB 153	360.9	7.75	<LOD	<LOD	<LOD	-
	PCB 166	360.9	7.31	<LOD	<LOD	<LOD	-
	PCB 169	360.9	7.41	<LOD	<LOD	<LOD	-
	PCB 180	395.3	7.72[a]	<LOD	<LOD	<LOD	-
	PCB 206	464.2	9.14	1.6	10.7	3.5×10^{-12}	21
	OH-PCB 61[e]	308.0	NA	<LOD	<LOD	<LOD	-
Other							
	TPP	326.3	4.59	36.8	245.3	1.1×10^{-10}	21
	EDP	362.4	5.73	2.5	16.7	6.9×10^{-12}	23
	IPyr*	276.3	6.58	1.1	7.3	3.9×10^{-12}	22
	B(a)P	252.3	6.13	69.3	462.0	2.7×10^{-10}	18-22
	B(b)F*	252.3	5.78	3.7	24.7	1.5×10^{-11}	17-23
	DNOP*	390.6	8.10	4.4	29.3	1.1×10^{-11}	18-23
	Pyrene	202.3	4.88	7.5	50.0	3.7×10^{-11}	17-20
	Fluorene*	166.2	4.18	5.7	38.0	3.4×10^{-11}	19-23
	Mesitylene*	120.2	3.42	69.3	462.0	2.7×10^{-10}	17-23
	Azobenzene*	182.2	3.82	251.1	1674.0	1.4×10^{-9}	17-22
	4-Octylphenol[e]	206.3	5.30	<LOD	<LOD	<LOD	-

Note: compounds were analysed in one pooled AF samples, n=1; NA - not available; * - targeted analysis of compounds selected from the analysis of 350 environmental chemicals; underscore – compounds present in the extraction blank above LOD; ^ - experimental Log *P* values based on octanol/water partition coefficient were retrieved from PubChem or literature; a - Braekevelt et al. 2003; b - Watanabe and Tatsukawa 1990; c - Swann et al. 1981; d - Howard and Meylan 1997; e - Dusza et al. 2019.

DR, (anti-)AR and (anti-)ER activity of detected compounds

Dioxin-like activity was found for 13 compounds with EC50 values ranging from 9.5 x 10^{-12} M for the most potent compounds (2,3,7,8-TCDD and 1,2,3,7,8-PCDD) to >1.5 x 10^{-5} M for the least potent compound (BDE 99) (Table 2). In terms of androgenic activity, only one compound (mesitylene) was a weak AR agonist, with EC50 of 6.4 x 10^{-5} M, whereas 23 compounds were AR antagonists. The majority of AR antagonists had low to moderate anti-AR potency while two, namely BDE 100 (0.1 μM) and p,p'-DDD (0.7 μM), were highly potent (Table 2). Estrogenic potency was found for 15 compounds, with EC50 values ranging from 2.0 x 10^{-6} M (o,p'-DDT) to >1.5 x 10^{-5} M (BDE 28). Additionally, 9 compounds were ER antagonists, from which 2,3,7,8-TCDD, IPyr, B(b)F and 6-OH-BDE 47 were reported as highly potent with IC50 values of 1.0 x 10^{-8} M, 3.0 x 10^{-7} M, 4.0 x 10^{-7} M and 5.0 x 10^{-7} M, respectively (Table 2).

Table 2. Compounds detected in nonpolar fractions of amniotic fluid by targeted GC-HRMS analysis and their dioxin-like (DR), androgenic (AR), anti-androgenic (anti-AR), estrogenic (ER) and anti-estrogenic (anti-ER) potencies measured as half-maximal effect (EC50) or inhibition (IC50) concentration, as reported in *in vitro* reporter gene bioassays.

Compound	EC50 or IC50 (M) based on Reported Gene Assays					Fraction number
	DR (EC50)	AR (EC50)	anti-AR (IC50)	ER (EC50)	anti-ER (IC50)	
2,3,7,8-TCDD	1.1 x 10^{-11}[a]	-	-	-	1.0 x 10^{-8}[h]	23
1,2,3,7,8-PCDD	9.5 x 10^{-12}[a]	NA	NA	NA	NA	19-20
1,2,3,4,7,8-HCDF	9.6 x 10^{-11}[a]	NA	NA	NA	NA	21-22
OCDF	4.0 x 10^{-8}[b]	NA	NA	NA	NA	17
BDE 3	-	-	5.9 x 10^{-5} ^	-	7.5 x 10^{-5}^	21-22
BDE 28	-	-	3.1 x 10^{-6}[c]	>1.5 x 10^{-5}[c]	-	17-20
BDE 47	-	-	1.0 x 10^{-6}[c]	1.2 x 10^{-5}[c]	-	21-22
BDE 99	>1.5 x 10^{-5}[c]^	-	7.8 x 10^{-6}[c]	-	-	22
BDE 100	-	-	1.0 x 10^{-7}[c]	7.0 x 10^{-6}[c]	-	20
BDE 153	6.0 x 10^{-7}[c]	-	1.3 x 10^{-5}[c]	-	-	17-18
BDE 183	2.0 x 10^{-6}[c]^	-	>1.5 x 10^{-5}[c]	-	6.4 x 10^{-6}[c]	20
BDE 209	-	-	-	-	-	22-23
6-OH-BDE 47	1.3 x 10^{-6}[c]	-	2.8 x 10^{-6}[c]	-	5.0 x 10^{-7}[c]	19-20
3-OH-BDE 157	NA	NA	NA	NA	NA	19-20
p,p'-DDT	-	-	1.0 x 10^{-6}[d]	5.2 x 10^{-6}^	-	17
o,p'-DDT	-	-	3.3 x 10^{-6}[e]	2.0 x 10^{-6}	-	19-22
p,p'-DDD	-	-	7.0 x 10^{-7}[d]	2.8 x 10^{-5}^	-	17-22
o,p'-DDD	-	-	9.9 x 10^{-6}^	6.3 x 10^{-6}^	-	17-22
Endrin	-	-	3.2 x 10^{-5}^	2.3 x 10^{-5}^	-	21-22
Fonofos	-	-	2.9 x 10^{-5}^	-	-	17
Heptachlor	-	-	-	-	-	20
γ-HCH	-	-	4.0 x 10^{-6}[d]	NA	-	22
β-HCH	-	-	-	2.8 x 10^{-6}^	-	17-22
δ-HCH	NA	-	5.1 x 10^{-5}^	NA	-	17-20

Table 2. *[continued]*

Compound	EC50 or IC50 (M) based on Reported Gene Assays					Fraction number
	DR (EC50)	AR (EC50)	anti-AR (IC50)	ER (EC50)	anti-ER (IC50)	
Triclosan	-	-	1.3×10^{-5}	2.0×10^{-6}^	6.9×10^{-5}	17-18
Phenol, 2,4,5-trichloro-	-	-	4.3×10^{-5}	-	9.2×10^{-5}	17-22
PCB 18	NA	NA	NA	NA	NA	17-22
PCB 105	4.0×10^{-7}[b]	NA	NA	NA	NA	21-22
PCB 114	4.0×10^{-7}[b]	NA	NA	NA	NA	22-23
PCB 138	NA	NA	1.0×10^{-6}[f]	NA	1.6×10^{-5}[f]^	18-22
PCB 206	NA	NA	NA	NA	NA	21
TPP	-	-	7.7×10^{-6}^	7.3×10^{-6}^	-	21
EDP	-	-	-	-	-	23
Pyrene	-	-	2.5×10^{-6}^	-	-	17-20
Fluorene	-	-	-	-	-	19-23
Mesitylene	-	6.4×10^{-5}	-	-	-	17-23
Azobenzene	-	-	-	4.8×10^{-5}^	-	17-22
IPyr	4.0×10^{-8}[g]	-	4.2×10^{-6}^	-	3.0×10^{-7}^	22
B[a]P	6.0×10^{-7}[a]	-	1.1×10^{-6}^	3.4×10^{-5}^	2.6×10^{-6}^	18-22
B[b]F	1.0×10^{-8}[a]	-	-	4.2×10^{-5}^	4.0×10^{-7}^	17-23
DNOP	-	-	-	-	-	23

Note: NA, not available; ^, partial agonist/antagonist i.e., maximum activity below 100% of the activity of the positive control; a, own data; b, based on TEF values (WHO 2005); c, Hamers et al. 2006; d, Misaki et al. 2015; e, Aït-Aïssa et al. 2010; f, Hamers et al. 2011; g, Vondráček et al. 2017; h, Legler et al. 1999; all other data was retrieved from ToxCast bioassays: TOX21_AhR_LUC_Agonist, TOX21_AR_LUC_MDAKB2_Agonist TOX21_ERA_LUC_VM7_Agonist, TOX21_ERA_LUC_VM7_Antagonist_0.1nM_E2. Details of each bioassay used are given in Supplementary Material Table S1.

Contribution of compounds to the observed bioassay activity

Total analytical TEQs of the detected DR agonists corresponded to 3.0 ng TCDD-EQs/L AF (Table 3). Three compounds, namely 2,3,7,8-TCDD, 1,2,3,4,7,8-HCDF, 1,2,3,7,8-PCDD, contributed 47 %, 17 % and 35 % to the estimated total analytical TEQ, respectively (Table 3). Total bioassay TEQ of the active nonpolar AF fractions corresponded to 10.0 ng TCDD-EQs/L AF, from which approximately 30 % could be explained by the total analytical TEQ (Table 4). Approximately 40 % and 48 % of the bioassay activity observed in F19 and F20 respectively was explained by the dioxin-like compounds measured in these fractions, whereas the activity observed in F23 could be explained almost entirely (97 %) by the respective analytical TEQ. For the most DR-active AF fraction (F21, Figure 2A), only 8 % of the activity observed was explained by the dioxin-like compounds measured in this fraction. Three DR-active compounds, namely PCB 114, B[a]P and B[b]F were also detected in the extraction blank (Table S2), nevertheless, their levels in the extraction blank were low (close to LODs) and thus their contribution to the observed activity in the AF fraction was minimal (<1%, Table 3).

Table 3. Analytical Toxic Equivalents (TEQs) of individual chemicals based on GC-HRMS analysis of the nonpolar fractions of the full-term amniotic fluid (AF), expressed in ng TCDD equivalents/L AF (ng TCDD-EQs/L).

		Analytical TEQs (ng TCDD-EQs/L)							∑ TEQ per compound	% contribution to ∑ analytical TEQs
	REPs*	F17	F18	F19	F20	F21	F22	F23		
2,3,7,8-TCDD	1.0	-	-	-	-	-	-	1.4	1.4	47
1,2,3,7,8-PCDD	1.0	-	-	0.3	0.7	-	-	-	1.0	35
1,2,3,4,7,8-HCDF	0.1	-	-	-	-	0.3	0.2	-	0.5	17
OCDF	3×10^{-4}	6×10^{-4}	-	-	-	-	-	-	6×10^{-4}	<1
BDE 99	1×10^{-6}	-	-	-	-	-	1×10^{-6}	-	1×10^{-6}	<1
BDE 153	1×10^{-5}	1×10^{-4}	3×10^{-6}	-	-	-	-	-	1×10^{-4}	<1
BDE 183	4×10^{-6}	-	-	-	8×10^{-6}	-	-	-	8×10^{-6}	<1
6-OH-BDE 47	7×10^{-6}	-	-	9×10^{-5}	-	-	-	-	9×10^{-5}	<1
PCB 105	3×10^{-5}	-	-	-	-	1×10^{-6}	2×10^{-6}	-	3×10^{-6}	<1
PCB 114	3×10^{-5}	-	-	-	-	-	1×10^{-5}	9×10^{-5}	1×10^{-4}	<1
B[a]P	2×10^{-5}	-	4×10^{-5}	3×10^{-5}	1×10^{-4}	8×10^{-4}	2×10^{-4}	-	1×10^{-3}	<1
B[b]F	6×10^{-3}	3×10^{-3}	4×10^{-4}	4×10^{-4}	6×10^{-4}	1×10^{-3}	2×10^{-3}	1×10^{-2}	2×10^{-2}	1
∑ TEQs		**4×10^{-3}**	**4×10^{-4}**	**0.3**	**0.7**	**0.3**	**0.2**	**1.4**	**3.0**	**100**

Note: *, relative potency (REP) values were based on our own data or data retrieved from the literature as indicated in Table 2 and Supplementary Material Table S1.

Table 4. Toxic Equivalents (TEQs) based on the reporter gene activity (bioassay TEQs) and GC-HRMS analysis (analytical TEQs) of the nonpolar fractions of full-term amniotic fluid (AF) expressed in ng TCDD equivalents/L AF (ng TCDD-EQs/L).

Fraction number	Bioassay TEQs (ng TCDD-EQs/L)*	Analytical TEQs (ng TCDD-EQs/L)	Explained (%)
F17	1.0	4×10^{-3}	<1
F18	-	4×10^{-4}	-
F19	0.8	0.3	40
F20	1.5	0.7	48
F21	3.3	0.3	8
F22	2.0	0.3	13
F23	1.5	1.4	97
∑	**10.0**	**3.0**	**30**

Note: *, agonistic activity of AF fractions was measured with DR-GFP reporter gene assay as described in the method section.

The androgenic activity observed in the F21 and F23 fraction was not explained by any of the compounds measured in this study, and the contribution of the only weak AR agonist, mesitylene (Table 1 & 2), to the observed AR-activity was negligible. The total analytical Anti-AR-EQs measured in the nonpolar AF fractions corresponded to 673.3 ng Flu-EQs/L AF (Table 5). The individual chemicals o,p'-DDD, BDE 100 and p,p'-DDD contributed 48 %, 30 % and 11 %, respectively to the total analytical Anti-AR-EQ. The total bioassay Anti-AR-EQ corresponded to 336.1 µg Flu-EQs/L AF, from which <1 % could be explained by the total analytical Anti-AR-EQ (Table 6).

The ER potencies of the detected compounds were much lower than that of the positive control E2, with the REP values between 6 to 8 orders of magnitude below E2. Consequently, the calculated total analytical EEQ was low and corresponded to 0.4 pg E2-EQs/L AF (Table 7). o,p'-DDD contributed 59 % to the total analytical EEQ, followed by triclosan (15 %) and p,p'-DDD (7 %). The total bioassay EEQ corresponded to 1.8 ng E2-EQs/L from which <1 % could be explained by the total analytical EEQ (Table 8). The anti-ER activity was not observed in any of the fractions tested (Figure 2B).

Non-targeted GC-HRMS analysis: filtering and screening of the Identified Unknowns

A total of 14,110 features were found in all the fractions, including solvent and extraction blanks, from which 3,243 were match identified with NIST/in house database. After applying the data filtering strategy (see Supplementary Material S1.3) the resulting chemical list contained 977 unique compounds: 73 Identified Knowns and 904 Identified Unknowns. The remaining 2,428 compounds fell into the Ambient Background Noise and were eliminated because these features were likely solvent or sample preparation artefacts.

Table 5. Analytical Anti-Androgenic Equivalents (Anti-AR-EQs) of individual chemicals based on GC-HRMS analysis of the nonpolar fractions of the full-term amniotic fluid (AF), expressed in ng flutamide equivalents/L AF (ng Flu-EQs/L).

		Analytical Anti-Androgenic EQs (ng Flu-EQs/L)							∑ Anti-AR-EQs per compound	% Contribution to ∑ Analytical Anti-AR-EQs
	REPs*	F17	F18	F19	F20	F21	F22	F23		
Flutamide	1.00	-	-	-	-	-	-	-	-	-
BDE 3	0.01	-	-	-	-	1.1	0.2	-	1.3	<1
BDE 28	0.41	1.4	0.6	0.5	10.8	-	-	-	13.3	2
BDE 47	1.29	-	-	-	-	0.8	0.5	-	1.3	<1
BDE 99	0.17	-	-	-	-	-	0.2	-	0.2	<1
BDE 100	13.40	-	-	-	201.8	-	-	-	201.8	30
BDE 153	0.10	0.7	2×10^{-2}	-	-	-	-	-	0.7	<1
BDE 183	0.10	-	-	-	0.2	-	-	-	0.2	<1
6-OH-BDE 47	0.46	-	-	6.3	-	-	-	-	6.3	1
p,p'-DDT	0.20	0.2	-	-	-	-	-	-	0.2	<1
o,p'-DDT	0.15	-	-	0.3	0.6	0.2	0.1	-	1.2	<1
p,p'-DDD	0.26	9.7	11.5	15.6	29.0	5.3	0.4	-	71.4	11
o,p'-DDD	0.60	19.2	24.9	127.1	119.1	19.9	12.2	-	322.3	48
Endrin	0.02	-	-	-	-	0.2	0.7	-	0.9	<1
Fonofos	0.02	3×10^{-2}	-	-	-	-	-	-	<0.1	<1
Heptachlor	0.01	-	-	-	-	-	-	-	-	<1
Lindane	0.07	-	-	-	-	-	0.2	-	0.2	<1
δ-Lindane	0.01	2.0	1.1	1.3	0.5	-	-	-	5.0	1
Triclosan	0.17	4.4	3.4	-	-	-	-	-	7.8	1
TPP	0.01	-	-	-	-	0.3	-	-	0.3	<1
Pyrene	0.02	0.1	4×10^{-2}	-	0.1	-	-	-	0.2	<1
B[a]P	0.56	-	1.3	1.1	3.6	25.6	7.4	-	38.9	6
∑Anti-AR EQs		**37.6**	**42.8**	**152.1**	**365.7**	**53.4**	**21.7**	-	**673.3**	**100**

Note: *, relative potency (REP) values were retrieved from the literature as indicated in Table 2 and Supplementary Material Table S1.

Table 6. Anti-Androgenic Equivalents (Anti-AR-EQs) based on reporter gene activity (bioassay Anti-AR-EQs) and GC-HRMS analysis (analytical Anti-AR-EQs) of the nonpolar fractions of full-term amniotic fluid (AF), expressed in ng flutamide equivalents/L AF (ng Flu-EQs/L).

Fraction number	Bioassay Anti-AR-EQs (ng Flu-EQs/L)*	Analytical Anti-AR-EQs (ng Flu-EQs/L)	Explained (%)
F17	6.9×10^4	37.6	<1
F18	9.4×10^4	42.8	<1
F19	5.4×10^4	152.2	<1
F20	$7.5.x\ 10^4$	365.7	1
F21	2.5×10^4	53.4	<1
F22	1.9×10^4	21.8	<1
F23	-	-	ND
∑	**33.6×10^4**	**673.3**	**<1**

Note: *, antagonistic activity of AF fractions was measured with co-exposure to 150 nM of DHT (positive control) in AR-Luc reporter gene assay, as described in the method section.

The strategy for filtering and prioritization of Identified Unknowns is shown in Figure S1. The heatmap (Figure S1), showing relative peak intensities of the Identified Unknowns (calculated as z scores), was generated for a visual inspection of the chemical complexity of each fraction and corroboration of the correlation between compound elution and bioassay activity. Only compounds with RSI/Rev Dot Product Score above 700 were included in the generation of the heatmap and further analysis. This threshold was based on the observed RSI/Rev of the Identified Knowns, which for the majority of compounds fell between 700 and 999. Further, the lipophilicity of compounds was used to filter chemicals based on the correlation between their predicted log*P* (Kowwin and OPERA) and their elution profile. Based on the elution profile of the reference compounds (log*P* ≥ 4.0 observed in ≥ F17, Dusza et al. 2019), only compounds with log*P* ≥ 4.0 were included in the subsequent analysis. For the 67 targeted compounds, a high correlation was found between experimental and predicted log*P* values estimated with both Kowwin (r =0.95) and OPERA (r=0.92) software (Figure S2). For a few compounds, such as BDE 209 and B(b)F, Kowwin overestimated, whereas OPERA underestimated the log*P* values (Figure S2). To ensure that no compound was eliminated from further analysis based on an inaccurate log*P* prediction, both tools were used to predict log*P* values of the Identified Unknowns, and any inconsistencies in the predictions were checked against online data sources (e.g., PubChem). In general, a good correlation (r=0.725) was found between the log*P* from the two prediction tools nevertheless, after the discrepancy check, 27 additional compounds were included in further analysis.

Table 7. Analytical Estrogenic Equivalents (EEQs) of individual chemicals based on GC-HRMS analysis of the nonpolar fractions of the full-term amniotic fluid (AF), expressed in ng estradiol equivalents/L AF (ng E2-EQs/L).

		Analytical EEQs (ng E2-EQs/L)							∑ EEQs per compound	% Contribution to ∑ Analytical EEQs
	REP	F17	F18	F19	F20	F21	F22	F23		
E2	1.0	-	-	-	-	-	-	-	-	-
BDE 28	3×10^{-7}	9×10^{-7}	4×10^{-7}	3×10^{-7}	7×10^{-6}	-	-	-	9×10^{-6}	2
BDE 47	3×10^{-7}	-	-	-	-	2×10^{-7}	1×10^{-7}	-	3×10^{-7}	<1
BDE 100	6×10^{-7}	-	-	-	9×10^{-6}	-	-	-	9×10^{-6}	2
p,p'-DDT	6×10^{-7}	5×10^{-7}	-	-	-	-	-	-	5×10^{-7}	<1
o,p'-DDT	2×10^{-6}	-	-	3×10^{-6}	6×10^{-6}	2×10^{-6}	1×10^{-6}	-	1×10^{-5}	3
p,p'-DDD	1×10^{-7}	4×10^{-6}	5×10^{-6}	6×10^{-6}	1×10^{-5}	2×10^{-6}	2×10^{-7}	-	3×10^{-5}	7
o,p'-DDD	5×10^{-7}	2×10^{-5}	2×10^{-5}	1×10^{-4}	1×10^{-4}	2×10^{-5}	2×10^{-6}	-	3×10^{-4}	59
Endrin	1×10^{-7}	-	-	-	-	2×10^{-6}	5×10^{-6}	-	7×10^{-6}	2
β-HCH	1×10^{-6}	8×10^{-7}	1×10^{-6}	2×10^{-6}	1×10^{-6}	2×10^{-6}	2×10^{-6}	-	9×10^{-6}	2
Triclosan	2×10^{-6}	4×10^{-5}	3×10^{-5}	-	-	-	-	-	7×10^{-5}	15
TPP	4×10^{-7}	-	-	-	-	2×10^{-5}	-	-	2×10^{-5}	4
Azobenzene	6×10^{-8}	2×10^{-6}	-	3×10^{-6}	8×10^{-6}	2×10^{-6}	8×10^{-7}	1×10^{-7}	2×10^{-5}	4
B[a]P	9×10^{-8}	-	2×10^{-7}	2×10^{-7}	6×10^{-7}	4×10^{-6}	1×10^{-6}	-	6×10^{-6}	1
B[b]F	7×10^{-8}	4×10^{-8}	5×10^{-9}	5×10^{-9}	7×10^{-9}	2×10^{-8}	3×10^{-8}	2×10^{-7}	3×10^{-7}	<1
∑ ER-EQs		**6×10^{-5}**	**6×10^{-5}**	**1×10^{-4}**	**1×10^{-4}**	**5×10^{-5}**	**2×10^{-5}**	**3×10^{-7}**	**4×10^{-4}**	**100**

Note: *, relative potency (REP) values were retrieved from the literature as indicated in Table 2 and Supplementary Material Table S1.

Table 8. Estrogenic Equivalents (EEQs) based on reporter gene activity (bioassay EEQs) and GC-HRMS analysis (analytical EEQs) of the nonpolar fractions of full-term amniotic fluid (AF) expressed in ng estradiol equivalents/L AF (ng E2-EQs/L).

Fraction number	Bioassay EEQs (ng E2-EQ/L)*	Analytical EEQs (ng E2-EQ/L)	Explained (%)
F17	0.3	6×10^{-5}	<1
F18	0.2	6×10^{-5}	<1
F19	-	1×10^{-4}	-
F20	0.2	1×10^{-4}	<1
F21	0.4	5×10^{-5}	<1
F22	0.1	2×10^{-5}	<1
F23	0.7	3×10^{-7}	<1
∑	**1.8**	**4×10^{-4}**	**<1**

Note: *, agonistic activity of AF fractions was measured with ER-Luc reporter gene assay as described in the method section.

The final list of Identified Unknowns consisted of 121 unique compounds, of which squalene and heptadecane were the only endogenous metabolites. These compounds were further evaluated for their endocrine activity using experimental data available through various ToxCast and Tox21 HTS bioassays (CompTox Chemicals Dashboard, Table S3), and ERBA and ARBA experimental databases (OECD QSAR Toolbox). Of the 121 compounds, 47 were found on the CompTox Chemicals Dashboard, of which only 7 were tested for their endocrine activity in ToxCast/Tox21 HTS bioassays. From the 7 compounds, 4 were weak ER agonists (EC50 >1 μM) and one compound, allethrin, was both a weak ER and AR antagonist (EC50 6-7 $x10^{-5}$ μM, Table S5). Additionally, five compounds were also tested in ERBA and ARBA of which 4 were inactive and one, benzoin isobutyl ether, was weakly active in both assays (Table S5). There were no Identified Unknowns that matched PubChem or OECD QSAR Toolbox experimental databases for activation of the AhR. Compounds were further estimated for their biological activity using various QSARs and profiling tools with endocrine endpoints (Table S4). Here, 69 compounds were found active in one or more QSARs (Supplementary Excel File). Compounds were further prioritized based on commercial availability. The resulting 14 compounds listed in Table 9 were purchased, and chemically and biologically confirmed with GC-HRMS and reporter gene bioassay analysis, respectively.

Table 9. The IC20/IC50 (antagonism) or EC20/EC50 (agonism) of the candidate compounds confirmed in AR-Luc, ER-Luc and DR-GFP reporter gene assays. The maximum activity (MAX) was calculated as % of the highest activity observed in the concentration-response curve of the positive control flutamide, E2 and TCDD, respectively.

Compound (fraction nr.)	AR-antagonism			ER-agonism			DR-agonism		
	IC20 (M)	IC50 (M)	MAX (%)	EC20 (M)	EC50 (M)	MAX (%)	EC20 (M)	EC50 (M)	MAX (%)
E2	ND	ND	ND	1.0×10^{-12}	3.0×10^{-12}	100	ND	ND	ND
Flutamide	1.4×10^{-7}	1.0×10^{-6}	100	ND	ND	ND	ND	ND	ND
TCDD (F23)	-	-	-	ND	ND	ND	4.8×10^{-12}	11.4×10^{-12}	100
Allethrin (F20)	3.0×10^{-6}	7.7×10^{-6}	100	-	-	-	-	-	-
Methyl oleate (F23)	-	-	-	-	-	-	-	-	-
Tridecyl benzoate (F22)	-	-	-	-	-	-	3.0×10^{-5}	-	42
p,p'-Ditolylamine (F19)	2.4×10^{-5}	3.7×10^{-5}	95	1.6×10^{-6}	4.7×10^{-6}	78	-	-	-
Benzoin isobutyl ether (F23)	3.1×10^{-5}	3.4×10^{-5}	100	1.1×10^{-6}	-	33	-	-	-
Diphenyl isophthalate (F22)	-	-	-	6.9×10^{-7}	8.2×10^{-7}	163	-	-	-
Cholesterol n-propionate (F23)	1.1×10^{-5}	1.9×10^{-5}	100	2.4×10^{-7}	3.9×10^{-7}	95	-	-	-
BTT (F21)	7.6×10^{-6}	1.4×10^{-5}	100	-	-	-	-	-	-
NABD (F20)	3.4×10^{-6}	6.9×10^{-6}	100	-	-	-	-	-	-
BNP[α] (F22)	-	-	-	-	-	-	-	-	-
BFBT[α] (F21)	1.0×10^{-5}	3.1×10^{-5}	72	1.3×10^{-6}	2.2×10^{-6}	113	-	-	-
TTCP[α] (F18)	-	-	-	-	-	-	1.8×10^{-5}	-	46
BMD[α] (F21)	3.3×10^{-6}	5.2×10^{-6}	100	-	-	-	-	-	-
CAB[α] (F20)	5.9×10^{-6}	1.4×10^{-5}	100	2.2×10^{-6}	9.7×10^{-6}	57	-	-	-

Note: Abbrev.: BTT, 6-Benzyl-3-(thiophen-3-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole; NABD, N,N'-Di-acridin-9-yl-benzene-1,4-diamine; BNP, 4,6-Bis(4-ethoxybenzylthio)-5-nitropyrimidine; BFBT, 1H-Benzoimidazole, 1-(4-fluorobenzyl)-2-p-tolyloxymethyl-; TTCP, [1,2,4]Triazolo[3,4-b][1,3,4]thiadiazole, 3-(4-chlorophenyl)-6-(4-pyridinyl)-; BMD, 1H-Benzimidazole, 1,2-diphenyl-; CAB, Cyclohexanone, 2-(α-anilinobenzyl)-; ND, not determined; NA, not applicable; -, not active; *α*, compounds not confirmed chemically.

Chemical and biological confirmation of candidates

The 14 prioritized candidates were tested for both agonistic and antagonistic potency (Figure 3). Nine compounds showed anti-androgenic activity, with IC50 values ranging from 1.4 x 10^{-5} M (CAB) to 5.2 x 10^{-6} (BMD). For all but one compound (BFBT), full antagonism was observed (Table 9). In general, tested candidates were 5- to 37-fold less potent than the reference antiandrogen flutamide. Additionally, four compounds i.e., BMD, NABD, BFPT, and allethrin, exhibited cytotoxicity in the concentration range tested (Figure S3). No compounds showed androgenic activity, except for diphenyl isophthalate, which was AR active, but only when co-exposed with the positive control DHT, showing a 234 % increase in luminescence as compared to DHT alone (Figure S4).

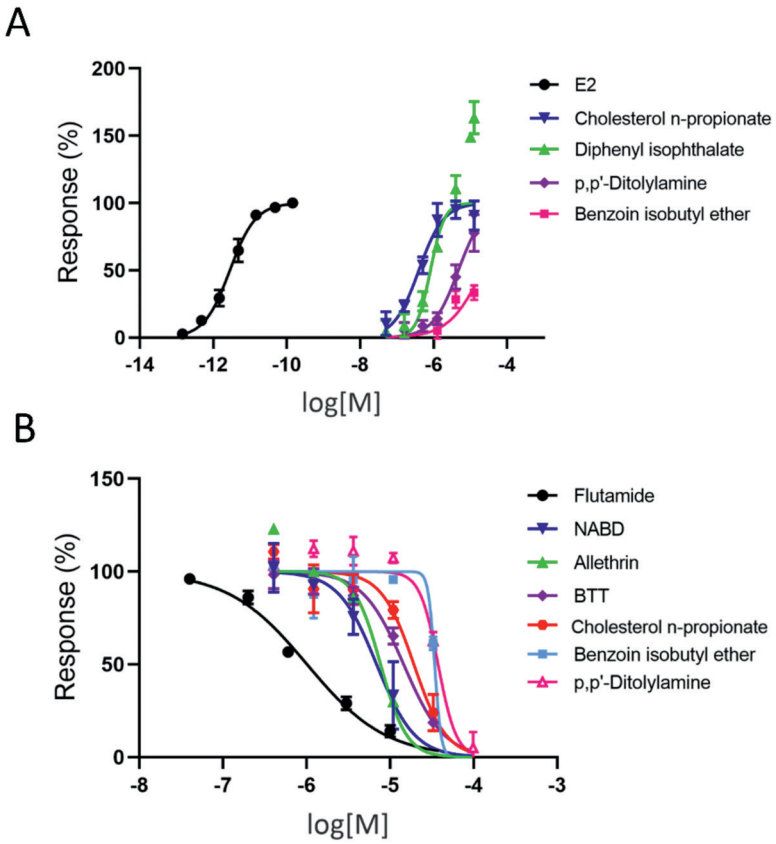


Figure 3. Estrogenic (A) and anti-androgenic (B) activity of novel identified compounds in AF fractions, measured in ER-Luc and AR-Luc reporter gene assay, respectively. In the ER-Luc assay, the % response is expressed relative to the maximum induction by 50 pM E2. In the AR-Luc assay, cells were co-exposed with DHT and the antagonistic response is expressed relative to 150 pM DHT, n=3.

Estrogenic activity was observed for 6 compounds: p,p'-ditolylamine, benzoin isobutyl ether, diphenyl isophthalate, cholesterol n-propionate, BFBT, and CAB, of which diphenyl isophthalate and cholesterol n-propionate were the most potent, with EC50 of 8.2 x 10^{-7} M and 3.9 x 10^{-7} M, respectively (Table 9). The REP of the tested candidates ranged from 10^{-5} to 10^{-6} of the potency of E2. Here, diphenyl isophthalate showed maximum agonistic activity of 163 % as compared to the highest activity observed for the positive control E2, and a 217 % increase in luminescence activity when co-exposed with E2 (Table 9, Figure S4). None of the tested compounds showed ER antagonistic activity. Additionally, two compounds (i.e., tridecyl benzoate and TTCP) were partial AhR agonists, showing weak dioxin-like activity. For these compounds only EC20 values could be derived (Table 9). The presence of 9 out of 14 candidates in AF fractions was confirmed with GC-HRMS and high-purity standards by comparing their exact masses, mass spectra and retention times to the data obtained during the non-targeted analysis (Table 9).

Discussion

Prenatal exposure to nonpolar EDCs is still largely unexplored, with the majority of research restricted to the analysis of well-known priority pollutants in maternal matrices, which are used as surrogates for the fetal environment [17]. This study is the first to investigate a wide range of known and novel nonpolar EDCs in AF, an *in utero* matrix representative of direct fetal exposure. Previously, using an effect-directed analysis (EDA) approach, we discovered significant (anti-)estrogenic, (anti-)androgenic and dioxin-like activity in nonpolar fractions of full-term AF which could not be attributed to the presence of natural endogenous hormones [35]. The discovery of endocrine activity in nonpolar fractions was noteworthy given the aqueous nature of AF and the very low concentrations generally reported for nonpolar compounds in this matrix. In this study, through targeted and non-targeted GC-HRMS analysis and an innovative weight-of-evidence approach, we identified known and novel EDCs that, at least partially, were responsible for the observed endocrine activity.

Known and Novel Nonpolar EDCs in AF

The targeted analysis of nonpolar AF fractions revealed a wide range of nonpolar EDCs from multiple chemical classes, many of which had never before been measured in AF samples (Table 1). The presence of these compounds in AF undoubtedly demonstrates placental transfer and fetal exposure. Many of the 42 compounds detected, such as organochlorine pesticides (OCPs), PCBs and dioxin and furans, are known as persistent organic pollutants (POPs), recognized for their high toxicity, slow degradation, bioaccumulation and long biological half-lives. Although the production and use of these POPs have been banned or severely restricted (e.g. under the Stockholm Convention, 2001),

they are continuously and frequently detected in maternal and fetal tissues and thus their legacy persists [33,34,53,54].

Three OCPs namely, o,p'-DDD, p,p'-DDD and δ-HCH, were the most abundant compounds detected in AF during the targeted analysis. DDT is omnipresent in the environment and is usually detected in >90 % of samples including maternal matrices [55–57]. Typically, DDE is the most abundant metabolite of DDT measured in maternal serum, placenta or cord blood [33,58,59]. Considering that AF is largely a product of the fetal metabolic activity, the higher concentrations of o,p'-DDD and p,p'-DDD detected in AF could be indicative of specific *in utero* metabolism and/or toxicokinetics. The reports on the presence of these compounds in AF are limited, however, one recent study corroborated our findings i.e., p,p'-DDD was measured in AF but not in maternal serum [33]. Other OCPs, such as heptachlor and endrin, were also detected but were present at much lower levels (Table 1).

Although Penta- and Octa-BDEs have been largely phased out, Deca-BDE is still produced and used [60]. The presence of these compounds in maternal blood, cord blood and placenta, has been previously demonstrated [61–63]. It has been suggested that the higher relative bromination of BDEs may decrease transplacental transport due to increased molecular weight, resulting in higher concentrations found in e.g., maternal blood rather than cord blood [64,65]. In this study, Penta-, Octa-, Deca-BDE and higher brominated BDEs (e.g., BDE 209) were detected in AF above LODs, with the latter detected at a higher concentration than some of the more common lower brominated BDEs, such as DBE 47. This suggests that despite the high bromination, BDE 209 pass effectively the placenta barrier. It is possible that the higher brominated compounds accumulate *in utero* e.g., due to low placental and fetal clearance, however, this is still largely unexplored [63,66]. To our knowledge, only one other study measured BDEs in AF, and the congeners and concentrations reported were similar to the ones reported here [32].

Only 5 out of 14 PCB congeners analysed were detected above LODs (Table 1). The low concentration range measured in this study (low ng/L range) fell within the concentration range reported in AF by other authors [33,34,67–69] and in general, was much lower than that reported in maternal serum [54,70]. The presence of the higher chlorinated PCBs in our samples (e.g., PCB 206, 138), was corroborated by a few other studies [34,63,68,71], confirms their bioavailability and transplacental transport.

PAHs such as IPyr, B[a]P and B[b]F were detected in this study, as well as a small number of other studies that analysed human AF [72–74]. Maternal exposure to PAHs and their ability to cross the placenta and reach cord blood has already been demonstrated [75–77]. However, to our knowledge, this study is the first to detect the most potent, highly toxic dioxins i.e., 2,3,7,8-TCDD, 1,2,3,7,8-PCDD and 1,2,3,4,7,8-HCDF in human AF samples (Table 1 & 2). Although the past decades have seen a steady decrease in exposure to these compounds in the general population [78], considering their high toxic potency

(EC50 within pM range), the concentration measured here might be of toxicological significance. Taken together, maternal exposure to the chemicals detected in AF including dioxin and furans, OCPs, BDEs and PCBs have been linked, amongst other things, with impaired fetal neurodevelopment, metabolism and growth, changes in steroid hormone levels and increased prevalence of hormone-sensitive cancers, raising further concern for their contribution to the fetal origins of adult disease [79–84].

Approximately 30 % of the dioxin-like activity (Table 4) and only a small fraction of the estrogenic and (anti-)androgenic activity observed in the nonpolar fractions could be explained by the compounds discussed above (Table 6 & 8), indicating the presence of potential novel, potent EDCs in the AF. Following our non-targeted suspect screening workflow, 121 unique compounds were tentatively identified in AF (Supplementary Excel File) including such diverse compounds as hexyl pivalate and octyl benzoate (fragrance agents), octyl methacrylate (adhesive and components of coatings), neryl isovalerate (flavouring agent) and betamethadol (synthetic opioid analgesic). Squalene and heptadecane were the only two identified endogenous metabolites. This could be explained by the fact that the majority of endogenous compounds in the human body (e.g., hormones, fatty acids, amino acids, carbohydrates, cyclic amines) are polar or semi-polar [85] and therefore would not elute in the nonpolar fractions analysed in this study. Only 8 out of the 121 Identified Unknowns were previously experimentally tested for their endocrine activity (Table S5). Further evaluation of their endocrine activity using *in silico* prediction and profiling tools resulted in a total of 69 additional suspects (Supplementary Excel File). To our knowledge, none of these compounds was previously measured in AF samples, and for only a few of them, limited information on production and use was found. In a recent study, Wang *et al*. (2021) performed non-targeted screening of paired maternal and cord serum samples and tentatively identified 55 compounds previously not reported in human samples. There was no overlap between the compounds identified by Wang *et al*. (2021) and this study, which is not surprising considering that Wang *et al*. (2021) focused mainly on polar industrial chemicals. Collectively, these studies show that there is a vast knowledge gap regarding *in utero* exposure to both polar and nonpolar environmental pollutants that calls for further investigation into their origins and possible health effects [86].

From the 69 compounds with potential endocrine activity, 14 were chosen for further evaluation, based on the toxicity profile and commercial availability (Table 9). From the six compounds confirmed to have estrogenic activity, diphenyl isophthalate, a plasticizer and a flame retardant, was one of the most active, showing 10^{-5} of the potency of E2. Interestingly, diphenyl isophthalate showed supramaximal responses (i.e., induction of luminescence at higher levels than the positive control E2), as well as, high androgenic activity in AR-Luc assay, but only after co-exposure to positive control DHT (Figure S4). The mechanism behind the supramaximal responses is still not fully understood. The

unexplained androgenic activity observed in AF fractions may at least in part, stem from its mixture activity, which is typically overlooked when using REP values of individual compounds. The information about diphenyl isophthalate in the literature is scarce and to our knowledge, this is the first report on its presence in human samples. The other potent ER agonists measured in AF were cholesterol n-propionate and p,p'-ditolylamine. The chemical structure of cholesterol n-propionate closely resembles estradiol, whereas p,p'-ditolylamine (an amine bound to two phenyl groups) resembles the known endocrine disruptor bisphenol A (BPA), which may explain their estrogenic activity. Both compounds also acted as weak anti-androgens (Table 9). The information about this compound in the literature is scarce, however, some online sources report them as industrial chemicals used mainly in semiconductors (www.tcichemicals.com). Similarly, benzoin isobutyl ether showed both ER and anti-AR activity (Table 9). Online sources report that it is used mainly as a coating auxiliary agent [87]. For the other active compounds confirmed in AF, limited to no information about their source or use could be found.

Strengths and limitations of this study

We developed a robust method for the analysis and identification of novel nonpolar EDCs in AF. The use of AF indisputably demonstrates placental transfer and fetal exposure and in general, might be a better approximation of fetal body burden to environmental pollutants than maternal matrices. Additionally, the wide range of nonpolar compounds detected in this study demonstrates that AF can be successfully used for the analysis of even highly lipophilic compounds. It should be noted that this study used a small number of AF samples, and larger epidemiological investigations are needed to better understand the extent of exposure to these compounds in the general population.

The analytical methods applied in this study, i.e. GC-HRMS is the method of choice for non-targeted screening of nonpolar compounds, which usually remain undetected with softer ionization methods like electron spray ionization (ESI) [88]. It provides trace-level detection of highly nonpolar compounds (BDE 209, log*P* 9.97, Table 1), and allows for the non-targeted detection of thousands of volatile substances. Matching spectral information with the compound's retention time provides much improved tentative identifications [88]. In addition, Log*P* values increase the accuracy of chromatographic retention time prediction, narrowing the number of candidates that could elute in the nonpolar fraction during the chromatographic fractionation [35]. The two log*P* prediction tools used in this study, Kowwin and OPERA, showed a high correlation between experimental and predicted log*P* values for the compounds detected during targeted analysis (Figure S2). Nevertheless, discrepancies were found for approximately 20% of the Identified Unknowns. Cross-checking the inconsistencies with external databases (e.g., PubChem) greatly improved the compound selection process.

The use of curated and freely available resources such as the CompTox Chemicals Dashboard, which contains a wealth of information for thousands of chemicals improved the identification and prioritization of suspected EDCs from the list of tentatively identified compounds [37,89]. The ongoing advances in *in vitro* technologies, and the increase in the availability of HTS data, continuously improve modelling algorithms and predictive performances of QSAR *in silico* models. The reported sensitivity, specificity and accuracy of all QSARs used in this study were high (>80%, Table S4) and our data (i.e., the confirmed activity of 12 out of 14 suspected EDCs) validated the applicability of QSAR models for profiling of novel EDCs. Accordingly, there is a high degree of confidence that the list of remaining suspected EDCs that could not be tested in this study (Supplementary Excel File) contains biologically active compounds that could contribute to the remaining, unexplained activity observed in the AF fractions.

Regarding potential methodological limitations in this study, lipid adjustment is noteworthy. The low concentration range of nonpolar compounds reported in AF, including this study, may be attributed to the low concentration of lipids present in the full-term AF [41]. Lipid adjustment is a common practice in order to compare the inter and intra-individual differences in lipophilic toxicant concentration [90,91]; however, it is not without pitfalls. For highly lipophilic, nonpolar compounds, tissue distributions are more closely related to the lipid composition, rather than total lipids [92,93]. The AF lipid profile is highly specific and differs significantly from the maternal plasma [42]. The differences in ratio and composition of polar (e.g., phospholipids) to neutral lipids (e.g., cholesterol, triglycerides) in AF and maternal plasma might therefore influence nonpolar EDC distribution, however, this is still largely unexplored. Moreover, a mixture of developmental waste products and small organic constituents (cells, nutrients, growth factors and proteins) present in AF provide additional matter to which nonpolar compounds may sequester [29]. In particular, as binding to plasma protein is a competitive process between exogenous compounds and endogenous ligands, specifically free fatty acids [28], lower fatty acids concentration in AF as compared to maternal serum might increase EDC binding to proteins and other organic matter present in AF. This illustrates that measurements adjusted for lipid content may not yield meaningful values. Consequently, extrapolation of AF concentration to maternal matrices, based on lipid adjustment, is challenging and should be performed with caution.

Additional methodological limitations may lie in the bioassays used to measure endocrine activity, or in the EDA approach. Although dioxin-like activity in AF fractions could be partially attributed to the analytical TEQ measured, the observed ER and AR activity was largely unexplained by their respective analytical EQs (Table 6 & 8). It is possible that the unexplained (anti-)androgenic activity, to some degree, stems from the use of MDA-kb2 cells which stably express an androgen-responsive luciferase reporter gene construct that responds to compounds that activate not only the AR but also the gluco-

corticoid receptor (GR) [94]. Thus, the contribution of GR-active compounds to the observed androgen activity cannot be excluded. Furthermore, in our EDA approach, though chromatographic separation ensures the reduction of the chemical complexity of the sample, there could be masking effects of co-eluting agonists and antagonists [95], or unknown mixture effects resulting in significantly lower or higher bioassay activity than calculated based on the individual REP values [96], as shown with diphenyl isophthalate (Figure S4). Higher-resolution fractionation may further decrease chemical complexity, but at the same time could result in lower bioassay sensitivity due to compounds being split over multiple fractions. Such trade-offs are inherent to the EDA approach, especially when measured compounds are expected to be present at low levels.

Conclusions

While several thousand man-made substances have been detected to date in the environment, the number of compounds with endocrine disrupting properties that the fetus might be exposed to is still largely unknown. The workflow used in this study i.e., the combination of targeted and non-targeted GC-HRMS analysis of AF together with the application of curated and freely available in vitro experimental data and in silico prediction tools, provided a powerful tool to identify known and novel nonpolar EDCs in the fetal environment. Although we measured a wide variety of known EDCs in AF the remaining, the largely unexplained endocrine activity observed in the nonpolar fractions indicates the presence of potential, novel active compounds. The non-targeted analysis together with the innovative, weight of evidence data mining approach, allowed for the identification of additional suspected EDCs, for which limited to no information on their production and use was found. This study contributes to a better understanding of the fetal exposome and shows that the fetus may be directly exposed to many more EDCs than previously thought, including novel unknown compounds. Further research is needed to better understand the source, nature and extent of exposure to these compounds and their possible effects on fetal development and long-term health.

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Supplementary Materials

S1. Materials and Methods

S1.1. Chemicals and reagents

The following certified reference standards of purity ≥97% were obtained from AccuStandard (New Haven, CT): Furan Mix, Dioxin Mix, PBDE Congeners of Primary Interest Calibration Mix, Pesticide Mix 1, Pesticide Mix 2, AccuGrand 8270 Semi-Volatile Standard (AG01), Organochlorine Pesticides, Pesticide/Herbicide Mix, Triphenyl phosphate, WHO/NIST/NOAA Congener List, PCB Congeners Mix 2, Pesticide/Herbicide Mix, Internal Standard (Phenanthrene-d10 and Chrysene-d12), and Carbon Number Distribution Marker (n-hexane, n-heptane, n-octane, n-decane, n-dodecane, n-hexadecane, n-heneicosane, n-octacosane, and n-pentatriacontane). Dichloromethane, methanol and water (HPLC grade) were purchased from Fisher Scientific (Waltham, MA). Benzoic acid, tridecyl ester (>95% purity) was purchased from Angene (Nanjing, China). 6-benzyl-3-thiophen-3-yl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (BTT, >92% purity) and 4,6-Bis(4-ethoxybenzylthio)-5-nitropyrimidine (BNP, >92% purity) were purchased from Interbioscreen Ltd. (Montenegro). 1H-benzoimidazole,1-(4-fluorobenzyl)-2-p-tolyloxymethyl- (BFPT); [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole,3-(4-chlorophenyl)-6-(4-pyridinyl)- (TTCP) and N,N'-Di-acridin-9-yl-benzene-1,4-diamine (NABD) were purchased at >90% purity from Vitas-M Laboratory, Ltd. (Hong Kong, China). 1H-Benzimidazole, 1,2-diphenyl- (BMD, >95% purity) were purchased from Carbosynth LLC (Compton, UK) and cyclohexanone, 2-(α-anilinobenzyl)- (CAB, >95% purity) from Chemat (Gdansk, Poland). All other reference compounds, if not specified otherwise, were purchased from Sigma Aldrich (Poole, Dorset, UK) at the highest commercially available purity.

S1.2. Reporter gene bioassay

The (anti-)estrogen (ER-Luc) receptor-mediated luciferase reporter gene assay (VM7Luc4E2 human breast carcinoma) [1], the (anti-)androgen/glucocorticoid (AR-Luc) receptor-mediated luciferase reporter gene assay (MDA-kb2 human breast carcinoma) [2], and the aryl hydrocarbon (DR-GFP) receptor enhanced green fluorescent protein reporter gene assay (H1G1.1c3 mouse hepatoma) [3] were used to test the endocrine activity of AF fractions and candidate compounds. Cells were seeded on 96-well plates and exposed to AF fractions, candidate EDCs, solvent (DMSO) control, extraction blank, positive control concentration-response curves (17β-estradiol (E2), dihydrotestosterone (DHT) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) for ER- and AR-Luc and DR-GFP, respectively) in three independent experiments. In the anti-ER/AR/DR assays, cells were co-exposed to the fractions and positive controls at 4 pM, 150 pM and 30 pM of E2, DHT and TCDD, respectively. After 24 h of exposure, ER-Luc and AR-Luc cells were lysed, lucif-

erase reagent was added and the luciferase activity was measured in a luminometer (LUMIstar Optima, BMG Labtech GmbH). The fluorescence activity in the DR-GFP cells was measured in the intact cells using a fluorescent plate reader (POLARstar Galaxy, BMG Labtech GmbH) with 485 nm and 510 nm excitation wavelength and an emission wavelength, respectively. Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Reporter gene activity of each fraction or candidate EDC, corrected for background luminescence or fluorescence, was compared to the highest activity observed in the concentration-response curve of the positive control express either as % relative luminescence units (% RLUs) for the ER and AR-Luc or % relative fluorescence units (% RFUs) for the DR-GFP. The maximum activity (Max) observed for the candidate compound was expressed as % of the highest activity observed in the concentration-response curve of the positive control. In each co-exposure assay (anti-ER/AR/DR activity), the activity of the positive control was set at 100% and the decrease in luminescence/fluorescence was expressed as relative to the positive control. The concentration-response curves of the positive controls and the candidate compounds were fit with the four-parameter sigmoidal Hill equation (GraphPad Software Inc., San Diego, CA). Compound activity was reported as a concentration at which 50% of the maximum activation (EC50) or inhibition (IC50) of the luminescence/fluorescence, was interpolated from the linear region of the sigmoidal equation.

S1.3. Non-targeted GC-HRMS data filtering strategy

A data filtering strategy, based on a calibration standard containing 350 compounds was performed using MATLAB (vR2018a). The script exported the identified features for all samples to the PubChem database to retrieve International Chemical Identifiers (InChi) and CAS numbers. The InChi numbers found in all samples were compared to a list of InChi identifiers that the calibration standard contained. If the InChi identifier was found in the samples and in the calibration standard list, then the chemicals were flagged as “InChi match”. If an InChi identifier was in the samples and not in the calibration list, the chemical was flagged as “no InChi match”. The calibration standard was also evaluated for feature area and classified as “contains feature” or “does not contain feature.” The identified chemical data was then sorted into four categories: (1) Identified Knowns, which were flagged as “InChi match” and “contains feature” (i.e., positive identification, the compound was found in the standard and the samples), (2) Identified Unknowns, which were flagged as “no InChi match” and “does not contain feature (i.e., identification, the compound was identified and the samples contained it, but the standard did not contain it), (3) compounds flagged as “InChi match” and “does not contain feature” (i.e., the samples and the standard contained it, but it was not detected in the standard), and (4) ambient background noise, which was flagged as “no InChi match” and “contains feature” (i.e., a compound was identified, but it was categorized as GC background noise or solvent contamination).

Table S1. Details of various reporter gene assays used to retrieve dioxin-like (AhR), (anti-)androgenic (AR) and (anti-) estrogenic (ER) half-maximal effect (EC50) and inhibitory concentration (IC50) values for compounds detected in the nonpolar fractions of a full-term amniotic fluid.

				Key reference compound			Key antagonist	
Source	**Bioassay**	**Cell line**	**Endocrine pathway**	**Abbrev.**	**EC50 (pM)**	**Co-exposure (pM)**	**Abbrev.**	**EC50 (M)**
1	DR-GFP	H1G1.1c3 (mouse hepatoma)	AhR	TCDD	11	30	ND	ND
2	DR-CALUX	H4IIE (rat hepatoma)	AhR	TCDD	8	15	PCB 128	3.0×10^{-6}
1	AR-LUC	MDA-kb2 (human breast carcinoma)	(anti-)AR	DHT	52	150	Flutamide	1.0×10^{-6}
3	AR-CALUX	U-2 OS (human osteoblast)	(anti-)AR	DHT	100	100	Flutamide	2.3×10^{-7}
4	AR-LUC	MDA-kb2 (human breast carcinoma)	(anti-)AR	DHT	150	100	Flutamide	5.1×10^{-7}
2	AR-CALUX	U-2 OS (human osteoblast)	(anti-)AR	DHT	NR	164	Flutamide	1.3×10^{-6}
5	AR-CALUX	U-2 OS (human osteoblast)	(anti-)AR	DHT	130	130	Flutamide	1.3×10^{-6}
6	AR-LUC	MDA-kb2 (human breast carcinoma)	(anti-)AR	R1881	ND	500	Flutamide	5.9×10^{-7}
1	ER-LUC	VM7Luc4E2 (human breast carcinoma)	(anti-)ER	17β-E2	3	4	ND	ND
2	ER-CALUX	T47D (human breast cancer)	(anti-)ER	17β-E2	4	6	ICI 182.780	1.4×10^{-10}
6	ER-LUC	VM7 (human breast cancer)	(anti-)ER	17β-E2	ND	100	ICI 182.780	1.0×10^{-8}*

Note: 1, own data; 2, Hamers et al. 2006; 3, Misaki et al. 2015; 4, Aït-Aïssa et al. 2010; 5, Sonneveld et al. 2005; 6, ToxCast; *, EC50 determined after co-exposure with 500nM 17β-E2.

Table S2. Compounds quantified during targeted GC-HRMS analysis of nonpolar amniotic fluid fractions.

	Compound	CAS nr.	Analytical Column (m)	Retention Time (min)	Quantifying Ion (*m/z*)	Confirming Ion 1 (*m/z*)	Confirming Ion 2 (*m/z*)	LOD (ng/mL)	Extraction Blank (ng/mL)	Solvent Blank (ng/mL)	Calibration Curve (R^2)
Furans and Dioxins	2,3,7,8-Tetrachlorodibenzofuran (2,3,7,8-TCDF)	51207-31-9	15	5.95	305.8981	303.90	307.90	0.035	ND	ND	0.999
	2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD)	1746-01-6	15	6.05	321.893	319.90	323.89	0.032	ND	ND	0.980
	1,2,3,7,8-Pentachlorodibenzofuran (1,2,3,7,8-PCDF)	57117-41-6	15	6.78	339.859	341.86	337.86	0.124	ND	ND	0.994
	1,2,3,7,8-Pentachlorodibenzo-p-dioxin (1,2,3,7,8-PCDDF)	40321-76-4	15	7.06	355.8542	353.86	357.85	0.037	ND	ND	0.985
	1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HCDF)	70648-26-9	15	7.86	373.8201	375.82	371.82	0.119	ND	ND	0.964
	1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin (1,2,3,4,7,8-HCDD)	39227-28-6	15	8.12	389.8153	391.81	387.82	0.027	ND	ND	0.955
	1,2,3,4,6,7,8-Heptachlorodibenzofuran (1,2,3,4,6,7,8-HCDF)	67562-39-4	15	8.97	407.7812	409.78	411.78	0.034	ND	ND	0.933
	1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin (1,2,3,4,6,7,8-HCDD)	35822-46-9	15	9.37	423.7766	425.77	427.77	0.028	ND	ND	0.962
	Octachlorodibenzofuran (OCDF)	39001-02-0	15	10.57	443.7392	441.74	445.74	0.027	ND	ND	0.923
	Octachlorodibenzo-p-dioxin (OCDD)	3268-87-9	15	10.61	459.7344	457.74	461.73	0.016	ND	ND	0.952
Bromodiphenyl Ethers (BDEs)	4-Bromodiphenyl ether (BDE 3)*	101-55-3	30	18.91	249.9809	328.00	325.98	0.017	ND	ND	0.931
	2,4,4'-Tribromodiphenyl ether (BDE 28)	41318-75-6	15	5.49	405.8018	407.80	245.97	0.008	ND	ND	0.981
	2,2',4,4'-Tetrabromodiphenyl ether (BDE 47)	5436-43-1	15	6.67	325.8762	485.71	483.71	0.015	ND	ND	0.973
	2,2',4,4',5-Pentabromodiphenyl ether (BDE 99)	60348-60-9	15	8.12	403.7873	405.79	563.62	0.022	ND	ND	0.988
	2,2',4,4',6-Pentabromodiphenyl ether (BDE 100)	189084-64-8	15	7.72	403.7873	405.79	563.62	0.015	ND	ND	0.973
	2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE 153)	68631-49-2	15	9.61	483.6951	481.70	485.69	0.006	ND	ND	0.980
	2,2',4,4',5,6'-Hexabromodiphenyl ether (BDE 154)	207122-15-4	15	9.04	483.6951	481.70	485.69	0.027	ND	ND	0.928
	2,2',3,4,4',5,5'-heptabromodiphenyl ether (BDE 180)	446255-26-1	15	12.02	561.6071	563.60	559.61	0.041	ND	ND	0.995
	2,2',3,4,4',5',6-Heptabromodiphenyl ether (BDE 183)	207122-16-5	15	11.14	561.6065	563.60	559.61	0.011	ND	ND	0.983
	2,2',3,3',4,4',5,5',6,6'-Decabromodiphenyl ether (BDE 209)	1163-19-5	15	22.07	799.3395	797.34	801.34	0.068	ND	ND	0.977

Table S2. *[continued]*

	Compound	CAS nr.	Analytical Column (m)	Retention Time (min)	Quantifying Ion (*m/z*)	Confirming Ion 1 (*m/z*)	Confirming Ion 2 (*m/z*)	LOD (ng/mL)	Extraction Blank (ng/mL)	Solvent Blank (ng/mL)	Calibration Curve (R^2)
Pesticides, Fungicides, and Herbicides	Dichlorodiphenyltrichloroethane (p,p'-DDT)	50-29-3	30	28.04	235.0076	165.07	237.00	0.007	ND	ND	0.972
	2,4'-dichlorodiphenyltrichloroethane (o,p'-DDT)	789-02-6	30	26.97	235.0076	165.07	237.00	0.023	ND	ND	0.983
	Dichlorodiphenyldichloroethylene (p,p'-DDE)	72-55-9	30	25.55	245.9997	248.00	317.93	0.326	ND	ND	0.989
	2,4'-Dichlorodiphenyldichloroethylene (o,p'-DDE)	3424-82-6	30	24.69	245.9997	248.00	317.93	0.026	ND	ND	0.986
	Dichlorodiphenyldichloroethane (p,p'-DDD)	72-54-8	30	26.71	235.0076	165.07	237.00	0.063	ND	ND	0.976
	2,4'-Dichlorodiphenyldichloroethane (o,p'-DDD)	53-19-0	30	25.73	235.0076	165.07	237.00	0.078	ND	ND	0.999
	Mirex	2385-85-5	30	32.61	271.8098	273.81	236.84	0.045	ND	ND	0.988
	Endrin	72-20-8	30	26.23	262.8566	244.95	262.86	0.112	ND	ND	0.977
	Fonofos*	944-22-9	30	20.08	108.9872	137.02	80.96	0.015	ND	ND	0.915
	Heptachlor	76-44-8	30	21.88	271.8098	273.81	236.84	0.063	ND	ND	0.989
Pesticides, Fungicides, and Herbicides	γ-Lindane (γ-HCH)	58-89-9	30	19.89	180.9373	182.93	218.91	0.069	ND	ND	0.995
	α-Lindane (α-HCH)	319-84-6	30	18.85	180.9373	182.93	218.91	0.029	ND	ND	0.984
	β-Lindane (β-HCH)	319-85-7	30	19.70	180.9373	182.93	218.91	0.031	ND	ND	0.993
	δ-Lindane (δ-HCH)	319-86-8	30	20.76	180.9373	182.93	218.91	0.064	ND	ND	0.983
	cis-Chlordane	5103-71-9	30	24.94	372.8256	374.82	376.82	0.060	ND	ND	0.996
	trans-Chlordane	5103-74-2	30	24.60	372.8256	374.82	376.82	0.079	ND	ND	0.998
	trans-Nonachlor	39765-80-5	30	25.03	408.7863	406.79	299.86	0.089	ND	ND	0.994
	Hexachlorobenzene	118-74-1	30	18.95	283.8095	285.81	281.81	0.087	0.021	0.006	0.986
	Phenol, 2,4,5-trichloro-*	95-95-4	30	13.06	195.9242	197.92	96.98	0.027	0.189	ND	0.962

Table S2. *[continued]*

	Compound	CAS nr.	Analytical Column (m)	Retention Time (min)	Quantifying Ion (*m/z*)	Confirming Ion 1 (*m/z*)	Confirming Ion 2 (*m/z*)	LOD (ng/mL)	Extraction Blank (ng/mL)	Solvent Blank (ng/mL)	Calibration Curve (R^2)
Polychlorinated biphenyls(PCBs)	2,2',5-Trichlorobiphenyl (PCB 18)*	37680-65-2	30	20.17	186.0229	255.96	257.96	0.011	ND	ND	0.975
	2,3,5,6-Tetrachlorobiphenyl (PCB 65)	33284-54-7	30	23.02	289.9212	219.98	289.92	0.076	ND	ND	0.971
	3,4,4',5-Tetrachlorobiphenyl (PCB 81)*	70362-50-4	30	25.53	289.9219	219.98	289.92	0.017	0.006	ND	0.938
	2,3,3',4,4'-Pentachlorobiphenyl (PCB 105)*	32598-14-4	30	27.29	325.8799	323.88	327.88	0.002	0.002	ND	0.891
	2,3,4,4',5-Pentachlorobiphenyl (PCB 114)*	74472-37-0	30	26.80	325.8799	323.88	327.88	0.002	0.003	ND	0.903
	2,3',4,4',5-Pentachlorobiphenyl (PCB 118)	31508-00-6	30	26.49	325.8799	323.88	327.88	0.071	ND	ND	0.961
	3,3',4,4',5-Pentachlorobiphenyl (PCB 126)	57465-28-8	30	28.52	327.8771	323.88	327.88	0.053	ND	ND	0.953
	2,2',3,4,4',5'-Hexachlorobiphenyl (PCB 138)*	35065-28-2	30	28.08	325.8604	359.84	361.84	0.014	0.013	ND	0.905
	2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153)	35065-27-1	30	27.15	359.8409	289.9	361.84	0.059	ND	ND	0.975
	2,3,4,4',5,6-Hexachlorobiphenyl (PCB 166)	41411-63-6	30	28.58	359.8409	361.84	357.84	0.019	ND	ND	0.963
	3,3',4,4',5,5'-Hexachlorobiphenyl (PCB 169)	32774-16-6	30	32.10	359.8409	361.84	289.90	0.057	ND	ND	0.955
	2,2',3,4,4',5,5'-Heptachlorobiphenyl (PCB 180)	35065-29-3	30	31.06	323.8643	393.80	395.80	0.101	ND	ND	0.995
	2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl (PCB 206)	40186-72-9	30	36.02	463.7208	461.72	465.72	0.044	ND	ND	0.981
Others	Triphenyl Phosphate (TPP)	115-86-6	30	28.79	325.0625	169.06	326.07	0.062	0.080	ND	0.968
	2-Ethylhexyl diphenyl phosphate (EDP)	1241-94-7	30	29.21	249.0313	251.05	250.04	0.048	0.053	0.378	0.995
	Indeno[1,2,3-cd]pyrene (IPyr)*	193-39-5	30	41.22	276.0934	274.08	277.09	0.009	0.002	ND	0.921
	Benzo[a]pyrene (B(a)P)	50-32-8	30	40.69	252.0934	250.08	253.09	0.070	0.073	0.024	0.990
	Benzo[b]fluoranthene (B(b)F)*	205-99-2	30	40.50	252.0933	250.08	253.09	0.002	0.003	ND	0.945
	Di-n-octyl phthalate (DNOP)*	117-84-0	30	37.8	149.0232	167.03	279.16	0.024	ND	ND	0.998
	Pyrene	129-00-0	30	24.93	202.0776	200.06	203.08	0.039	0.013	0.143	0.904
	Fluorene*	86-73-7	30	20.14	165.0699	166.08	163.05	0.039	0.030	ND	0.972
	Mesitylene*	108-67-8	30	6.05	115.0699	105.07	120.05	0.017	0.020	ND	0.951
	Azobenzene*	103-33-3	30	17.14	152.0620	77.04	182.07	0.041	0.343	ND	0.935

Note: * - targeted analysis of compounds selected from the analysis of 350 environmental chemicals

Table S3. ToxCast/Tox21 high-throughput *in vitro* screening (HTS) assays used during the non-targeted screening of "Identified Unknowns" in the nonpolar amniotic fluid fractions.

Assay name	Source	Gene	Cell name	Assay type	Assay subtype	Active/ Tested	Exposure
TOX21_AhR_LUC_Agonist	TOX21	AhR	HepG2	reporter gene	luciferase induction	804 / 8305	24
TOX21_AhR_LUC_Agonist_viability	TOX21	AhR	HepG2	reporter gene	cytotoxicity, ATP content	863 / 8305	24
OT_AR_ARELUC_AG_1440	Odyssey Thera	AR	CHO-K1	reporter gene	luciferase induction	153 / 1857	24
OT_AR_ARSRC1_0960	Odyssey Thera	AR, SRC	HEK293T	binding	protein fragment complementation	418 / 1857	16
TOX21_AR_BLA_Agonist_ratio	TOX21	AR	HEK293T	reporter gene	beta lactamase induction	614 / 8305	24
TOX21_AR_BLA_Antagonist_ratio	TOX21	AR	HEK293T	reporter gene	beta lactamase induction	1746/8305	24
TOX21_AR_BLA_Antagonist_viability	TOX21	AR	HEK293T	reporter gene	beta lactamase induction	1142/8305	24
TOX21_AR_LUC_MDAKB2_Agonist	TOX21	AR	MDA-kb2	reporter gene	luciferase induction	432/8305	24
TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881	TOX21	AR	MDA-kb2	reporter gene	luciferase induction	1654/7871	24
TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881_viability	TOX21	AR	MDA-kb2	reporter gene	cytotoxicity, ATP content	805/7871	24
TOX21_AR_LUC_MDAKB2_Antagonist_10nM_R1881	TOX21	AR	MDA-kb2	reporter gene	luciferase induction	1094/8305	24
TOX21_AR_LUC_MDAKB2_Antagonist_10nM_R1881_viability	TOX21	AR	MDA-kb2	reporter gene	cytotoxicity, ATP content	904/8305	24
TOX21_AR_LUC_MDAKB2_Agonist_3uM_Nilutamide	TOX21	AR	MDA-kb2	NA	artifact detection	267/7871	24
TOX21_AR_LUC_MDAKB2_Agonist_3uM_Nilutamide_viability	TOX21	AR	MDA-kb2	NA	cytotoxicity, ATP content	857/7871	24
OT_ER_ERaERa_1440	Odyssey Thera	ESR1	HEK293T	binding	protein fragment complementation	171/1857	24
OT_ER_ERaERb_1440	Odyssey Thera	ESR1 ESR2	HEK293T	binding	protein fragment complementation	330/1857	24
OT_ER_ERbERb_1440	Odyssey Thera	ESR2	HEK293T	binding	protein fragment complementation	255/1857	24
TOX21_ERa_BLA_Agonist_ratio	TOX21	ESR1	HEK293T	reporter gene	beta lactamase induction	503/8305	24
TOX21_ERa_BLA_Antagonist_ratio	TOX21	ESR1	HEK293T	reporter gene	beta lactamase induction	1244/8305	24
TOX21_ERa_BLA_Antagonist_viability	TOX21	ESR2	HEK293T	reporter gene	cytotoxicity, ATP content	373 / 8305	24
TOX21_ERb_BLA_Agonist_ratio	TOX21	ESR2	HEK293T	reporter gene	beta lactamase induction	168 / 7871	24
TOX21_ERb_BLA_Agonist_viability	TOX21	ESR2	HEK293T	reporter gene	cytotoxicity, ATP content	1334 / 7871	24
TOX21_ERb_BLA_Antagonist_ratio	TOX21	ESR2	HEK293T	reporter gene	beta lactamase induction	1689 / 7871	24
TOX21_ERb_BLA_Antagonist_viability	TOX21	ESR2	HEK293T	reporter gene	cytotoxicity, ATP content	1300 / 7871	24
TOX21_ERA_LUC_VM7_Agonist	TOX21	ESR1	VM7	reporter gene	luciferase induction	1324 / 8305	22
TOX21_ERA_LUC_VM7_Antagonist_0.1nM_E2	TOX21	ESR1	VM7	reporter gene	luciferase induction	1162 / 7871	48
TOX21_ERA_LUC_VM7_Antagonist_0.1nM_E2_viability	TOX21	ESR1	VM7	reporter gene	cytotoxicity, ATP content	685 / 7871	48
TOX21_ERA_LUC_VM7_Antagonist _0.5nM_E2	TOX21	ESR1	VM7	reporter gene	luciferase induction	986 / 8305	22
TOX21_ERA_LUC_VM7_Antagonist _0.5nM_E2_viability	TOX21	ESR1	VM7	reporter gene	cytotoxicity, ATP content	769 / 8305	22
TOX21_ERA_LUC_VM7_Agonist_10nM_ICI182780	TOX21	ESR1	VM7	NA	artifact detection	168 / 7871	22
TOX21_ERA_LUC_VM7_Agonist_10nM_ICI182780_viability	TOX21	ESR1	VM7	NA	cytotoxicity, ATP content	813 / 7871	22

Note: *, AhR = aryl hydrocarbon receptor; AR = androgen receptor; SRC = tyrosine-protein kinase Src; ER = estrogen receptor; ESR1 = estrogen receptor 1; ESR2 = estrogen receptor 2;

Table S4. QSARs and profiling tools used for the *in silico* evaluation of ER, AR and DR (AhR) activity of the Identified Unknowns uncovered through the non-targeted screening of the nonpolar AF fractions.

OECD QSAR Toolbox (Version 4.3)
1. *Estrogen receptor (ER) binding*: ER binding profiling scheme was developed to predict ER binding using structural and parametric data extracted from literature and supported by experimental data. Chemicals are categorized as very strong, strong, moderate, weak and non-binders based on their molecular weight (MW) and structural characteristics (OECD QSAR Toolbox, https://www.qsartoolbox.org).
OPERA (Version 2.5)
1. CERAPP (Collaborative Estrogen Receptor Activity Prediction Project): A large scale modelling project developed in collaboration with 17 groups in the United States and Europe. CERAPP combines multiple models trained on a set of 1,677 chemical structures from high-throughput screening data provided by US EPA. CERAPP is applied to evaluated chemicals for binding, agonist, and antagonist ER activity (Mansouri et al. 2016). *2. CoMPARA (Collaborative Modelling Project for Androgen Receptor Activity):* A large scale modelling project developed in collaboration with 25 international groups that contributed 91 predictive models for binding, agonist, and antagonist AR activity. CoMPARA models were trained on a set of 1,746 from 11 ToxCast™/Tox21 high-throughput in vitro screening assays, and have an averaged predictive accuracy of approx. 80% (Mansouri et al. 2020).
Danish (Q)SAR database (2015)
1. *ER alpha binding (human in vitro):* Combines a battery of three models: MultiCASE CASE Ultra model for binding to the human Estrogen Receptor alpha (hERalpha) in vitro; Leadscope Enterprise model for binding to the hERalpha in vitro, and SciMatics SciQSAR model for binding to the hERalpha in vitro. All three models used training data set consisted of 595 compounds (284 positives and 311 negatives) tested for ER binding in a cell-free assay containing the hERalpha. 2. *ER alpha activation (human in vitro):* Combines a battery of three models: MultiCASE CASE Ultra model for activation of the human Estrogen Receptor alpha (hERalpha) in vitro; Leadscope Enterprise model for activation of the hERalpha in vitro; and SciMatics SciQSAR model for activation of the hERalpha in vitro. All three models used training data set consisted of 481 compounds (195 positives and 286 negatives) tested for their ER activity in MCF-7 cells. 3. *Androgen receptor (AR) antagonism (human in vitro)*: Combines a battery of three models: MultiCASE CASE Ultra model for Androgen Receptor (AR) antagonism (human vector) in vitro; Leadscope Enterprise model for AR antagonism (human vector) in vitro, and SciMatics SciQSAR model for AR antagonism (human vector) in vitro. All three models used training data set consisted of 874 compounds (231 positives and 643 negatives) tested for their AR antagonism of human androgen receptor in Chinese Hamster Ovary cells. 4. *Arylhydrocarbon (AhR) activation (human in vitro):* Two models are available within Danish QSAR database for prediction of AhR activation: rational model trained with data on 832 actives and 3,328 inactives and a random model with a proportion of inactives chosen entirely by random. Both models show good statistical performance, with the rational model having external validation sensitivity of 85.1% and specificity of 97.1%, compared to the random model with sensitivity 89.1% and specificity 91.3% (Klimenko et al. 2019).

Table S5. Experimental data retrieved for the Identified Unknowns. EC50 values for (anti-)estrogenic and (anti-)androgenic activity measured in high-throughput ToxCast/Tox21 bioassays, retrieved from EPA Dashboard. The % estrogen binding affinity (ERBA) and androgen binding affinity (ARBA) retrieved from OECD QSAR Toolbox.

	ToxCast/Tox21				OECD QSAR	
	ER[a] Agonists	ER[a] Antagonist	AR[b] Agonists	AR[b] Antagonist	Relative ERBA	Relative ARBA
Compound	EC50 (M)	AC50 (M)	EC50 (M)	AC50 (M)	%	%
Allethrin	_	7.1×10^{-5}	-	6×10^{-5}	-	ND
Isopentyl benzoate	4.9×10^{-5}	-	-	-	-	ND
Heptadecane	4.1×10^{-5}	-	-	-	ND	ND
Methyl oleate	4.6×10^{-5}	-	-	-	ND	ND
Diphenyl isophthalate	1.4×10^{-5}	-	-	-	-	ND
Squalene	-	-	-	-	-	-
Pentyl valerate	-	-	-	-	ND	ND
Benzoin isobutyl ether	ND	ND	ND	ND	0.000995	0.00209

Note: a, TOX21_ERa_LUC_VM7 assay; b, TOX21_AR_LUC_MDAKB2 assay; -, no activity; ND, not determined

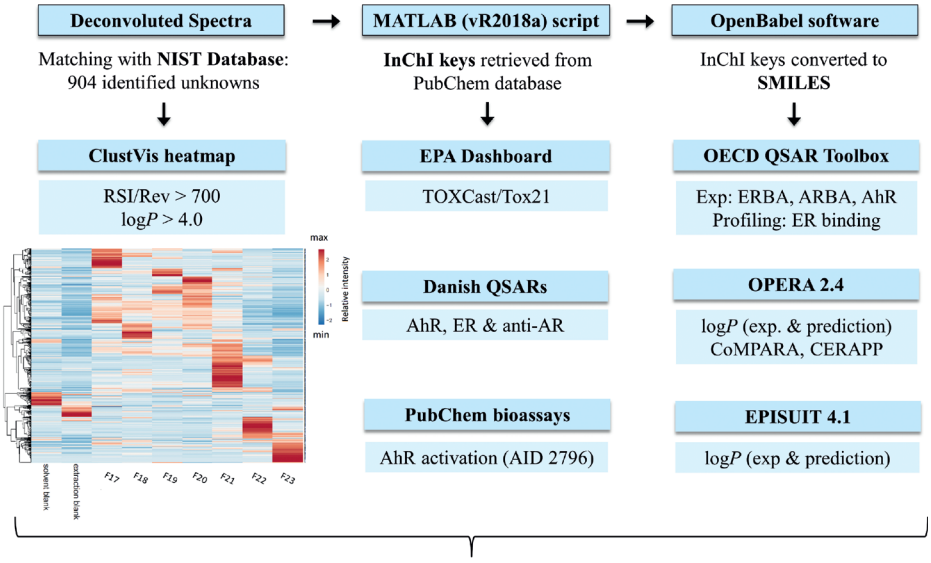


Figure S1. Strategy for filtering and prioritization of novel EDCs from the unknown compounds identified through the non-targeted GS-HRMS analysis.

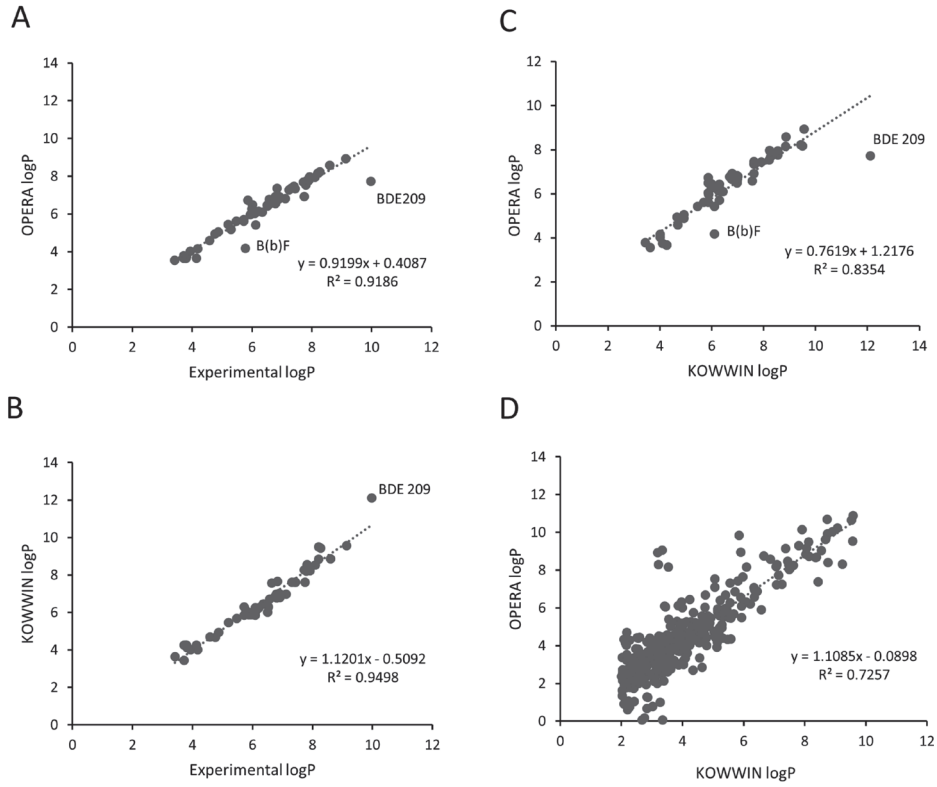
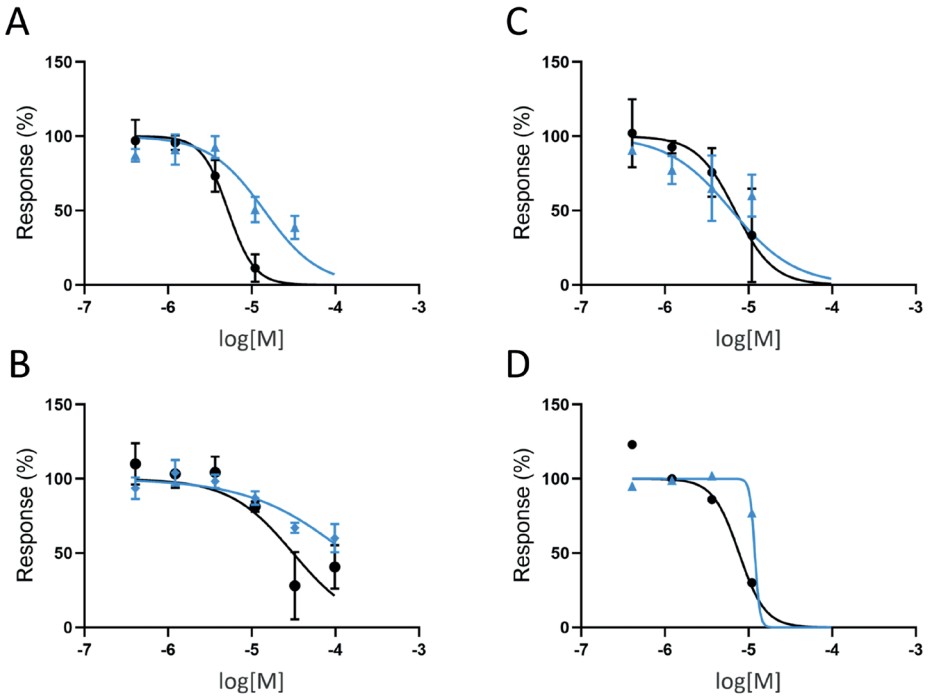


Figure S2. Correlation between experimental and OPERA (A), experimental and KOWWIN (B), and OPERA and KOWWIN (C) predicted logP values for the compounds measured during the targeted analysis of the nonpolar fractions of amniotic fluid, and a correlation between OPERA and KOWWIN log*P* values predicted for a subset of Identified Unknowns (D).



	EC50 (M)	
	AR-antagonism	AR-cytotoxicity
BMD	5.2×10^{-6}	1.4×10^{-5}
NABD	6.9×10^{-6}	6.3×10^{-6}
BFPT	3.1×10^{-5}	1.4×10^{-4}
Allethrin	7.7×10^{-6}	1.2×10^{-5}

Figure S3. Comparison of AR-antagonistic activity (black circles) and cytotoxicity (blue triangles) of 1H-benzimidazole, 1,2-diphenyl- (BMD), N,N'-di-acridin-9-yl-benzene-1,4-diamine (NABD), 1-(4-fluorobenzyl)-2-p-tolyloxymethyl (BFPT) and allethrin, measured with AR-Luc assay and MTT assay, respectively, as described in the method section. Half maximal effective concentration (EC50) is given in molar units (M), n=3 except for allethrin, n=1.

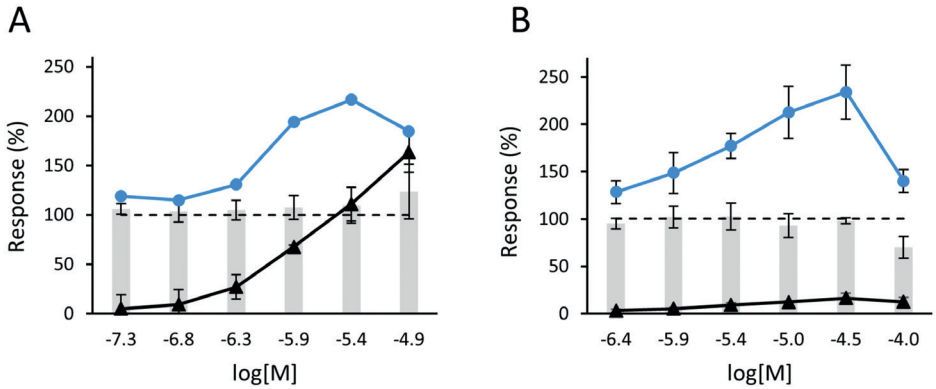


Figure S4. Diphenyl isophthalate agonistic (black triangles), antagonistic (blue circles) and cytotoxic activity (grey bars) measured in ER-Luc (A) and AR-Luc (B) reporter gene assays. The % of the agonistic response was measured as relative to the maximum induction by positive control E2 (A, n=3) or DHT (B, n=3). The antagonistic activity was measured with co-exposure to E2 (4 pM, n=1) and DHT (150 pM, n=3) and the decrease in luminescence was expressed as relative to the respective positive control. Cytotoxicity was measured with MTT assay, n=3.

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PART III

MICRO- AND NANOPLASTICS:

CONTAMINANTS OF EMERGING CONCERN FOR EARLY LIFE HEALTH

4

Experimental human placental models for studying uptake, transport and toxicity of micro- and nanoplastics

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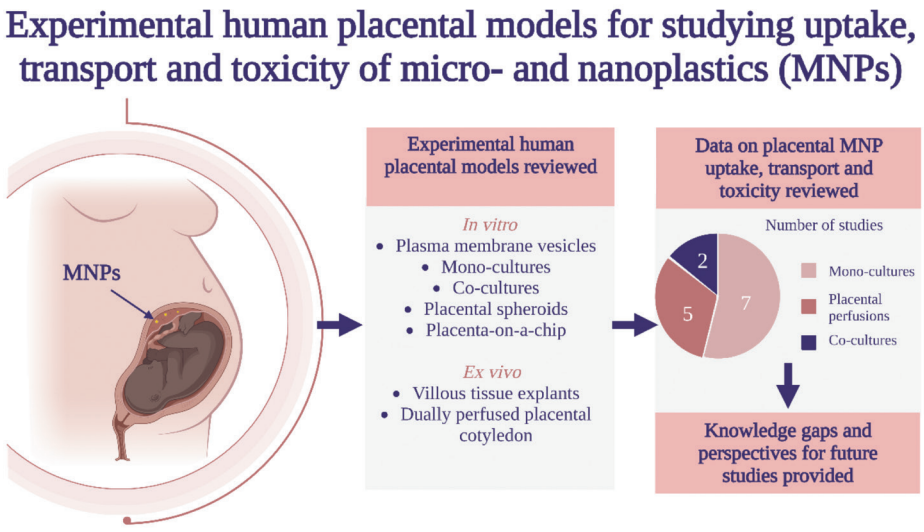
Science of The Total Environment (Accepted)

Abstract

Micro- and nanoplastics (MNPs) are ubiquitous in the environment and have recently been found in human lungs, blood and placenta. However, data on the possible effects of MNPs on human health is extremely scarce. The potential toxicity of MNPs during pregnancy, a period of increased susceptibility to environmental insults, is of particular concern. The placenta provides a unique interface between maternal and fetal circulation which is essential for *in utero* survival and healthy pregnancy. Placental toxicokinetics and toxicity of MNPs are still largely unexplored. Practical and ethical considerations limit research options in humans, and extrapolation from animal studies is challenging due to marked differences between species. Nevertheless, diverse *in vitro* and *ex vivo* human placental models exist e.g., plasma membrane vesicles, mono-culture and co-culture of placental cells, placenta-on-a-chip, villous tissue explants, and placental perfusion that can be used to advance this research area. The objective of this concise review is to recapitulate different human placental models, summarize the current understanding of placental uptake, transport and toxicity of MNPs and define knowledge gaps. Moreover, we provide perspectives for future research urgently needed to assess the potential hazards and risks of MNP exposure to maternal and fetal health.



Graphical abstract

Abbreviations

ABC = ATP binding cassette
ABCB1 = P-glycoprotein
ABCG2 = Breast cancer resistance protein
CdTe-COOH = Carboxylic acid functionalized CdTe quantum dots
CRH = Corticotrophin-releasing hormone
CTs = Cytotrophoblasts
CuO = Copper oxide
DDT = Dichlorodiphenyltrichloroethane
ECM = Extracellular matrix
ER = Estrogen receptor
EVCTB = Extravillous cytotrophoblast
EVTs = Extravillous trophoblasts
Fluo-PS = Fluorescent polystyrene
GPMVs = Giant plasma membrane vesicles
hCG = Human chorionic gonadotropin
hPL = Human placental lactogen
HPVEC = Human placental vascular endothelial cells
HUVEC = Human umbilical vein endothelial cells
IL = Interleukin
MNPs = Micro- and nanoplastics
MPs = Microplastics
NPs = Nanoplastics
PAH = Polycyclic aromatic hydrocarbons
PCB = Polychlorinated biphenyls
PGH = Placental growth hormone
PL = Placental lactogen
PR = Progesterone receptor
PS = Polystyrene
ROS = Reactive oxygen species
ST = Syncytiotrophoblast
TiO_2 = Titanium dioxide
TLR = Toll-like receptor
VCTs = Villous cytotrophoblasts

Introduction

The environment is widely polluted with microplastics (MPs, <5 mm) and nanoplastics (NPs, <1 µm), collectively called micro- and nanoplastics (MNPs) [1,2]. MNPs are synthetic polymers that are formed by weathering, mechanical abrasion, and photodegradation of plastic products and waste [3,4]. MNPs are also intentionally produced for various purposes such as drug delivery systems and cosmetics (e.g., sunscreens) [4,5]. Thus, environmental exposures are typically to a mixture of MNPs of various sizes, polymer types and chemical compositions. Exposure to MNPs in humans occurs mainly through ingestion and inhalation [3,6]. Recently, MNPs have been found in lung tissue and blood confirming internal human exposure [7–9] and raising concerns about their effects on human health [10]. Of special concern is the potential toxicity of MNPs during pregnancy. The fetus is highly susceptible to toxicity due to intense and strictly regulated cell proliferation, apoptosis, cell differentiation and cell migration during organogenesis. The so-called developmental windows of susceptibility define periods of heightened vulnerability to environmental toxicants, during which disturbance of pre- or postnatal development could contribute to adult-onset disease [11,12]. In the case of MNPs, direct developmental toxicity may arise from particles in maternal blood that cross the placental barrier and damage fetal tissues [13]. However, developmental toxicity may also arise from the damage to the placenta itself, which may affect not only fetal but also maternal health during pregnancy.

The placenta is a multifaceted temporary organ at the interface of maternal and fetal circulation, crucial for fetal growth and development. Contrary to the old belief that the placenta forms an impermeable layer that protects the fetus from harmful substances present in maternal blood, it is now known that a large variety of environmental pollutants with diverse molecular structures can cross the placenta. Transplacental transport of licit and illicit drugs, xenobiotics, as well as engineered metallic and carbonaceous nanoparticles is now well documented [14–17]. More recently, (ultra)fine particles from air pollution have been detected in placental tissue, notably ambient black carbon particles [18] and nanoparticles from diesel exhausts [19]. Carbon and metal-bearing nanoparticles were also recently found in placental phagocytes [20]. Epidemiological data indicate that exposure to (ultra)fine particles during pregnancy is associated with various complications such as pre-eclampsia [21,22], pre-term birth [23], low birth weight [24] and stillbirth [25]. They may also predispose the fetus to malformations such as congenital heart defects [26] and diseases later in life such as respiratory diseases [27]. Recently, MNPs have been detected for the first time in human placental samples and meconium [28,29]. This demonstrates that environmental (ultra)fine particles including MNPs reach placental cells and that a human fetus may be exposed *in utero*. Considering the central role of the placenta in fetal and maternal wellbeing during pregnancy, there is an urgent need to understand the potential placental toxicokinetics and toxicity of MNPs.

Due to practical and ethical considerations, *in vivo* studies with rodents are the most commonly used to study placental function and development. So far, only a few studies have investigated the transplacental transport of MNPs. In vivo rodent studied have shown that MNPs can cross the placental barrier and accumulate in the internal organs of the offspring [17,30–32]. It is hypothesized that consequent toxicity, such as metabolic disturbance, alterations of behaviour, neurotoxicity and increased mortality, are mediated through changes in gene expression, oxidative stress, inflammation and cell death [33]. The placenta is, however, the most species-specific organ with outstanding evolutionary diversity. The functional (e.g., expression of transporter proteins), as well as anatomical (e.g., the number of trophoblast cell layers) differences between species are important factors determining placental permeability to nutrients, chemicals and particles, making extrapolation from rodent models to humans challenging [34–36]. The use of models relevant to humans, derived from human placental tissue, such as primary cells or cell lines of placental origin, are therefore considered superior to using other mammalian models when analysing various aspects of placental biology and toxicity, including uptake and toxicity of MNPs. So far, a limited number of studies employing human *in vitro* placental cell models and *ex vivo* placental perfusion have investigated transplacental transport of MNPs, and MNP toxicity and the mechanisms underlying toxicity have rarely been addressed. The objective of this concise review is to summarize the current state of the science of placental MNP research, with a focus on the available *in vitro* and *ex vivo* placental models relevant to humans. First, we summarize the development, physiology and morphology of the human placenta. Next, we critically evaluate different models of the human placenta and recapitulate the current understanding of placental toxicokinetics and toxicodynamics of MNPs. Lastly, we highlight areas that require further scrutiny including potential toxicity endpoints and key components to consider when investigating the placental MNP exposure.

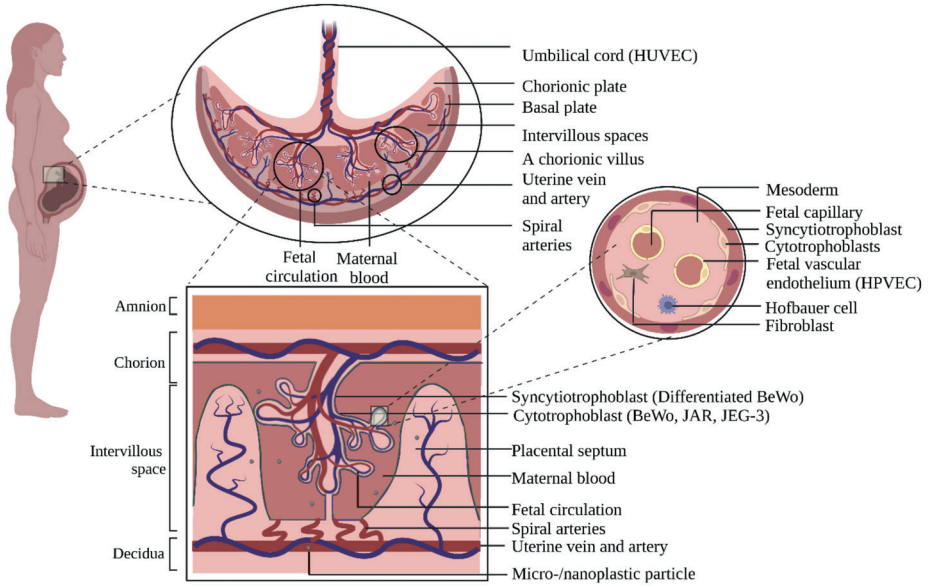


Figure 1. Schematic overview of the morphology of the human placenta and chorionic villi. The different *in vitro* cell models representative of various placental cell types are given in brackets. This figure was created using BioRender.com

Human placenta

Placental development and physiology

The placenta is the first organ that forms during human embryogenesis. It develops from the trophectoderm (i.e., early trophoblast cells surrounding the blastocyst) in the first weeks of pregnancy [37]. Human placentation differs from that of other mammals and is orchestrated by highly regulated temporal and spatial interactions between extraembryonic mesenchymal cells and trophoblastic cells [38]. The trophoblastic cells engage in diverse functions throughout gestation, ranging from attachment, invasion, and vascular remodelling to hormone production and nutrient transport. Implantation and placental development have been extensively described elsewhere [39–43]. Trophoblastic cell lineage has two distinctive cell populations, the undifferentiated cytotrophoblasts (CTs) and the fully differentiated syncytiotrophoblast (ST). The undifferentiated CTs either differentiate into villous cytotrophoblasts (VCTs) and further fuse to form multinucleated ST (with a lost capacity to divide) or gain a migratory phenotype to become extravillous trophoblasts (EVTs). Molecular details of the regulation of the differentiation processes are very complex and still being studied [40]. At two weeks after implantation, EVTs begin an aggressive invasion into maternal stroma and remodelling of the resident blood vessels, a process unique to human placentation [44]. At the same time, VCTs start to form primary chorionic villi and proteolytic enzymes continue to break down the extracellular

matrix (ECM) to generate intervillous space. By approximately 8 weeks postconception, the blueprint of the placenta is established and maternal blood starts to flow through the placental villi [40,45]. The placenta and placental villi continue to change and grow to ensure adequate nutritional supply throughout the developmental stages of the fetus [40].

At full-term, the human placenta is a large discoid organ that is 2-2.5 cm thick, approximately 15-20 cm in diameter and weighs up to 600 grams [46,47]. On one side, there is a chorionic plate with an umbilical cord that connects to the fetus. On the other side, there is a basal plate that is attached to the maternal endometrium (Figure 1). Villous trees emerging from the chorionic plate begin with stem villi that branch into 3-5 intermediate villi and then further into 10-12 terminal villi (<20 μm diameter), richly vascularized by a fetal capillary network [48,49]. These villous trees float in an intervillous space filled with maternal blood and are separated laterally by incomplete placental septa. At full-term, maternal blood (about 30% of the maternal cardiac output) from remodelled spiral arteries flows through the intervillous space at a flow rate of 600-700 mL/min, bathing villous trees with a total surface area of 12-14 m^2 [14,50,51]. It is here, at the cellular barrier of the villous tree, that the exchange of nutrients, gases and waste products between maternal and fetal blood takes place, and where the exogenous particles such as MNPs present in maternal blood can interact with the placental trophoblastic structures.

Placental barrier

The term “placental barrier” refers to the cell layers that separate maternal blood in the intervillous space from the fetal capillaries in the core of the chorionic villi (Figure 1) and harbour important molecular structures supporting placental functions, such as specialized transporter proteins. The cell layer in direct contact with maternal blood is a layer of ST with multiple nondividing nuclei and without lateral cell membranes forming thus one continuous layer [51–53]. The plasma membrane of the ST is highly polarized and consists of two horizontal membranes: the brush border membrane facing maternal blood and the basal membrane, which can be distinguished by their protein, receptor, and transporter compositions [54]. In the villous stroma, under the ST, are some remaining CTs that retain the capacity to divide, and by cellular fusion give rise to ST (Figure 1). Behind ST and CTs lies the connective tissue containing fibroblasts and Hofbauer cells, the placental macrophages [14,53,55]. The last layer of the placental barrier in the chorionic villi is the endothelium of the fetal capillaries.

This placental barrier in chorionic villi undergoes dynamic morphological changes during pregnancy that may influence placental MNP toxicokinetics. The ST layer becomes thinner, fetal capillaries move towards the periphery of the villi, CTs become flat rather than cubic, and their relative cell mass in relation to ST diminishes [56]. The average thickness of the barrier in the first trimester is approximately 20-30 μm and by the end of the

third trimester, the thickness is reduced considerably to as little as 5 µm, to allow increased exchange between the mother and the fetus [39,52,57,58].

Placental functions

One of the most important placental functions is the transport of nutrients and solutes from maternal to fetal circulation, through the placental barrier. Various mechanisms of transplacental transport exist including passive diffusion, active transport, pinocytosis and phagocytosis [14]. Most compounds with low molecular mass (<500 Da) simply diffuse across the placental tissue through the lipid membrane or protein channels, driven mainly by a concentration gradient. There are, however, some exceptions, such as immunoglobulin G (IgG, 160 Da) which is transported mainly via pinocytosis [59]. IgG is the most common antibody in the human body and its transplacental transfer takes place through binding to a specialized neonatal Fc receptor (FcRn). The FcRn-mediated transcytosis shuttles IgG in both directions across the ST layer [60]. Pinocytic extracellular vesicles of various sizes (macrovesicles, microvesicles and exosomes) that carry protein, lipids, DNA, and RNA, and originating from ST, provide a means of feto-maternal communication and transmission of macromolecules between cells. Trophoblastic cells are also active phagocytes. They are involved in histotrophic phagocytosis during tissue remodelling throughout implantation and placentation, but also participate in innate immune defence by removing bacteria or other components of the ECM during inflammation [61–63]. Active placental transport occurs against a concentration gradient and requires protein membrane carriers. The major influx and efflux transporter families in the human placenta are the solute carrier (SLC) and ATP-binding cassette (ABC) transporters [64]. There are over 30 types of specialized transporter proteins present in placental cells, with varying expressions depending on the stage of pregnancy [15,54]. The ABC efflux transporters, especially the p-glycoprotein (ABCB1) and the breast cancer resistance protein (BCRP or ABCG2) are abundant in the brush border of syncytiotrophoblast facing maternal blood and are important in protecting the fetus from foreign and potentially teratogenic compounds [15]. In addition, the human placenta contains enzymes for xenobiotic metabolism yielding metabolites with an altered capacity to be transported [65]. Overall, the blood flow, surface area, membrane thickness and protein binding are important parameters influencing the uptake and transport of exogenous compounds, and possibly MNPs, at the placental barrier.

Besides transport, the placenta has many other functions including biosynthesis and metabolism of cholesterol, steroid hormones and growth factors essential for the maintenance of pregnancy and fetal development [51,66]. The communication between the mother, placenta and fetus is largely regulated by the placenta through the effects of placental hormones liberated to both maternal and fetal blood, and the exchange of other important molecules between the circulations [40,67]. The numerous bioactive molecules, such as chorionic gonadotropin and placental lactogen, are mainly secreted by the ST

[47,67,68]. Each placental hormone is tightly regulated in space and time by several factors, including other placental hormones. This delicate hormonal interplay is important for a wide variety of processes that are vital for the establishment and maintenance of pregnancy such as implantation, invasion of trophoblast, placental angiogenesis, hormone synthesis, maternal and fetal metabolic regulation, fetal growth and myometrium contractility, among others (for a comprehensive review see Costa 2016) [67]. The placenta is also an active immunological organ [69,70]. Specifically, the interplay between the ST and maternal immune cells actively inhibits infections and protects the fetus from rejection by the maternal immune system [71]. Also, maternal long-chain fatty acids, by inducing cell proliferation, differentiation and angiogenesis, play a crucial role in placental and fetal growth and development [72]. Special placental proteins exist to ensure transcellular and transplacental transfer of fatty acids, including transport proteins in the cell wall (FAT, FATP, p-FABPpm, & FFARs), and the intracellular fatty acid-binding protein FABP. Disruption of these diverse placental functions has been implicated in various adverse pregnancy outcomes such as placental insufficiency, intrauterine growth restriction, preterm birth, pre-eclampsia and loss of pregnancy. However, all in all, placental functions during various stages of pregnancy remain poorly understood and studied [40]. Very little is known about the placental toxicity of environmental factors, such as MNPs, and their potential impact on maternal and fetal health during pregnancy. Nevertheless, diverse *in vitro* and *ex vivo* human placental models exist and can be used to advance this research area.

Human placental models

Various *in vitro* and *ex vivo* models of the human placenta exist including mono-cultures, co-cultures, 3D models, placenta-on-a-chip, explant cultures and *ex vivo* human placental perfusion models, reflecting different levels of placental organisation and complexity (Figure 2). These models have been successfully used to investigate diverse aspects of placental biology, such as xenobiotic metabolism, steroid metabolism and transfer of drugs, endogenous and exogenous compounds and nanoparticles. The main characteristics of each model are presented in Table 1 and summarized below, moving from less to more complex models based on their biological organisation. The use of these models in MNP research is discussed in the later sections.

Plasma membrane vesicles

Isolated plasma membrane vesicles from the human placenta have been used to characterize the expression and function of membrane transporter proteins, and to study transport mechanisms of substrates across individual plasma membranes, either the maternal-facing microvillous brush border membrane or the fetal-facing basal membrane [73,74]. Various inhibitors of specialized membrane transporter proteins can be used to study

specific transport mechanisms in the apical and basal membranes separately [75]. Nevertheless, the purity of the isolated membrane vesicles has to be considered, as cross-contamination of the contralateral membrane can occur. So far, the microvillous membrane vesicles have not been used in MNP research. Although such cell-free *in vitro* models may lack crucial constituents important for particle uptake [74]. Recently giant plasma membrane vesicles (GPMVs, size range in the lower µm) isolated from various cell lines (HepG2, Caco-2 and HeLa) have been suggested to represent a useful substitute for a cellular model [76]. Carboxylated polystyrene (PS) particles of 40 and 100 nm were efficiently transferred to GPMVs [76]. Nevertheless, isolated microvillous membrane vesicles are relatively small (about 0.15 µm in diameter) and the size range of MNPs that can be studied using this method is rather limited.

Placental mono-cultures

From all the different placental cell types, the trophoblast is probably the most studied. Different cell lines were developed to study trophoblast functions including gestational choriocarcinoma, immortalized trophoblasts and CT-derived stem cells, however, their use in MNP research so far has been limited. The most commonly used placental cell lines are described in Table 1. Three malignant human choriocarcinoma cell lines, BeWo, JEG-3 and JAR, are commonly used *in vitro* models to investigate diverse aspects of trophoblast function [77]. The BeWo and JEG-3 cell lines have been used to study endocrine function since they produce multiple hormones e.g., human chorionic gonadotropin (hCG), progesterone, and androgens [78,79]. However, multiple genes are differentially expressed between BeWo and JEG-3 suggesting that they may vary in their function [77]. While the JAR cells also produce hormones, they are not frequently used to study endocrine functions. The most frequently used cell model for placental transport studies is the BeWo b30 which is a subclone of the BeWo cell line. This cell line forms a confluent monolayer on a permeable membrane and develops polarized membranes with different expression of growth factors and transporter proteins in apical and basal membranes [78]. Although BeWo is a carcinoma cell line and there are marked differences in solute carriers and transporter proteins between BeWo cells and trophoblasts *in situ,* transport studies with BeWo b30 cells are usually in agreement with the *ex vivo* results [80,81]. Another trophoblastic cell line, the 3A-sub-E cell line, has been used to study chemokine receptors and promoter activity, such as the ABCB1 promoter which encodes the placental efflux transporter P-glycoprotein [82,83].

Freshly isolated primary trophoblasts are less frequently used than choriocarcinoma cell lines since they are difficult to isolate and culture, have a short lifespan and are often contaminated by different cell types [84]. Moreover, primary CTs can spontaneously differentiate into functional syncytium (followed by a loss of epithelial cell-cell junctions) and therefore a successful establishment of a confluent monolayer from primary CTs is chal-

lenging [78,85]. Nevertheless, the use of highly viable purified CTs, optimized culture conditions and semipermeable inserts can aid the formation of a tight monolayer [86,87]. Two commonly used cell lines derived from primary cells are ACH-3P and HRT-8/Svneo (Table 1). ACH-3P cells may be applied to study autocrine and paracrine regulation of trophoblast formation [88–90], while HRT-8/Svneo cells may be used to study e.g., extravillous cytotrophoblast (EVCTB) invasion and proliferation. More recently, trophoblast stem cells were derived from human villous CTs and human blastocysts [91]. These long-term stem cell cultures may give rise to CTs, EVT and ST-like cells that show transcriptomes similar to those of the corresponding primary trophoblasts, providing a new powerful model to study human trophoblast development and function.

The two most common vascular endothelial cell lines applied in placental research are human umbilical vein endothelial cells (HUVEC) and human placental vascular endothelial cells (HPVEC) [92,93] (Table 1). HUVECs represent macrovascular endothelial cells while HPVECs represent microvascular endothelial cells involved in the formation of the placental vascular network, and thus marked phenotypic and physiologic differences exist between these cell lines [93]. The HPEC-A2 cell line is another SV-40-transformed placental venous endothelial cell line, however, it has limited commercial availability and thus is not commonly used in placental research.

Placental mono-cultures allow for low-cost and high-throughput investigation of different aspects of placental biology under controlled and simplified conditions. They can be applied to study transplacental transport (e.g., using the Transwell ® system) or to investigate other functional endpoints (using a standard well plate format). However, as they lack the physiological complexity of the multi-layered cell structures of placental villi (e.g., trophoblasts, mesenchymal, Hofbauer cells and fibroblasts) they do not account for the complex placental cell-cell interactions.

Placental co-cultures

Co-culture models may improve the physiological relevance of *in vitro* models by mimicking the human anatomical architecture of placental tissue and by facilitating cell-cell interactions [94]. Two different co-culture model types are used, namely direct co-culture and indirect co-culture [95]. In direct co-culture, distinct cell types are in physical contact with each other which allows direct communication between them. In the indirect co-culture model, the distinct cell types are physically separated from each other, and the communication between them is facilitated through a culture medium (Figure 2). Co-culture models may show altered protein expression and hormone secretion in comparison with mono-cultures. For example, co-culture of BeWo or JEG-3 cells with the human adrenocortical cell line H295R resulted in higher secretion of progesterone, estradiol, estriol and estrone in comparison with H295R, JEG-3 or BeWo cells cultured alone [77,96]. Both direct and indirect co-culture models may also be applied in a Transwell ® system to determine

the transplacental transport of compounds or particles over multiple cell barriers. However, in the co-culture models, the distinct cell types often require different media and supplements to ensure optimal culture conditions. Thus, finding the right medium composition is often challenging. So far co-culture models have rarely been applied to MNP research.

Placental spheroids

Placental spheroids are more comparable to the placenta *in situ* than mono-, or co-cultures since spheroids have multiple cell layers and recreate a 'natural-like'-microenvironment for the cells [97,98]. The cell-cell interactions in spheroids induce changes in cellular morphology and functions, showing continuous proliferation, differentiation, and changes in gene and protein expression which are more representative of the placenta *in situ* [99]. Placental spheroids can be formed using transfected cell lines, primary or progenitor cells, and by combining different cell types e.g., endothelial precursor cells, cytotrophoblasts and villous stromal cells [100,101]. For example, Turco et al. (2018) established trophoblast organoids capable of differentiating to ST and EVTs, that formed complex structures which anatomically and functionally resembled placental villi [102]. Spheroids can be formed using round-bottom nonadherent well plates or the hanging drop technique [103]. Both techniques can be used in 96-well plates, which allows for high throughput screening [104]. Depending on the cell types used to form spheroids, this model can be used to study cell adhesion, proliferation, placental vascular development and trophoblast invasion [98,100]. Nevertheless, using spheroids is not without challenges. For instance, it is difficult to exchange culture medium, prevent necrotic damage and control the size and exact composition of the cellular components of spheroids [101].

Placenta-on-a-chip

The emergence of the 'placenta-on-a-chip' model, combining microfluidics and diverse human placental cells in 3D structures, provides a novel promising tool that exhibits organotypic features of the placenta [105]. This model has been already used to study placental translocation, inflammatory responses and complex cellular interactions [106–110]. In the placenta *in situ*, the ECM is an important component of the tissue microenvironment since it regulates cellular adhesion, migration and invasion [111,112]. Therefore, the use of an ECM membrane can be physiologically relevant for creating the placental barrier *in vitro* [106]. Matrigel®, fibronectin or gelatine, have been used to represent the ECM in the placenta-on-a-chip model [108,113–115]. On top of the ECM diverse placental cell types can be grown. Human villous mesenchymal fibroblasts have been used in a combination with the BeWo b30 cells to study the translocation of PS particles across the placental barrier [116]. Other cell lines can be included in this model, such as endothelial or immune cells, creating co-culture microtissues and further reducing the gap between *in vitro* and

in vivo conditions [117]. However, similar to co-cultures, the different cell types in the placenta-on-a-chip model may require a specific medium/microenvironment [105]. Furthermore, these models are labour-intensive since it is essential to continuously monitor cell-cell communication and their microenvironment for secreted metabolites or hormones, and, cannot be used for high throughput screening. Nevertheless, the potential of the placenta-on-a-chip model has already been demonstrated in the fields of reproductive biology and toxicology (e.g., assessment of engineered nanoparticles for drug delivery), reviewed recently by Young and Huh (2021) and Shojaei et al. (2021).

Villous tissue explants

Placental villous tissue explants from term placentas are most commonly used, although explants from the first trimester (from elective abortions) have also been utilised [119]. Villous explants from term placentas have been used to evaluate villous metabolism, endocrine function and transport of amino acids and other small molecules [120–122], whereas early placental tissue explants have been used to study placentation, invasion of EVTs, trophoblast proliferation and differentiation [123,124]. Villous explants from both early and term placenta have been used to study the effects of xenobiotics on transporter expression [125] and endoplasmic reticulum (ER) stress [126]. These models maintain the topology of chorionic villi (i.e., contain microvillous membrane of the syncytium, mesenchymal stromal cells, endothelial cells, blood cells and Hofbauer cells) and represent a functional unit of the placenta. However, the tissue has to be carefully dissected from the placenta soon after delivery and assessed for structural integrity and viability after handling and during culture (syncytial detachment, necrosis and other tissue degeneration are often reported) [127]. The production of hormones, growth factors, cytokines and proteins may show significant inter- and intra-placental variation [119,128,129]. Additionally, different culture conditions (use of diverse supports and inserts, different oxygen levels and culture media composition) can have marked effects on the explant function [130–132]. Lastly, villous explant cultures under static conditions, the most commonly used approach, differ significantly from the *in vivo* situation as they lack the beneficial effects of fluid flow which creates stress on placental villi, specifically on ST [133,134]. Human placental villous explants under flow culture systems show better structural and biochemical integrity as compared to a static culture [133]. To our knowledge, villous explants have so far not been used to study the uptake or toxicity of MNPs in villous structures.

Dually perfused placental cotyledon

Placental cotyledons are functional units of the placenta, containing chorionic villi separated by placental septa. Since its development by Panigel et al., in 1967, the *ex vivo* perfusion of a placental cotyledon is still considered the golden standard for the evaluation of placental clearance and transport of various endogenous compounds [136,137], phar-

maceutical drugs [138,139] and environmental pollutants [140] under dynamic, near-physiological conditions. This *ex vivo* model takes advantage of the full complexity of the intact human term placental tissue and generally shows a good correlation with *in vivo* data [141]. Usually, only a single cotyledon is used and the transfer can be analyzed over time. After perfusion, samples can be taken from placental tissue, which can be processed further for biomarkers (e.g., using genomic or proteomic methods). However, the perfusion time is limited and mainly term placentas are used, because perfusion of the earlier placenta is extremely difficult [142]. Additionally, considering that placental physiology changes significantly during pregnancy, the term placenta does not represent the earlier developmental phases [15,141]. Similar to villous explants, placental perfusion conditions (such as the composition of perfusates and gas mixtures used to oxygenate the tissue, flow rates in maternal and fetal circulations and perfusate volumes) may significantly affect the transport function. Placental viability must be closely monitored during perfusions and interindividual variation in transplacental kinetics, including potential consequences of polymorphisms, must also be considered [143]. Human placental perfusion has been used to study the transplacental transfer of various nanoparticles (reviewed by Aengenheister et al. (2021)), but only a limited number of studies have investigated MNPs of which all were PS particles.

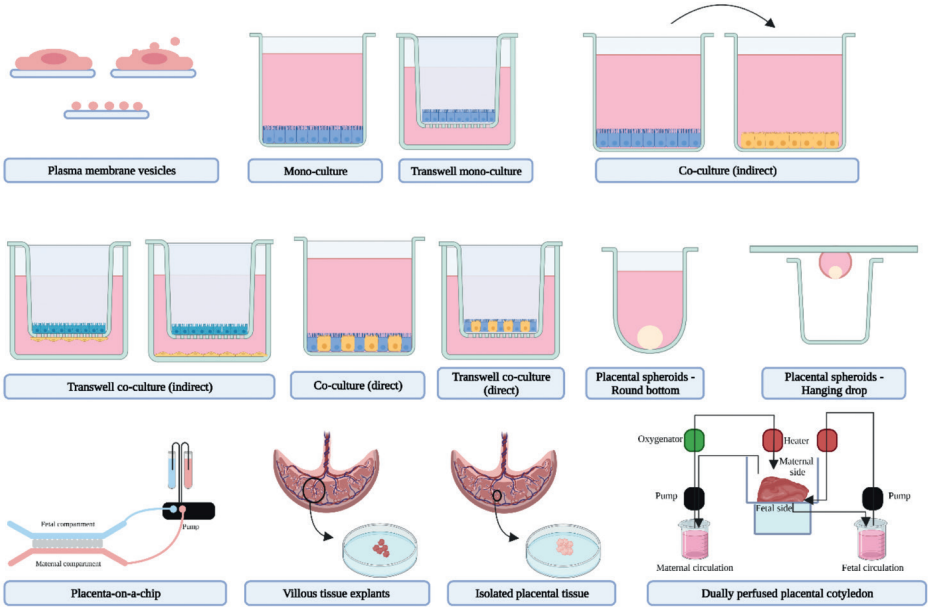


Figure 2. Diverse *in vitro* and *ex vivo* human placental models with increasing order of complexity (from a molecule, cell, tissue to organ) used to study diverse aspects of placental development and functions, including transplacental transport. On a molecular level, plasma membrane vesicles are used. These can be isolated from human placental tissue to study specific transporter proteins. On a cell level, mono- and co-cultures models can be used. Here, diverse placental epithelial and endothelial cells are cultured in a well-plate or a Transwell ® system. Co-cultures can be used in the "indirect" model where no physical contact between different cell types exists, or in the "direct model" where the different cell types are in direct contact. On a tissue level, placental spheroids can be used. Placental spheroids may consist of one or multiple cell types that are cultured in a round bottom well or using a hanging drop technique to recreate a more 'natural-like' microenvironment of the cells. More complex is the placenta-on-a-chip model which combines diverse human placental cells and microfluidics in 3D structures. Moreover, placental tissue explants, such as villous tissue explants can be isolated from the placenta and cultured *in vitro*. On an organ level, placental cotyledon representative of a whole functional unit of the placenta can be dually perfused to study maternal and fetal clearance of various molecules under dynamic, near-physiological conditions.

Table 1: Most commonly used human placental cell models, their characteristics and experimental considerations.

Cell line	*Origin*	*Characteristics*	*Considerations*	*Used to study*	*Ref.*
		Epithelial			
BeWo	Choriocarcinoma cells	- Repr: cytotrophoblast - Hormones: PGH, P4, E1, E2, hCG, hPL, leptin - Transport: Passive diffusion, carrier systems - Immune: Cytokine and IL excretion	- Do not form monolayer - Used in 3D models - Lower expression of chemokines as compared to JEG-3	Adhesion, endocrine function and syncytialization	[79,144–147]
BeWo b30	Choriocarcinoma cells	- Repr: cytotrophoblast - Hormones: PGH, P4, E1, E2, hCG, hPL, leptin - Transport: Passive diffusion, carrier systems - Immune: Cytokine and IL excretion	- Form a tight monolayer - Lower expression of chemokines as compared to JEG-3	Transport, endocrine function and syncytialization	[144,146,147]
JEG-3	Choriocarcinoma cells	- Repr: cytotrophoblast; extravillous cells - Hormones: P4, E1, E2, hCG, hPL, leptin - Transport: passive diffusion and active transport - Immune: Cytokine and IL excretion	- Do not form a monolayer - Used in 3D models - A different transport mechanism for glucose than in BeWo, JAR or ACH-3P cells	Proliferation, invasive potential and endocrine function	[145–149]
JAR	Choriocarcinoma cells	- Repr: cytotrophoblast - Hormones: P4, E1, E2, hCG, hPL - Transport: passive diffusion and active transport - Immune: Cytokines	- Do not form a monolayer - Do not express HLAG or IGF2(R)	Migration and invasion	[150]
3A-Sub-E	Term villous trophoblastic cells	- Repr: term villous trophoblast - Hormones: hCG, able to transform androgens to E1 and E2 - Transport: No information - Immune: No information	- Unlimited life span - Rescued from senescence - SV40 transfected - Not often used in research	Apoptosis, cytogenic features	ATCC website
ACH-3P	AC1-1 with primary first trimester trophoblasts	- Repr: first trimester trophoblasts - Hormones: hCG - Transport: No information - Immune: HLA-G positive and negative cell subpopulations	- Do not form a monolayer - Polyclonal cell line - Mixed cell populations	Trophoblast invasion and differentiation	[90]

Table 1: *[continued]*

Cell line	*Origin*	*Characteristics*	*Considerations*	*Used to study*	*Ref.*
			Epithelial		
HTR-8/Svneo	Transfected cells of chorionic villi explants	- Repr: extravillious CTBs - Hormones: hCG - Transport: No information - Immune: No information	- Not immortal: 12–15 passages - SV40 transfected - Mix cell population (epithelial/mesenchymal-like) - Original HRT-8 cells do not produce hCG, suggesting that HRT-8/SVneo are not representative of first trimester placenta in vivo	EVCTB invasion, proliferation, regulation, cytokine and hormone production	151,152
			Endothelial		
HUVEC	Umbilical vein endothelial cells	- Repr: Macrovascular endothelial cells - Hormones: No hormone production, express ER-β and PR-A - Transport: Passive and active transport - Immune: Cytokines and interleukin	- Used in 3D cultures - Form confluent monolayer - Umbilical cord endothelium - Sex differences in cells - Short life span *in vitro* - Phenotype can change	Regulation of endothelial cell function and angiogenesis	153–157
HPVEC	Placental vascular endothelial cells	- Repr: Microvascular endothelial cells - Hormones: No information - Transport: No information - Immune: No information	- Short life span in vitro - Expensive - Poor commercial availability	Vascular defects in placenta, placental barrier, angiogenesis and vasculogenesis	115
HPEC-A2	Placental venous endothelial cells	- Repr: Microvascular endothelial cells - Hormones: No information - Transport: Passive and active transport - Immune: No information	- Limited availability	Vascular defects in placenta, placental barrier angiogenesis and vasculogenesis	158

Abbreviations: ER, estrogen receptor; EVCTB, extravillous cytotrophoblast; E1, estrogen; E2, estradiol; hCG, human chorionic gonadotropin; HLA-G, human leukocyte antigen-G; hPL, human placental lactogen; IGF2, insulin-like growth factor 2; IL, interleukin; PGH, Placental growth hormone; PR, progesterone receptor; P4, progesterone;

In vitro placental PS uptake, transport and toxicity

Uptake

To date, the only MNPs investigated in the *in vitro* placental models were PS particles. Several methods have been used to confirm the uptake of PS particles by placental cells, namely light-, electron- and confocal microscopy. So far, only fluorescent PS particles (Fluo-PS), with a size range of 20 nm to 10 µm, have been investigated in placental epithelial and endothelial cells *in vitro* (Table 2). Fluo-PS uptake was investigated in BeWo b30, 3A-sub-E cells, HUVEC and HPEC-A2 cells and all, except the HPEC-A2, took up Fluo-PS particles in a size-dependent manner. However, the exact mechanisms of uptake remain unclear. HUVEC and HPEC-A2 are micro- and macrovascular endothelial cells showing phenotypical and physiological heterogeneity and marked differences in gene expression and cell signalling [93]. Moreover, other microvascular HPVEC cells, have been shown to lack fenestrations and pinocytotic activity [159]. These differences between macro- and microvascular cells may account for the observed difference in PS uptake; however, so far this has not been elucidated.

Transfer

The most studied aspect of *in vitro* placental MNP exposure is the transplacental transport of MNPs across the maternal-fetal interface (Table 2). For this, the Transwell ® system is commonly used, where cells are separated by a semi-permeable membrane. The Transwell ® model has been used to study the transport of a variety of substrates (e.g., caffeine, silver nanoparticles) over the placental barrier [109,160]. However, the transport of MNPs has been insufficiently investigated. Only five *in vitro* studies using Transwell ® inserts have examined PS transport across cell layers, of which three have used mono-cultures, one co-culture and one study used both (Table 2). These studies used several methods to determine permeability and monolayer formation (e.g., by measuring transepithelial/endothelial electrical resistance (TEER)), uptake (e.g., by electron microscopy) and transfer (e.g., by microplate reader) (Table 2).

The mono-cultures used BeWo b30, JEG-3, JAR, ACH-3P or HPEC-A2 cell lines, whereas the co-culture consisted of a combination of BeWo b30 and HPEC-A2 cells [56,161–163]. Due to their ability to form a tight monolayer, the BeWo b30 cells are the most frequently used trophoblastic cell line to study the time-dependent transplacental transfer of MNPs using the Transwell set-up [56,162–164].

The transplacental transport has been investigated using different sizes (20 nm to 10 µm) and concentrations (0.05 to 1000 µg/mL) of PS particles (Table 2). Additionally, one study investigated negatively and positively charged PS particles [162]. All PS particles tested were able to cross the cellular monolayers regardless of the cell type, except for the 70 nm [56] and the negatively charged 50 nm PS particles (Magasphere) [162]. Also, neither 50 nor 500 nm negatively charged PS beads were translocated over the BeWo b30/HPEC-A2 co-cul-

ture [161]. Kloet and coworkers (2015) argued that the different translocation rates for the negatively charged 50 nm PS particles are possibly due to the different chemical groups on the surface of the PS supplied by the different manufacturers. The particle surface moiety in addition to the surface charge is thus probably an important factor in the placental uptake and transport of MNPs. In the co-culture model developed by Aengenheister and coworkers (2018), fluo-PS beads (70 nm) did not cross the BeWo b30/HPEC-A2 layers, while the smaller beads (49 nm) did [56]. Overall, the *in vitro* transcellular transport of MNPs has been shown to be limited, with a maximum of 41 and 2% of initial concentration measured in the basolateral compartment in mono-culture and co-culture, respectively.

Toxicity

The MNP toxicity, similarly to uptake and transfer, was studied only with PS particles, in a total of 9 *in vitro* studies (Table 2). An overview of the different toxicity endpoints investigated to date is presented in Table 2. Cell viability was most commonly investigated, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) or MTS (3-(4,5-dimethylthiazol-2-yls)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolim) assay (Table 2). Only exposure to 50 and 100 nm (1 mg/mL) non-charged PS and positively charged 50 nm PS (20 ug/mL) particles resulted in a reduction of cell viability [162,164]. However, the tested concentration of 1 mg/mL is most probably too high to reflect real human exposure level (the total amount of MNPs measured in human blood was recently estimated at 1.6 µg/mL [7]). Additionally, exposure to 50 nm (>5 µg/mL) and 500 nm (0.01-10 µg/mL) PS particles in a co-culture model with BeWo b30 and HPEC-A2 cells increased mitochondrial activity [161]. Nevertheless, PS particles were not transferred over the barrier and did not affect barrier integrity. In 3A-sub-E cells, exposure to 20 nm (500 µg/mL) and 40 nm (200 µg/mL) PS particles resulted in an increased number of TUNEL-positive cells indicating DNA fragmentation upon PS exposure [31]. Additionally, 20 nm PS particles (200 µg/mL) reduced cell proliferation (BrdU assay) in these cells.

In HUVEC cells, cytotoxicity was observed after exposure to 50 nm PS particles with a concentration of 50 µg/mL [165–167]. In addition, all sizes (20-10000 nm, 1000 µg/mL) resulted in apoptosis, with higher apoptosis rates observed in cells treated with smaller PS particles [167]. Furthermore, exposure to 20, 100 and 10000 nm (1000 µg/mL) PS particles resulted in lactate dehydrogenase (LDH) leakage. Upregulation of reactive oxygen species (ROS) was also seen after exposure to 20, 50 and 100 nm PS particles, at high, most probably far-from-physiological concentrations (1000 µg/mL). In contrast, another study showed that exposure to 500 nm PS particles at lower concentrations (0-80 µg/mL) did not increase ROS levels [165]. More data is needed to assess whether PS particles cause increased ROS levels in endothelial cells. HUVEC tube formation was repressed in a concentration-dependent manner after exposure to 500 and 1000 nm PS particles [168], but not after exposure to 5000 nm PS particles indicating a size-dependent effect.

Table 2. Human *in vitro* studies on placental uptake, transfer and toxicity of polystyrene particles.

Cells	*Particle size (nm)*	*Conc. (µg/mL)*	*Exposure (h)*	*Assay*	*Main findings*	*Ref.*
				Epithelial cells		
BeWo b30	50, 100	0.1-1000	24	Transfer	500 µg/mL tested: all PS transferred (50 nm: 10%, 100 nm: 0.8%)	164
				Uptake (TEM)	Size-dependent uptake	
				MTT	Reduction in cell viability - only at 1 mg/mL	
BeWo b30	50 *(neg & pos charged)*	0.05-100	24	Transfer	10 µg/mL tested: No translocation of neg. PS from Megasphere	162
				MTT	pos PS; reduction in cell viability at concentrations >20 µg/mL	
BeWo b30, JEG-3, ACH-3P, JAR	50, 490	125 - 500	24	Transfer	500 µg/mL tested: Both size of PS transferred (BeWo: 41.74%, JAR: 49.22%, ACH-3P: 82.58%, JEG-3: 79.05%)	163
				MTT	No cytotoxicity	
3A-Sub-E	*20-500*	200-500	4	Uptake (TEM)	From the highest to the lowest uptake (10 µg/mL): 40, 100, 200, 20 and 500nm	31
				MTT	Cytotoxicity: only at 1 mg/mL	
				TUNEL	120 nm (500 µg/mL) and 40 nm (200 µg/mL) increased number of TUNEL positive cells)	
				BrdU	Reduced cell proliferation (20 nm, 200 µg/mL)	
				Endothelial cells		
HUVEC	500, 1000, 5000	20-100	72	MTT	500, 1000 nm: the higher the conc., the higher the cytotoxicity. The smaller the size, the higher the cytotoxicity. 5000 nm no cytotoxicity	168
				Tube formation	Repression of tube formation (500, 1000 nm – max 80 µg/mL)	
				DCFH-DA	ROS not detected	
				Immunoblotting	Suppression of cell migration- and angiogenesis	
				Annexin-V	No apoptotic cells	

Table 2. *[continued]*

Cells	*Particle size (nm)*	*Conc. (µg/mL)*	*Exposure (h)*	*Assay*	*Main findings*	*Ref.*
				Endothelial cells		
HUVEC	20-10000	50-1000	24	Uptake (confocal)	No uptake of particles >500 nm	167
				CCK8/JC1	Cytotoxicity: 20 nm (50, 250, 1000 µg/mL), 50 nm (250, 1000 µg/mL), 100-10000 (>1000 µg/mL)	
				Annexin-V	Increased apoptosis (all sizes, 1000 µg/mL)	
				LDH leakage	20, 100, 10000 nm (1000 µg/mL) increased LDH levels	
				DCFH-DA	Upregulation of ROS after exposure to 20,50 and 100 nm PS (1000 µg/mL)	
				ELISA	Increased levels of IL-1β, IL-6 and TNF-α (1000 µg/mL)	
HUVEC	100, 500	5-100	48	MTT	Cytotoxicity only with PS 500 nm > 50 µg/mL	166
				Uptake (FC)	In a size-, time- and concentration-dependent manner (both sizes, 10 and 25 µg/mL)	
				LDH	Increased LDH activity (both sizes, 10 and 25 µg/mL)	
				DCFH-DA	No ROS induction	
				Co-cultures		
BeWo b30/ HPEC-A2	49, 70	6.25-100	24	Transfer	Mono-culture: BeWo (49 nm 1.2%), HPEC-A2 (49 nm, 2%); Co-culture: BeWo + HPEC-A2 (49 nm 1.3%); exposure conc. 50 µg/mL	56
				MTS	No cytotoxicity	
BeWo b30/ HPEC-A2	50, 500 *(neg)*	0.01-100	24	Transfer	No transport	161
				Uptake (confocal)	Detected in BeWo b30, not in HPEC-A2 layer	
				MTS	Increased metabolic activity (50 nm: >5 µg/mL, 500 nm: 0.01-10 µg/mL)	

Note: The literature search was performed using several databases (PubMed, ScienceDirect, Elsevier) and a combination of the following key words: in vitro, placenta, microplastics, polystyrene, transfer, uptake, toxicity. The literature search was performed between February 2022 – May 2022. Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; DCFH-DA, 2'-7'dichlorofluorescein diacetate; FC, flow cytometry; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PS, polystyrene; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

Ex vivo placental PS uptake, transport and toxicity

To date, five studies have investigated transplacental MNP uptake and transport in the *ex vivo* placental perfusion model, all using term placentas, closed, recirculating perfusion model with separate circulations on fetal and maternal sides, and 3 to 6-hour perfusion duration (Table 3). Fluo-PS particles have been the only MNPs investigated, with perfusate concentrations of 25 or 40 µg/ml, particle sizes ranging from 50 to 534 nm and various PS surface functionalization i.e., plain, carboxylate or amine-modified. Overall, a size-dependent maternal-to-fetal translocation was observed for all PS particles investigated. Smaller particles have been transferred more readily than bigger particles (Table 3). Typically, the lower transfer rate corresponds to a higher accumulation of PS particles in the placental tissue, specifically in the ST layer [169–171]. It seems that PS particles larger than 250 nm accumulate mainly in the ST and do not translocate to the deeper layers of the chorionic villi, at least not after acute exposure (up to 6h), suggesting that ST may be a major barrier for the transplacental transport of at least larger particles [53]. This is further supported by data on engineered metallic and carbonaceous nanoparticles where accumulation in placental tissue, specifically in the outer layer of the chorionic villi, has also been observed [17,172,173]. Nevertheless, it is unknown whether longer perfusions would result in increased translocation of MNPs to the deeper layers of the chorionic villi. Fluo-PS transport in the reverse direction i.e., fetal-to-maternal, has also been investigated [169]. After 6h of perfusion, a concentration equilibrium between maternal and fetal circulations was not achieved and the PS beads showed a tendency for a higher transfer in the reverse direction. This demonstrates that passive diffusion may not be the main mechanism underlying the placental translocation of PS particles [169]. As previously mentioned, the trophoblast is actively involved in pinocytosis and phagocytosis of macromolecules, such as IgG, from the extracellular environment and has importance in the transport of therapeutic monoclonal antibodies such as infliximab [60]. Such endocytotic pathways were also indicated as potential energy-dependent mechanisms of cellular particle uptake [174]. Caveolae-mediated endocytosis of PS nanoparticles up to 100 nm in diameter in endothelial cells [175], and, caveolae-mediated endocytosis of pullulan acetate nanoparticles in BeWo b30 cells have been observed [176]. However, specific mechanisms of MNP uptake by placental trophoblast remain to be identified.

Surface charge and modification of MNPs probably also influence placental transport. Grafmüeller et al (2015b)[170] observed a higher transport of plain or carboxylated PS beads than amine-modified PS of similar sizes. The amine-modified PS particles were mostly found in the ST and the villous mesenchyme. Similarly, amine-modified titanium dioxide nanoparticles showed higher accumulation in placental tissue than carboxylated particles of similar size [177]. Based on these studies, transplacental transport of particles seems to decrease in order of plain>carboxylated>amine-modified. Furthermore, next to surface modification, serum proteins seem to influence the placental uptake and transport

of MNPs. Plasma proteins increased the transplacental transfer of unmodified 80 nm PS-particles and the protein corona composition significantly changed upon crossing the human placenta [178]. The key proteins of the corona formed in the plasma medium were albumin, immunoglobulins and apolipoproteins [178]. Human plasma-induced corona formation enhanced the transfer of PS particles compared to PS-corona formed by bovine serum albumin and dextran. Furthermore, human albumin significantly increased the transfer of PS-particles compared to immunoglobulin G-corona [178]. The presence of albumin in perfusion media was also shown to increase the placental transfer rate of hydrophobic compounds such as benzo(a)pyrene [179]. Moreover, it has previously been shown that caveolae-mediated endocytosis of nanoparticles is dependent on the albumin coating of particles [175]. Therefore, the fact that proteins increase the transplacental transfer of PS particles further indicates the involvement of endocytosis in the transplacental transport of MNPs. Considering that endocytosed nanoparticles tend to accumulate in lysosomes, lysosomal dysfunction and subsequent cell death may be a possible mechanism of MNP toxicity, as previously shown for gold, SiO2 NPs and amine-modified PS nanoparticles [180,181]. In summary, the polymer type, size, charge and functional groups on the particle surface can alter the biological reactivity of the particles which can affect the formation of the protein corona, cellular uptake and consequently toxicity of MNPs [178,182,183].

None of the five studies indicated that the viability and functionality of placental tissue are affected by PS particles during perfusion. No effects on barrier integrity (monitored with passive diffusion markers and/or absence of fetal to maternal leakage of perfusate), on human chorionic gonadotropin, leptin, or lactate production, or on glucose consumption have been observed (Table 3). Perfusion time is usually 6 hours or less and thus, no long-term effects can be studied in this model. So far, effects of MNP on other toxicity endpoints such as oxidative stress, inflammation markers or endocrine functions have not been studied in human placental perfusion.

Table 3. Human *ex vivo* placental perfusion studies on uptake, transfer and toxicity of polystyrene particles.

Particle size (nm) and concentration	*Perfusion conditions*	*Assay*	*Main findings*	*Ref.*
50, 80, 240 and 500 (25 µg/ml)	Closed (3 h); NCTC-135 medium/Earl's buffer with BSA (8 g/L)	Transfer (TEM)	Size-dependent transfer, 8.90 ± 1.80 µg/mL (50 nm) 7.47 ± 1.77 µg/mL (80 nm), 2.03 ± 0.29 µg/mL (240 nm) after 3h in the fetal perfusion medium	53
		Viability and functionality	Glucose, lactate, human hCG and leptin concentrations not affected	
80 (40 µg/ml)	Closed (6 h); DMEM/Earls buffer with BSA (5 g/L) or HSA (40 g/L) and human plasma (8.6%) or HSA (40 g/L) or IgG (10 g/L)	Transfer (SP, TEM)	After 6 h of perfusion significantly higher transfer in the plasma medium (23.2 ± 5.5) µg/ml) than in the buffer medium (15.5 ± 2.3) µg/ml)	178
		Shotgun proteomics (LC-MS/MS)	Human plasma-enhanced transfer of Fluo-PS particles compared to PS particles with a corona formed by BSA and dextran. Human albumin induced the transfer of Fluo-PS particles compared to corona formed by IgG	
		Quality and viability	No change in O_2 and CO_2 tension, glucose and lactate concentration	
50, 240; 50, 300 carboxylate-modified (25 µg/ml)	Closed (6 h); M199 medium/ Earl's buffer with BSA (10 g/L), bidirectional transfer	Transfer (TEM)	Transfer from fetal to maternal direction was significantly higher than from maternal to fetal. Increased translocation of plain Fluo-PS compared with carboxylated Fluo-PS in both directions. Higher transfer rate, lower tissue accumulation.	169
		Uptake (FM)	All PS particles accumulated in ST, regardless of the direction of perfusion	
		Viability and functionality	Glucose, lactate, hCG and leptin concentrations not affected	
60-534, plain, carboxylate, amine-modified (25 µg/ml)	Closed (6 h); M199 medium/ Earl's buffer with BSA (10 g/L)	Transfer (SP)	Transfer of plain and carboxylated Fluo-PS but not amine-modified particles	170
		Uptake (FM)	Amine-modified particles found in the ST and villous mesenchyme	
80 and 500 (25 µg/ml)	Closed (6 h); NCTC-135 medium/Earl's buffer with BSA (10 g/L)	Transfer (SP)	80 nm Fluo-PS crossed the placental barrier (after 3 h, 20-30% of the added amount transferred) while 500 nm Fluo-PS were retained in the placental tissue or maternal circuit	171
		Uptake (FM)	Particles accumulated in the villi	
		Viability and functionality	Glucose, lactate, hCG and leptin concentrations not affected	

Note: The literature search was performed using several databases (PubMed, ScienceDirect, Elsevier) and a combination of the following key words: in vitro, placenta, microplastics, polystyrene, transfer, uptake, toxicity. The literature search was performed between February 2022 – May 2022. Abbreviations: Fluo-PS, fluorescent polystyrene particles; BSA, bovine serum albumin; HSA, human serum albumin; hCG, human chorionic gonadotropin; ST, syncytiotrophoblast; TEM, transmission electron microscopy; FM, fluorescence microscopy; SP, spectrophotometer.

Future directions

There are numerous knowledge gaps concerning MNPs and human health, specifically potential effects on pregnancy and the developing fetus are largely unexplored [10]. Recently, MNPs have been detected in human blood, placental tissue and meconium. Since pregnancy and early life represent the most vulnerable period of human development, toxicity studies on the potential effects of MNPs on placental development and essential metabolic, endocrine and immune functions are urgently needed. Experimental data on the toxicokinetics and toxicodynamics of MNPs in the human placenta is scarce and based solely on PS particles. The results from both *in vitro* and *ex vivo* studies indicate that PS particles are taken up and transported through the placental barrier in a size- and dose-dependent manner, which calls for a comprehensive investigation of placental toxicokinetics and toxicity of other environmentally relevant MNPs, and consequent effects of such exposure on fetal and maternal health during pregnancy.

Choosing the right human placental model

To pursue human developmental toxicity studies with MNPs, human placental models are essential because of the interspecies variation in placental structure, physiology and cell biology. A variety of experimental models using human placental tissue (tissue explant cultures, human placental perfusion), and human placental cells (primary cells, human choriocarcinoma cell lines) either as simple cultures or co-culture models can provide valuable, human-relevant data. As described above, each model has advantages and disadvantages and has to be carefully chosen according to the aim and relevant research question being studied. The use of placental cell lines provides relatively simple, high-throughput and robust tools to investigate cell type-specific processes underlying placental transport and toxicity of MNPs. However, they lack physiological complexity. Tissue explant cultures and human placental perfusions more closely simulate the functional units of term placental tissue and could be useful in studying villous transport, endocrine, metabolic and immune functions [129,131,133,184] as well as the aetiology of placental diseases such as pre-eclampsia [128,185]. Moreover, villous explants from the first trimester can be used to study EVT proliferation and differentiation [119,123]. Recently, villous explants have been used in research on biogenic silver nanoparticles [186] and polyglycerol nanoparticles [55], and, a combination of villous explants with human placental perfusion has been successfully used to study the transplacental passage of PEGylated liposomes [187]. In placental perfusion, the transplacental passage of MNPs can be investigated in both directions, maternal-to-fetal and fetal-to-maternal [169]. This can elucidate the potential role of placental transporters in the transplacental transfer of MNPs, however, transport processes occurring at specific cell types cannot be differentiated using this method. Also, placental accumulation and potential toxic effects could be investigated by using various markers of toxicity and multi-omics approaches, such as RNA sequencing and

metabolomic profiling [167,188]. However, taking into account a large number of possible experimental conditions (the multitude of MNP polymer types, shapes and sizes), the most important consideration for tissue explant cultures and placental perfusion studies is the low throughput of these methods: they are laborious, time-consuming, and, morphological and functional characterization of these fragile tissues has to be performed at different time points to ensure robust results. Furthermore, no standardised perfusion protocol for MNP research exists [15,189]. Such experiments have to be thus planned and designed carefully to maximize their usefulness. Moreover, due to technical difficulties so far only acute exposures and term placentas have been used. How the morphological changes that occur during placental development may influence the MNP toxicokinetics in the early gestation also need to be considered. More advanced *in vitro* models such as hanging-drop or placenta-on-a-chip provide promising models for microphysiological recapitulation of the human placenta that can be used for high-throughput screening. This has been previously demonstrated in studies with nanoparticles, such as titanium dioxide (TiO_2), copper oxide (CuO) and cadmium telluride quantum dots (CdTe-COOH) [108,114]. Here, endpoints including oxidative stress, cell apoptosis, barrier integrity and permeability were investigated, as well as the effects on maternal immune cells.

Placental transport and placental toxicity

The ST layer seems to be the main barrier to transplacental PS transport, as demonstrated with the *ex vivo* perfusion studies where bigger particles showed higher retention in ST. The exact mechanisms involved in MNP uptake and transport have not been elucidated, but likely passive diffusion is not the key mechanism underlying placental translocation, at least for the larger spherical PS particles. It is worth noting that ST is actively involved in pinocytosis and phagocytosis of micro- and macrovesicles between maternal and fetal circulations. Although these endocytotic pathways have been considered too slow to have any significant effect on placental drug transport [14], their role in MNP transport has not yet been characterized. ST is the first cell layer in contact with the maternal blood and plays a major role in the metabolic, endocrine, and immune functions of the placenta. However, to our knowledge, no *in vitro* studies exist on MNP uptake and toxicity in this cell layer. The possible accumulation of MNPs in ST presents a potential hazard. For example, the trophoblastic cells and structures, specifically ST, contribute to the production of extracellular vesicles. These contain mainly deported multinuclear fragments and sub-cellular components (from 50 nm to 1 µm in size) that are shed by the syncytial surface throughout pregnancy. An increase in placental debris has been associated with the development of maternal pre-eclampsia [190]. Whether the uptake of MNPs in ST contributes to an increase in the production of extracellular vesicles is not known, but warrants further investigation.

While the body of evidence on the placental transport of MNPs is growing, data on toxicity and the effect of MNPs on placental function is scarce. However, other particles, such as fine particulate matter from ambient air pollution, have been shown to translocate from the respiratory tract to the placenta and contribute to various adverse pregnancy outcomes [18,21–24,27,191]. MNPs, including fibres and traffic-generated particles, are increasingly found in ambient air pollution further adding to the atmospheric particle burden [192]. Yet, it is unknown if atmospheric MNPs pose added or novel risks for the developing child and maternal health during pregnancy. To date, research on MNPs investigated mostly generic acute endpoints such as cell viability, cytotoxicity and barrier integrity with effects observed mainly at high, most probably far-from-physiological concentrations. Whereas, the physiological and pathological placental perturbations, including more subtle endocrine or immune-related effects of particle and chemical toxicity of MNPs at environmentally relevant exposure scenarios have received scant attention. A growing body of literature shows that maternal exposure to other particle types such as engineered nanoparticles may directly or indirectly affect maternal health, placental functions and fetal development, which may subsequently lead to a range of adverse pregnancy outcomes and health effects later in life [13]. Impairment of placental growth, placental oxidative stress and inflammation, activation of placental toll-like receptors (TLRs), and altered secretion of hormones and vascular factors have been previously identified upon placental exposure to such particles [13,193]. Clearly, there are strong indications that MNP could affect placental function, but more studies are urgently needed that address this.

Defining MNP exposure

Experimental placental MNP research has focused mainly on acute exposures (up to 24h) and tested only one polymer type and shape (commercially available and fluorescently labelled spherical PS particles) under a limited number of conditions. The toxicokinetics and toxicodynamics of MNPs, however, are dependent on many factors such as particle size and shape, surface charge, presence of eco- or bio-corona, as well as various chemicals they may carry, all of which are strongly influenced by environmental weathering processes [10,183,194–196]. It is evident that the environmental MNPs are very complex and differ significantly from the pristine spherical PS particles commonly used in the laboratory setting. For example, the spherical particles differ in their uptake and toxicity from the more environmentally relevant irregular-shaped particles [197,198]. Weathering and degradation processes increase oxygen-containing functional groups on the particle surface influencing aggregation potential, interaction with cellular membranes, adsorption of co-contaminants, biological activity and likely consequent toxicity of MNPs [195,199].

Environmental MNPs may also act as vectors and transfer bacterial pathogens and exogenous compounds present inside the particles or on their surface [194]. For example,

a recent study identified more than ten thousand compounds associated with plastic particles, including monomers, additives and processing aids [200]. Consequently, in addition to particle toxicity, MNPs may leach plastic additives, such as bisphenol A and phthalates, as well as brominated flame retardants, antioxidants, UV stabilizers and synthetic dyes [194,200]. Moreover, hazardous environmental compounds e.g., dichlorodiphenyltrichloroethane (DDT) [201], polychlorinated biphenyls (PCBs) [202], heavy metals [203,204] and polycyclic aromatic hydrocarbons (PAHs) [205,206] have been shown to adsorb to MNPs and form an eco-corona surrounding the MNPs [207]. Many of these compounds are suspected endocrine disruptors, carcinogens and developmental toxicants. Animal studies suggest increased toxicity by combined exposure to plastic particles with contaminating chemicals than particles alone [208–210]. For example, an increase in the uptake and toxicity of nickel, lead and cadmium in the presence of MNPs has been reported in experimental animal models [211,212]. In humans, these heavy metals are well-known to cross the placenta, accumulate in fetal tissues and adversely affect placental functions and fetal development [213]. Interestingly, antagonistic interactions between MNPs and co-contaminants (e.g., cadmium, nickel, mercury) have also been observed in diverse animal models [196]. Upon uptake in the body, lipids, proteins, carbohydrates and nucleic acids can form a bio-corona around MNPs that are transported via the maternal blood to the placenta [207]. A bio-corona can play an important role in the transport of molecules and particles across the placental barrier and their toxicity [178,214]. The effect of weathering and formation of bio- and eco-coronas on MNP uptake and placental toxicity should be thoroughly investigated.

The identification of the drivers of toxicity of environmentally relevant MNPs is extremely challenging. This is mainly due to the scarcity of well-characterized relevant reference materials, challenges in isolating and characterizing environmental MNPs in complex environmental samples, and the lack of analytical methods for the detection and quantification of nonfluorescent particles (specifically in the nanosized range) in human samples as well as in laboratory setting. Such tools and relevant reference materials are urgently needed to study realistic exposure scenarios and to reduce the current uncertainty in the human risk assessment of MNPs.

This review shows that there are a number of suitable, human-relevant placenta models to characterize the toxicity of MNPs under experimental conditions that could be utilized now to address the most pressing knowledge gaps. These studies should: 1) include fit-for-purpose placental models; 2) focus on the placental transfer and placental function; 3) investigate environmentally relevant particles, i.e. polymers other than PS, weathered particles and MNPs with eco- and bio-corona; and 4) use harmonized reference materials and detection methods. The next step forward to better understand actual risks in humans would be to perform more comprehensive risk assessment studies. Here, longitudinal human biomonitoring and epidemiological studies with birth

cohorts are required in which maternal and fetal exposures and health outcomes should be further characterized.

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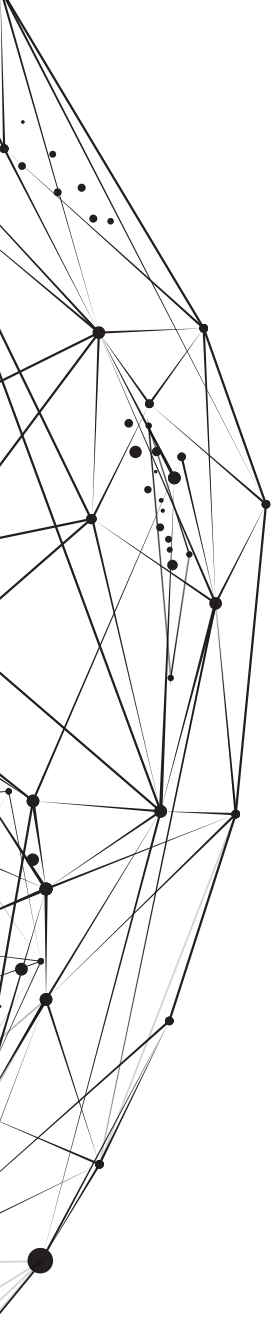

5

Uptake, transport and toxicity of pristine and weathered micro- and nanoplastics in human placenta cells

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Abstract

BACKGROUND: The first evidence of micro- and nanoplastic (MNP) exposure in the human placenta is emerging. However, the toxicokinetics and toxicity of MNPs in the placenta, specifically environmentally relevant particles, remain unclear.

OBJECTIVES: We examined the transport, uptake and toxicity of pristine and experimentally weathered MNPs in nonsyncytialized and syncytialized BeWo b30 choriocarcinoma cells.

METHODS: We performed untargeted chemical characterization of pristine and weathered MNPs using liquid chromatography high-resolution mass spectrometry to evaluate compositional differences following particle weathering. We investigated cellular internalization of pristine and weathered polystyrene (PS, 0.05-10 µm) and high-density polyethylene (HDPE, 0-80 µm) particles using high-resolution confocal imaging and three-dimensional rendering. We investigated the influence of particle coating with human plasma on the cellular transport of PS particles using a transwell set-up and examined the influence of acute MNP exposure on cell viability, damage to the plasma membrane and expression of genes involved in steroidogenesis.

RESULTS: Chemical characterization of MNPs showed a significantly higher number of unique features in pristine particles compared to weathered particles. Size-dependent placental uptake of pristine and weathered MNPs was observed in both placental cell types after 24 h exposure. Cellular transport was limited and size-dependent and was not influenced by particle coating with human plasma. None of the MNPs affected cell viability. Damage to the plasma membrane was observed only for 0.05 µm PS particles in the nonsyncytialized cells at the highest concentration tested (100 µg/ml). Modest downregulation of hsd17b1 was observed in syncytialized cells exposed to pristine MNPs.

DISCUSSION: Our results suggest that pristine and weathered MNPs are internalized and translocated in placental cells *in vitro*. Effects on gene expression observed upon pristine PS and HDPE particle exposure warrant further examination. More in-depth investigations are needed to better understand the potential health risks of MNP and chemicals associated with them under environmentally relevant exposure scenarios.

Introduction

Micro- and nanoplastics (MNPs) are global environmental pollutants with widespread exposure in the human environment e.g., food[1,2], water[3], air[4,5] and dust.[6]. Nevertheless, MNP toxicokinetics and toxicity in humans are still largely unexplored.[7] Recent animal studies have shown that ingested or inhaled MNPs can translocate across pulmonary and intestinal cell barriers to secondary organs, including placental and fetal tissues.[8–11] Moreover, epidemiological research indicates that fine particles (≤ 2.5 µm) from air pollution can reach the placenta *in situ* and increase the risk of pregnancy complications such as gestational diabetes and pre-eclampsia[12,13], pre-term birth or low birth weight.[14] These findings have raised concerns for potential adverse health effects of placental MNP exposure during pregnancy, a period of heightened vulnerability when even subtle environmental insults can have long-lasting effects on fetal development and health later in life.[15,16]

To date, a limited number of human *in vitro* placenta cell models[17–19] and *ex vivo* placenta perfusion studies[20–22] have demonstrated the transplacental transport of polystyrene MNPs. Recently, a study by Ragusa et al. (2021)[23] was the first to detect MNPs in human placenta samples. Although this research provides the first indication of placental exposure, understanding of placental toxicokinetics and toxicity of MNPs is limited. Gaining insight into these mechanisms is indispensable for a better understanding of possible adverse effects on placenta development and functioning and how these relate to fetal health.

The placenta is a unique and complex temporary organ that nourishes and protects the fetus. It is a multi-layered membranous structure that forms at the interface of maternal and fetal circulation and facilitates the exchange of gases, nutrients, metabolites and waste products.[24] Chorionic villi covered by villous trophoblasts (Figure 1A) are the main placental structures involved in the fetomaternal exchanges.[25] The inner layer of the villous trophoblasts is formed by stem-like cells called cytotrophoblasts.[26] These cells proliferate and differentiate by fusion to syncytiotrophoblasts, large multinucleated cells that form the top layer of the villous tree.[26] Syncytiotrophoblasts are in direct contact with maternal blood and thus are the main cells to facilitate the fetomaternal exchange.[27] Syncytiotrophoblasts also function as important endocrine cells involved in the biosynthesis and metabolism of a variety of hormones (e.g., leptin, human chorionic gonadotropin, progesterone, estrogens) crucial for the regulation of maternal and fetal physiology.[27,28] Growing evidence shows that disrupted functioning of the villous trophoblasts can lead to impaired endocrine, metabolic and transport functions of the placenta, resulting in a variety of pregnancy complications.[29–31] Although syncytiotrophoblasts are the first placental cells in contact with maternal blood and are crucial for fetomaternal exchange and placental functioning, to date, no data exist on the uptake or toxicity of

MNPs in syncytiotrophoblasts *in vitro*. The BeWo cell line derived from a human immortalized trophoblast, morphologically and functionally resembles villous cytotrophoblasts and is one of the most widely used placental cell models.[32] The BeWo b30 variant forms a confluent and polarized monolayer and has been successfully used in a variety of transport studies including transport of e.g., gold[33], silver[34], silica[35], iron [36] or polystyrene nanoparticles.[19]. The intercellular fusion and differentiation (syncytialization) of BeWo b30 can be successfully triggered by forskolin, resulting in the formation of endocrine active cells that resemble *in situ* human syncytiotrophoblasts.[37–40]

Particle size, morphology, surface charge and protein corona are factors that can influence the translocation of MNP over different cellular barriers.[41,42] Nevertheless, their role in transplacental MNP transport is still not well understood. Moreover, the key characteristics potentially driving toxicity, such as the presence of inherent or adsorbed contaminants, are largely understudied. Due to the challenges in capturing and isolating environmentally weathered MNPs, the majority of toxicity studies have used pristine, manufactured particles that may not be representative of real environmental exposures. The goal of this study was to examine both pristine and experimentally weathered MNPs, at different sizes, to investigate the influence of polymer type, size and weathering on the transport, uptake and toxicity in both, nonsyncytialized (undifferentiated) and syncytialized (differentiated) BeWo b30 cells. The model MNPs selected for this study were polystyrene (PS) and high-density polyethylene (HDPE) particles, two commonly used polymers present in a wide variety of consumer products. We investigated the cellular internalization of MNPs using high-resolution confocal imaging and three-dimensional rendering and developed a method for quantitative analysis of cellular uptake in both cell phenotypes. Moreover, we investigated the influence of human plasma protein corona on the cellular transport of MNPs in a transwell set-up. Furthermore, we investigated the influence of MNP exposure on several toxicity endpoints including cell viability, damage to the plasma membrane and expression of genes important in the placental biosynthesis of steroid hormones. Lastly, to gain the first insight into particle vs chemical toxicity, we performed an untargeted, small molecule characterization of pristine and weathered MNPs using liquid chromatography high-resolution mass spectrometry (LC-HRMS). This approach allowed us to test for the presence of co-transported pollutants and to evaluate compositional differences following particle weathering.

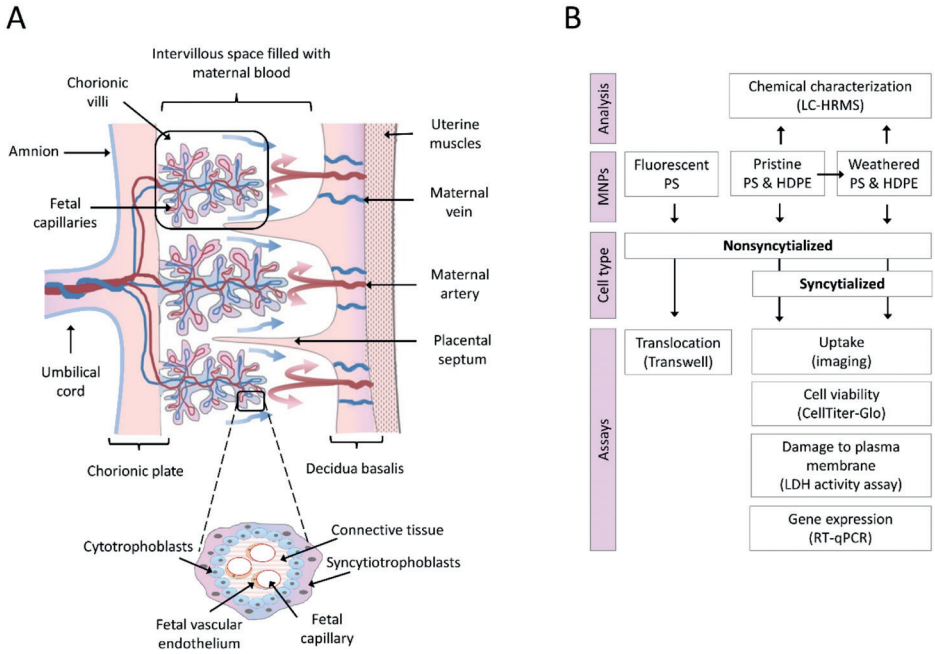


Figure 1. (A) Cross-section of early gestation human placenta showing chorionic villi covered by syncytiotrophoblasts and cytotrophoblasts. (B) Experimental design for analysis of translocation, uptake and toxicity of pristine and weathered micro- and nanoplastics (MNPs) in nonsyncytialized and syncytialized BeWo b30 cells. Note: HDPE, high-density polyethylene; HRMS, high-resolution mass spectrometry; PS, polystyrene.

Material and methods

An overview of the experimental design is presented in Figure 1B. A full list of reagents can be found in Supplemental Material, “Reagents”.

Pristine and weathered micro- and nanoplastics (MNPs)

Fluorescent and non-fluorescent MNPs of different sizes were used in this study. Fluoresbrite® YG polystyrene 0.05 µm **(**cat. nr. 17149), 0.2 µm (17151), 1.0 µm (17154) and 10.0 µm (18140-2) particles (Fluo-PS) were purchased from Polysciences Europe GmbH (Germany), delivered as 2.5% aqueous suspension. Non-fluorescent MNPs i.e. a high-density polyethylene (HDPE Abifor, Zürich, Switzerland) powder with nonuniformly shaped particles ranging 0–80 µm in size (Gaussian grain size distribution n: < 50 µm =35–45%, < 63 µm =60– 80%, < 80 µm =98–100%, Von Moos et al. 2012), and polystyrene microspheres at 0.05 µm (cat. nr. 08691), 0.2 µm (07304), 1.0 µm (07310) and 10.0 µm (17136, Polysciences Europe GmbH, Germany) were kindly provided by Deltares, the Netherlands. Stocks of pristine HDPE and PS particles were prepared by washing the particles in hexane and PBS, respectively, then the particles were centrifuged for 10 minutes at

4000 rpm and room temp. and resuspended in PBS (1% solids w/v). For weathering experiment the washed HDPE and PS were resuspended in environmental water (sampled alongside a 1-2 meter wide ditch at Tolakkerlaan, approximate coordinates 52°04'58.4"N 5°11'37.1"E, Utrecht, NL). The water was sampled in pre-sterilized 1L glass bottles, which were filled by opening the bottle 1-20 cm below the water surface, and then filtered through a standard paper filter to remove the bigger particles. The weathering was performed in a duplicate set-up, in glass bottles for 4 weeks at room temp. with exposure to sunlight and constant shaking (100 rpm), and particle concentration of 0.1% (w/v). After weathering, the particles were washed with PBS, separated from the liquid by decanting, pooled and resuspended in PBS (1% solids w/v). Because of the hydrophobic nature of HDPE, stocks in PBS were additionally supplemented with bovine serum albumin (BSA) to bring the particles into a homogenous suspension. BSA was dissolved in PBS, filtered through a 0.2 µm sterile Corning® syringe filter (431219, Sigma Aldrich) and added to a final concentration of 0.5% w/v (1:1 w/w BSA to HDPE). For all exposure studies, if not specified otherwise, serial dilutions of the highest MNP stocks (0.1, 1, 10, 100 ug/mL) were prepared in the exposure medium (culture medium without phenol red) and added to the cells so that the concentration of PBS did not exceed 1%.

Chemical characterization of pristine and weathered MNPs

A non-targeted, small molecule characterization was performed on MNP stock suspensions to evaluate chemical differences between pristine and weathered MNPs and to test for the presence of co-transported pollutants following weathering using previously described methods.[44] Briefly, MNP suspensions (pristine and weathered PS with 0.05 µm, 0.2 µm, 1µm and 10µm diameters) were prepared for analysis by diluting 50 ***µL*** of 10 mg/mL aliquots with 100 *µL acetonitrile* and centrifuging for 10 min at 16,100 x g. The supernatants were analysed (in triplicate) using dual-column (HILIC: Waters XBridge BEH Amide XP HILIC, 2.1mm x 50mm x 2.5µm; and C_{18}: Higgins Targa C18, 2.1mm x 50mm x 3µm) liquid chromatography paired with positive or negative mode electrospray ionization interfaced to a Thermo Scientific Q-Exactive HFX Orbitrap high-resolution mass spectrometer. Analyte separation for HILIC was accomplished using acetonitrile and formic acid; separation for C_{18} used an acetonitrile and ammonium acetate gradient. The mass spectrometer was operated at a resolution of 120,000 and a mass-to-charge ratio (*m/z*) range of 85-1275. Uniquely detected ions consisted of *m/z*, retention time, and ion abundance referred to as *m/z* features (all descriptors used in the analysis are explained further in Excel Table S1). High-resolution detection of *m/z* features was accomplished by a maximum injection time of 100 milliseconds and an Automatic Gain Control target of 1×10^6. Raw data files were extracted and aligned using apLCMS[45] with modifications by xMSanalyzer.[46] Detected signals with a fold-change of 5 or higher relative to the PBS buffer solution were annotated using xMSannotator by matching accurate mass *m/z* to

the plastics and plastic-related chemicals from PLASTICS|NORMAN: Database of Chemicals likely (List A, 906 chemicals) and possibly (List B, 3377 chemicals) associated with Plastic Packaging (CPPdb).[47] Compound annotation confidence was determined based on Schymanski et al. (2014) by matching accurate mass, isotope and adduct patterns to the combined plastic database; potential compound structures were ranked based on PubMed reference counts.[48,49] Initial differences between pristine and weathered MNPs were characterized using partial least squares discriminant analysis (PLS-DA). Next, a variable importance projection (VIP) score greater than 1.5 was used to identify signals that differed due to MNP weathering. To characterize the potential origin of plastic chemicals with VIP > 1.5, annotated compounds were assigned a primary use category based on a database of over 10,000 plastic-related chemicals (PlasticMap) recently complied by Wiesinger et al. (2021) from industrial, scientific, and regulatory data sources.[50] InChiKeys were used to avoid misclassifications due to differences in chemical names.

BeWo b30 culture and syncytialization

The BeWo b30 choriocarcinoma cell line (AddexBio, Cat. # C0030002, San Diego, USA) was kindly provided by Prof. Aldert Piersma and Conny van Oostrom (RIVM, the Netherlands). The cells were maintained in DMEM/F12 supplemented with 1% (v/v) P/S and 10% (v/v) fetal bovine serum (FBS), and incubated at 37 °C with 5% CO_2 and 95% relative humidity. Every 3 days, when reaching 70-90% confluency, cells were washed with PBS and subcultured using 0.05% trypsin-EDTA solution. Differentiation and intracellular fusion (syncytialization) were induced by treatment with forskolin 48h post-seeding when cells reached approximately 50% confluency. A stock solution of forskolin (10 mM) was prepared in DMSO and added to the cells in a culture medium to the final concentration of 10 µM at 0.1% DMSO. Medium with forskolin was refreshed daily for three consecutive days. An equal volume of DMSO was added as a vehicle control to the non-syncytialized BeWo cells. Two different cell culture media, MEM and DMEM/F12 were tested to ensure optimal conditions for BeWo b30 cell culture and syncytialization. The cells were seeded in a transparent 12-well plate, cultured, and differentiated in each test media; the cells were then stained and imaged as described below (n=2, with two technical replicates per plate). In general, undifferentiated cells showed a more consistent morphology when cultured with a medium containing high glucose DMEM/F12; i.e., cells formed a tighter confluent monolayer and showed clearer cellular boundaries, as indicated with β-actin (Sir-actin) and tight-junctions (ZO-1) staining (Figure S1). Therefore, DMEM/F12 was used in experiments throughout this study.

Cellular imaging and quantification of MNP uptake

For the visualization of the cells and quantification of the cellular uptake of MNPs, cells were seeded at a density of 2 x 10^5 cells/mL in a transparent 12 well-plate contain-

ing 18 mm glass covers pre-coated (for 2h at 37˚C) with collagen (type IV) from human placenta (50 µg/mL in 0.25% acetic acid), cultured and differentiated as above. On day 6 post-seeding, the nonsyncytialized and syncytialized cells were exposed for 24 h to fluorescent and non-fluorescent PS at 0.05, 0.2, 1 and 10 µm size at different concentrations in 1 mL of exposure media. To ensure an optimal non-toxic particle density during imaging, the concentrations used were chosen for each condition separately and kept within the non-toxic concentration range as indicated by cytotoxicity assays in this study. Exposed and not exposed cells (control) were fixed in 4% formaldehyde (10 min), washed with PBS and treated (for 1h at room temp.) with blocking buffer (3% BSA in PBS) containing DAPI (1 µM), SiR-actin labelling probe (1 µM) and ZO-1 antibody (1 µM) or Vybrant labelling dye (5 µM), to stain nucleus, β-actin and tight junction or plasma membrane, respectively. In the case of ZO-1, cells were subjected to the second round of staining with the secondary antibody (Alexa594, 10 µg/mL) for 1h at room temperature. After staining, the cells were washed with PBS and rinsed in MilliQ and the glass covers were mounted on a slide for imaging. Cells were imaged using Leica TCS SP8 STED 3X microscope with a pulsed (80 MHz) white-light laser (WLL), 405 nm DPSS laser, HyD/PMT detectors and spectroscopic detection. For imaging of 10 µm beads, we used HC PL APO 20x/075 IMM CORR CS2 (Leica 15506343) dry objective and all other conditions were imaged using HC PL APO 93 ×/1.30 GLYC motCORR STED (Leica 15506417) glycerol-immersion objective with corrective collar. Due to the absence of spectral crosstalk, all fluorescent channels were imaged simultaneously. For DAPI imaging we used 405 nm DPSS laser as the excitation source and detected emission in the 420-460 nm spectral range, for fluorescent beads we used 488 nm excitation (WLL) and 500-550 nm emission range, for Vybrant dye we used 561 nm excitation (WLL) and 570-620 nm emission, for ZO-1 we used 580 nm excitation (WLL) and 590-620 nm emission and for SiR-actin we used 633 nm excitation (WLL) and 640-750 nm emission. Non-fluorescent beads were imaged using transmitted light PMT with a 488 nm laser as a source. For 10 µm beads, the size of field-of-view (FOV) was about 415 µm and for all other conditions, it was in the range between 100-160 µm. The height of z-stacks was in the range of 10-30 µm, chosen per each sample individually to include the whole thickness of the cell layer and to ensure that the detected MNPs were within the cells and not only present on top of the cell boundary. The number of cells and particles per field of view was counted manually using the Cell Counter plugin for ImageJ [https://imagej.nih.gov/ij/plugins/cell-counter.html]. To accurately quantify and compare the particle uptake between the two cell types, the number of particles taken up was calculated per area (mm^2) occupied by the cells rather than per cell. Mean values of the particle uptake were calculated based on ≥ 3 FOV per sample from at least 2 independent experiments (n=2). To calculate an area occupied by the cells (adjusted cell density) in a FOV, we merged maximum intensity projections of the membrane (Vybrant dye) and actin (SiR-actin) channels, convolved it with a Gauss-

ian filter of 2 pixels (to remove noise), applied threshold manually and calculated background area using "Analyse Particles" function of ImageJ. Cell occupied area was calculated as the total area of FOV minus background area. Additionally, to image changes in cell morphology during syncytialization, the nonsyncytialized and syncytialized cells were stained with May–Grünwald–Giemsa following a standard protocol[51] and imaged with bright field microscopy.

Transwell experiments

Nonsyncytialized BeWo b30 cells were seeded at a density of 2 x 10^5 cells/mL, on 12-well polycarbonate Transwell® inserts with 12 mm diameter and 3 µm pore size (Corning®, Sigma Aldrich, Dorset, UK), containing 1.5 mL of culture medium on the basolateral and 0.5 mL on the apical side. The medium was replaced every second day. The formation of the monolayer and the monolayer integrity was monitored using transepithelial electrical resistance (TEER) measurements and sodium-fluorescein (Na-Flu) leakage assay.

TEER measurements

Monolayer formation was monitored with a Millicell-ERS (Electrical Resistance System), a volt-ohm-meter that measures membrane potential and resistance of cell monolayers in culture. TEER values were measured for 12 consecutive days in a transwell-plate equilibrated to room temperature and in the presence and absence of cells. The final TEER values were determined by subtracting the intrinsic resistance (insert membrane without cells) from the total resistance (insert membrane with cells) and were corrected for the surface area (Ω cm^2).

Na-Flu leakage assay

The barrier integrity was further evaluated with a fluorescein sodium salt (Na-Flu) leakage assay. A stock solution of Na-Flu (0.5 mM) was prepared in DMSO. At 4-, 6-, 8- and 11-days post-seeding 0.5 mL of 5 µM Na-Flu (0.1% DMSO) was added to the apical side, and 1.5 mL of medium only was added to the basolateral side. To avoid interference with fluorescence measurements, exposure medium (culture medium without phenol red) was used. After 3 h incubation, 100 µl of the sample was removed in triplicate from the basolateral side, transferred into a black 96-well plate and analysed using a fluorescent plate reader (POLARstar Galaxy, BMG Labtech GmbH) and 485 nm excitation and 520 nm emission wavelength, respectively. The final concentration of Na-Flu in the basolateral side was calculated using Na-Flu dilution series (0.1 nM - 10 uM), corrected for background fluorescence and volume and expressed as the average basolateral amount of the initial Na-Flu concentration in a percentage, based on three independent experiments (n=3).

Translocation study

The transport of Fluo-PS over the monolayer was measured by exposing cells on day 11 post-seeding. Fluo-PS at 0.05, 0.2 and 1 µm were added (50 µg) in exposure medium (0.5 mL) to the apical side of the transwell, and their transport to the basolateral side was monitored at different time points: 1, 2, 5, 24 and 48h, in three independent experiments (n=3). At each time point, 50 µL of medium from the basolateral side was transferred into a black 96-well plate and the fluorescence intensity was measured with a plate reader (Tecan Infinite M200, Tecan Trading AG), with excitation and emission wavelength set at 441 and 486 nm, respectively. Fluo-PS in three different conditions were used for the translocation studies: non-coated particles (NC), particles pre-coated with human plasma (P), and particles pre-coated with heat-deactivated human plasma (HDP). We chose plasma, not serum, as it contains clotting factors and thus resembles more closely maternal blood. A whole blood sample was obtained from a healthy donor with informed consent (University Medical Center Utrecht, NL). The plasma was isolated by centrifuging the whole blood (EDTA-treated) for 25 min at 500 x *g*, at room temperature. The collected plasma was stored at -20 ˚C. Heat-deactivated plasma was prepared by heating in a water bath at 56˚C for 1h. The coating was done by incubating the particles (5 µL of each highest stock) with plasma (1:4 particles to plasma ration, v/v), for 2h at 37˚C and constant shaking (at 100 rpm). The particles in the plasma (20 µL) were resuspended in the exposure medium before cell exposure to a final volume of 1 mL and particle concentration of 100 µg/mL, and final plasma concentration of 1.6%. The mass transported (ΔQ_n) into the basolateral side at each time point (t_n) was calculated with a calibration curve (0.01, 0.1, 1, 10, 100 µg/mL) prepared for each of the conditions, and corrected for blank sample (fluorescent intensity of non-exposed cells) and volume loss during sampling, using the following equation:

$$\Delta Q_n = C_n \cdot V_w + \sum_{j=1}^{n-1} C_n \cdot V_s$$

where C_n is the concentration of the sample measured at time t_n, V_w is the volume of the well sampled, V_s is the sampling volume (50 *µL), and the term* $\sum_{j=1}^{n-1} C_n \cdot V_s$ represents the cumulative mass removed by sampling during all the previous sampling periods.[52] The final amount transported was reported as a number of particles transported and as a percentage of the initial mass added to the apical well (50 µg). The method's limits of detection were calculated for each time point separately as 3 x standard deviation of the signal measured in the exposure medium only (n=3).

Cell viability

The viability of the cells was determined using CellTiter-Glo® luminescent assay, which measures adenosinetriphosphate (ATP) as an indicator of metabolically active cells. The amount of ATP present is directly proportional to the number of viable cells. Shortly, BeWo b30 cells were seeded at a density of 2 x 10^5 cells/mL (100 µl per well) in a black 96-well plate. Cells were differentiated and exposed to MNPs at 0.1, 1, 10 and 100 µg/mL on day 6 post-seeding as described above. The concentration range was chosen to encompass a wide range of concentrations used in previous studies[17,19], and falls within the quantifiable concentration of plastic particles found recently in human blood (1.6 µg/ml).[53] After 24h exposure, the plate was equilibrated to room temperature for approximately 30 minutes. After, 50 µL of medium from each well was transferred to a new 96-well plate and an equal volume of assay reagent was added. ATP disodium salt was used to generate a calibration curve. A stock solution of ATP (10 mM) was prepared in the exposure medium and the calibration curve was prepared by serial tenfold dilutions of the stock (5 nM - 5 µM) and added in parallel to each plate. Contents were mixed for 2 min on an orbital shaker to induce cell lysis and then allowed to incubate at room temperature for 10 minutes to stabilize the luminescent signal. Luminescence was measured using a plate reader (Tecan Infinite M200). Data was corrected for the background luminescence measured in the wells containing medium only and expressed as the mean percentage of the control (cells with no exposure), based on three independent experiments (n=3).

Lactate Dehydrogenase Activity Assay

Lactate dehydrogenase (LDH) activity assay was used to evaluate the damage to the plasma membrane. In short, 100 µL of BeWo b30 cell suspension (2 x 10^5 cells/mL) was added to each well of a transparent 96 well-plate. Cells were grown and differentiated as described above. At day 6 post-seeding, syncytialized and nonsyncytialized cells were exposed to MNPs at 0.1, 1, 10 and 100 *µg/mL* or a positive control Triton-X 100 (0.1%, v/v) in 100 µL of exposure medium. After 24 h exposure, 10 µL of medium from each well was sampled into another 96 well-plate containing 40 µL of LDH assay buffer. Also, a NADH standard curve (2.5 - 12.5 nmol/well) was added in parallel to each plate. Next, 50 ***µ***L of the Master Reaction Mix was added to each well, mixed for 2 min using a horizontal shaker and measured ($t_{initial}$) at 490 nm in a plate reader (Tecan Infinite M200). After the initial measurement, the plate was incubated at 37˚C. Subsequent measurements were taken every 5 min until the value of the most active sample was greater than the value of the highest standard. Data was corrected for the background absorbance measured in LDH assay buffer only and the LDH activity was calculated using the following formula:

$$LDH\ activity = \frac{B\ x\ sample\ dilution\ factor}{(reaction\ time)x\ V}$$

where B is the amount of NADH (nmole) generated between $t_{initial}$ and t_{final}, the reaction time is t_{final} - $t_{initial}$ (minutes), and V is the sample volume (mL). Mean LDH activity is reported as nanomoles/minute/milliliter (milliunit/milliliter), based on three independent experiments (n=3).

Gene expression analysis

The effects of MNPs on placental hormone synthesis were investigated with real-time quantitative PCR (RT-qPCR) using syncytialized BeWo b30 cells and a set of genes directly involved in the placental steroidogenesis (cyp19, cyp11a1, hsd3b1, hsd17b1, sts, see Table S1 for a detailed primer list). BeWo b30 cells were seeded in 24 well-plate (2 x 10^5 cells/mL), cultured, differentiated and exposed to MNPs at 0.1, 1, 10 and 100 µg/mL for 24 h, in three independent experiments (n=3), with three technical replicates per sample, per plate. After 24 h, the cells were washed with PBS and the RNA was extracted using Tri-reagent following the manufacturer's protocol. The quality and concentration of the extracted RNA were determined with Nanodrop 2000® (Thermo Scientific, NC, USA). RNA (0.6 µg) was reverse transcribed using an iScript cDNA synthesis kit and the cDNA was stored at -20˚C until further use. RT-qPCR was performed with a CFX96 real-time PCR Detection System (BioRad) using SYBR Green universal supermix, 10 times diluted cDNA and 400 nM of each primer in a final reaction volume of 15 µL. qPCR reaction conditions were as follows: 3 min 95*°C* followed by 40 cycles of 15 s 95*°C and* 45 s at 60*°C*. A melting curve was included in each run to check for primer dimer formation and/or formation of secondary products. The Cq (determined by regression) was normalized to two reference genes β-actin and YWHAZ using the BioRad CFX analysis software, and the log2 fold change in expression (ΔΔCq) was calculated relative to the control group (non-exposed cells).

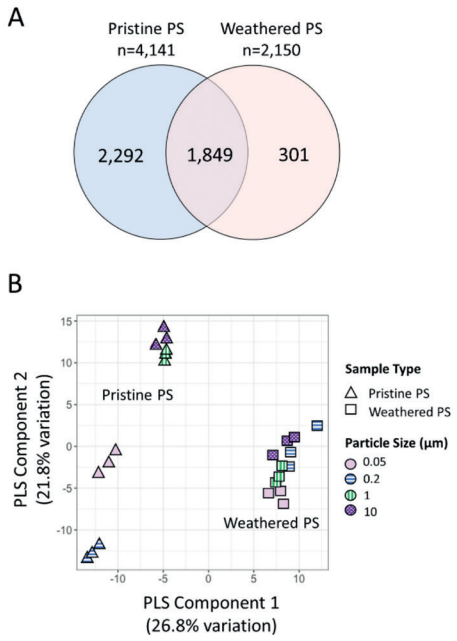
Statistical analysis

Differences in the rate of transport between NC, P and DP Fluo-PS particles over the nonsyncytialized BeWo b30 monolayer were investigated using the mixed-effects model and Tukey's multiple comparisons test. For any other statistical analysis, the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used. All statistical analyses were performed using GraphPad 8.3 (GraphPad Software Inc.) with $p \leq 0.05$ considered statistically significant.

Results

MNP chemical profiling

To mimic environmental weathering processes and to generate representative MNPs for the toxicological studies, PS and HDPE particles were weathered using the procedures described in the Method section. This process was hypothesized to alter the surface chemistry of the MNPs, and potentially increase the adsorption of organic compounds present in surface water. To gain insight into possible chemical changes occurring to the MNPs from the weathering processes, pristine and weathered PS were characterized for small molecule profiles using non-targeted HRMS. Initial analysis of nontargeted results showed 4,141 and 2,150 chemical signals, with peak intensities 5-fold or higher relative to the PBS buffer, detected for pristine PS and weathered PS, respectively (Figure 2A).



C

Plastic Map categories of annotated compounds with VIP > 1.5

Plastic Map Primary Use Category	Number of Annotated Chemicals (%)
Not present in Plastic Map*	13 (22.4%)
Catalyst	8 (13.8%)
Colorant	7 (12.1%)
Biocide	5 (8.6%)
Antioxidant	5 (8.6%)
Plasticizer	4 (6.9%)
Monomer	4 (6.9%)
Odor Agent	2 (3.4%)
Crosslinking Agent	2 (3.4%)
Intermediates	2 (3.4%)
Other Processing Aids	2 (3.4%)
Filler	1 (1.7%)
Antistatic Agent	1 (1.7%)
Lubricant	1 (1.7%)
Heat Stabilizer	1 (1.7%)

*Annotated compounds not listed in Wiesinger et al. (2021)

Figure 2. Differences in chemical profiles between pristine and weathered polystyrene (PS) particles. (A) Comparison of chemical signals detect in the pristine and weathered PS particles using liquid chromatography high-resolution mass spectrometry. (B) Partial least squares discriminant analysis (PLS-DA) of differentially expressed chemical signals between pristine and weathered PS particles. (C) Primary use categories assigned to the annotated compounds using PlasticMap database. Only compounds with high confidence annotations and variable importance projection (VIP) score > 1.5 were considered (Excel Table S1, S2 and S3).

A comparison of the two different PS formulations showed that pristine PS had the largest number of unique compounds, 301 were unique to the weathered PS particles and 1,849 chemical signals were overlapping between the two formulations (Figure 2A).

Potential identities of chemicals detected in the pristine and weathered particles were evaluated by annotating mass spectral signals using chemical databases that included plastic-related compounds, followed by PLS-DA to identify which annotated chemicals showed the greatest difference between pristine and weathered PS. Accurate mass annotation included matching based upon co-eluting adducts and isotopes to reduce false positives and identified 1062 plastic-related chemicals from which 182 were high confidence (level 3) annotations and 880 low confidence (level 4) annotations (Excel Table S2). The 182 plastic-related chemicals detected in pristine and weathered PS corresponded to 282 unique compounds (Excel Table S2). The number of compounds was higher than the number of detected signals because isomers could not be differentiated using this HRMS approach. Annotated compounds included multiple plastic additives, intermediates in polymer synthesis, hydrolysis products, and environmental chemicals (Excel Table S2). PLS-DA analysis showed significant differences in compound levels associated with pristine and weathered PS and suggested that for pristine PS particles, the differences could be mediated by particle surface area (Figure 2B). Primary use categories of compounds annotated with high confidence (level 3) were determined by comparison to a database of over 10,000 plastic-related chemicals (PlasticMap).[50] The primary use categories that differed between pristine and weathered particles (with VIP > 1.5) are listed in Figure 2C and included e.g. catalysts, colourants, biocides, antioxidants, plasticizers and monomers. Use categories for all annotated compounds are provided in Excel Table S3. Compounds present in the PLASTICS|NORMAN and Plastics Packaging Database that were not included in PlasticMap may represent plastic transformation products or environmental chemicals not directly linked to plastic manufacturing.

BeWo b30 syncytialization

Forskolin treatment resulted in a differentiated morphological phenotype evident after 72 hours, i.e., progressive nuclear aggregation followed by intercellular fusion (resulting in large multinucleated cells), loss of epithelial cell-cell junctions and thus loss of barrier integrity of the confluent monolayer (Figure 3 and S1). The tight junction protein ZO-1, a clear cell boundary marker in the nonsyncytialized cells, was notably less pronounced in the syncytialized cells (Figure S1). Therefore, for further imaging, a plasma membrane marker (Vybrant) was used instead. The syncytialized cells were distinct from the nonsyncytialized cells in morphology and size, i.e., consisted of large often multinucleated cells without clear cell boundaries (Figure 3). The adjusted cell density, calculated as the total area of FOV minus background area (Figure 4, Excel Table S4), showed that the syncytialized cells were on average approximately 3 times less dense than the nonsyncytialized cells, with a mean adjusted cell density of 1964 and 5559 per mm^2, respectively.

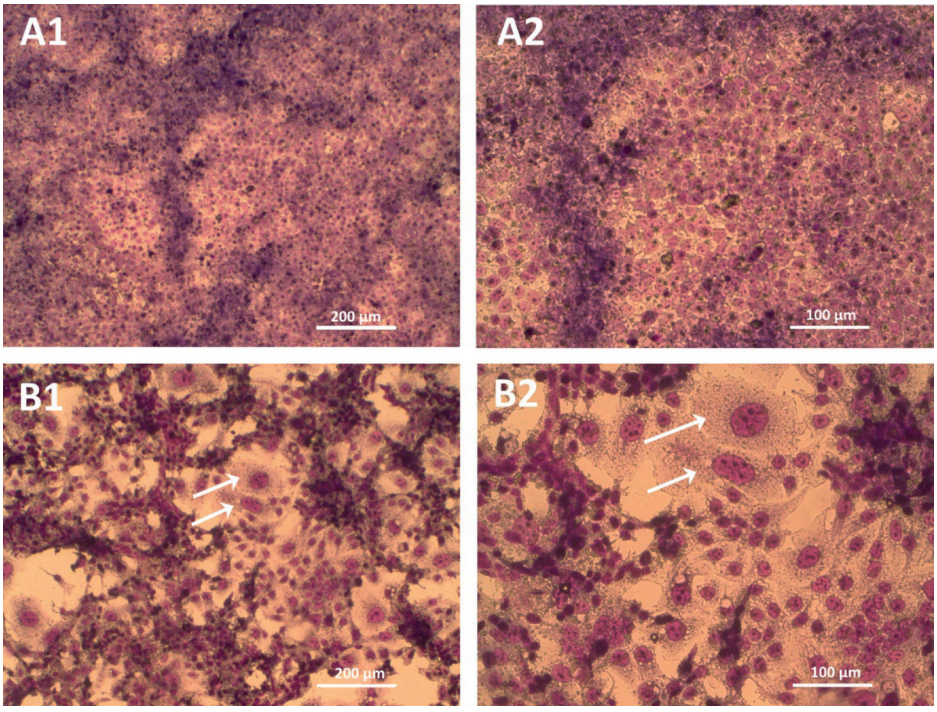


Figure 3. Morphology of the nonsyncytialized (A) and syncytialized (B) BeWo b30 cells at 10 x (1) and 20 x (2) magnification. Syncytialization was induced with 72 h treatment with forskolin (10 µM) as described in the "Materials and Methods" section. White arrows indicate multinucleated cells formed after syncytialization. Images were taken with bright field microscopy after May–Grünwald–Giemsa staining.

Uptake of MNPs

Representative images (still frames) of the internalization of Fluo-PS in the nonsyncytialized BeWo b30 cells are presented in Figure 5 (also see Supplemental Video files for the representative 3D videos). Only Fluo-PS particles at 0.2, 1, and 10 µm were investigated, as it was impossible to quantify individual 0.05 µm particles due to their low fluorescent signal. Non-fluorescent MNPs were also investigated, using transmitted light PMT. Here, only 1 µm and 10 µm pristine and weathered PS particles were quantifiable i.e., clearly visible and distinguishable from other cellular compartments (Figure 6). Moreover, for the non-fluorescent HDPE and PS particles, we observed weak auto-fluorescent in the red part of the spectrum (approximate excitation/emission wavelength of 590/650 nm, Figure S2). However, the auto-fluorescence intensity was too low in comparison with the background cellular auto-fluorescence in the same spectral range, to be able to use it for quantification of cellular uptake. Additional imaging using polarized light microscopy (PLM) indicated that HDPE particles were also in contact with the placenta cells and possibly internalized (Figure S3); however, cellular uptake was not quantifiable using this method.

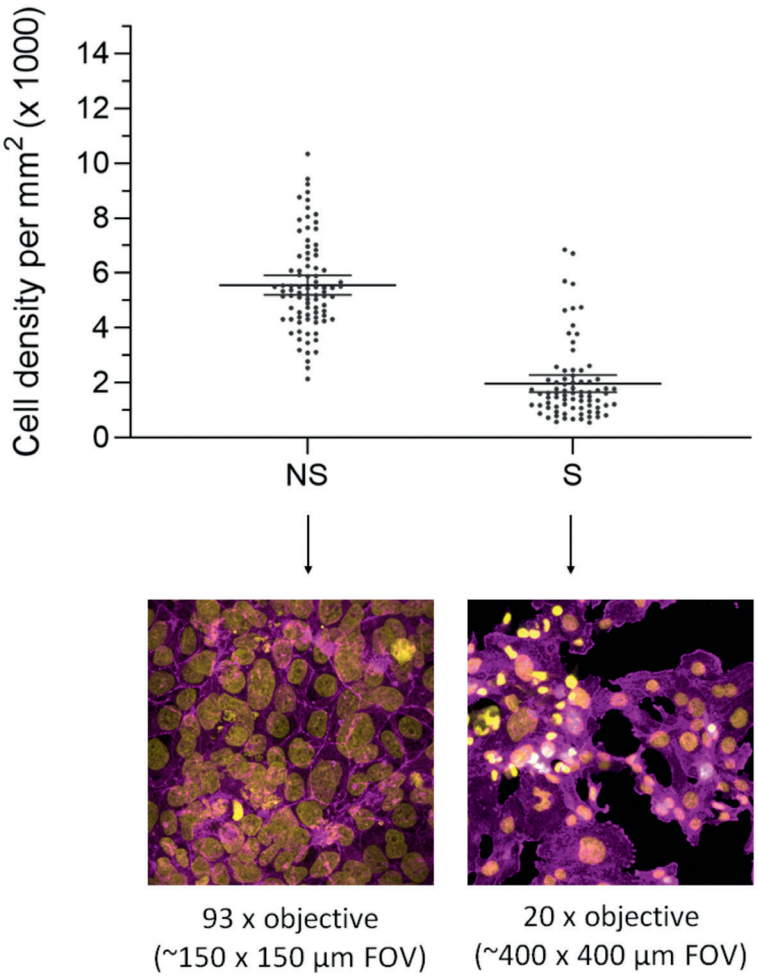


Figure 4. Adjusted cell density per area (mm^2) of the nonsyncytialized and syncytialized cells calculated as the total area of field of view (FOV) minus background area, based on DAPI (nucleus, yellow) and SiR-actin (β-actin, magenta) staining as described in the "Material and Methods" section. Each dot represents cell density calculated from one FOV. Bands represent the 95% confidence intervals of the respective mean (n=8, Excel Table S4).

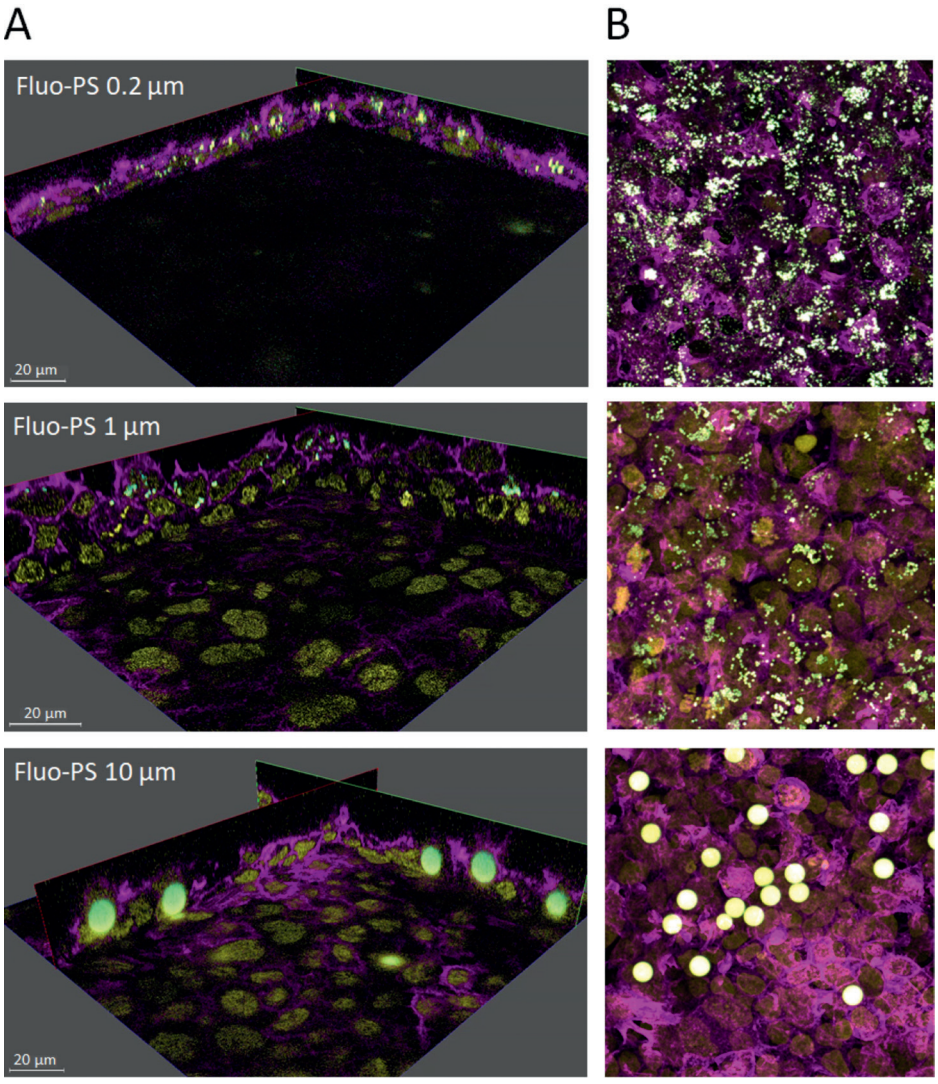


Figure 5. Representative images of the internalization of fluorescent polystyrene particles (Fluo-PS) in nonsyncytialized BeWo b30 cells. Cells were exposed for 24 h to 0.2, 1 and 10 µm Fluo-PS (fluo-green) at 25, 50 and 100 µg/mL respectively, stained for nucleus (DAPI, yellow) and β-actin (SiR-actin, magenta) and imaged with z-stacks using a Leica TCS SP8 STED 3X microscope and 20 x magnification. Images represent still frame (A) from 3D videos (available in the Supplemental Video files) complied from the z-stacks, and maximum projection image (B) of the respective sample.

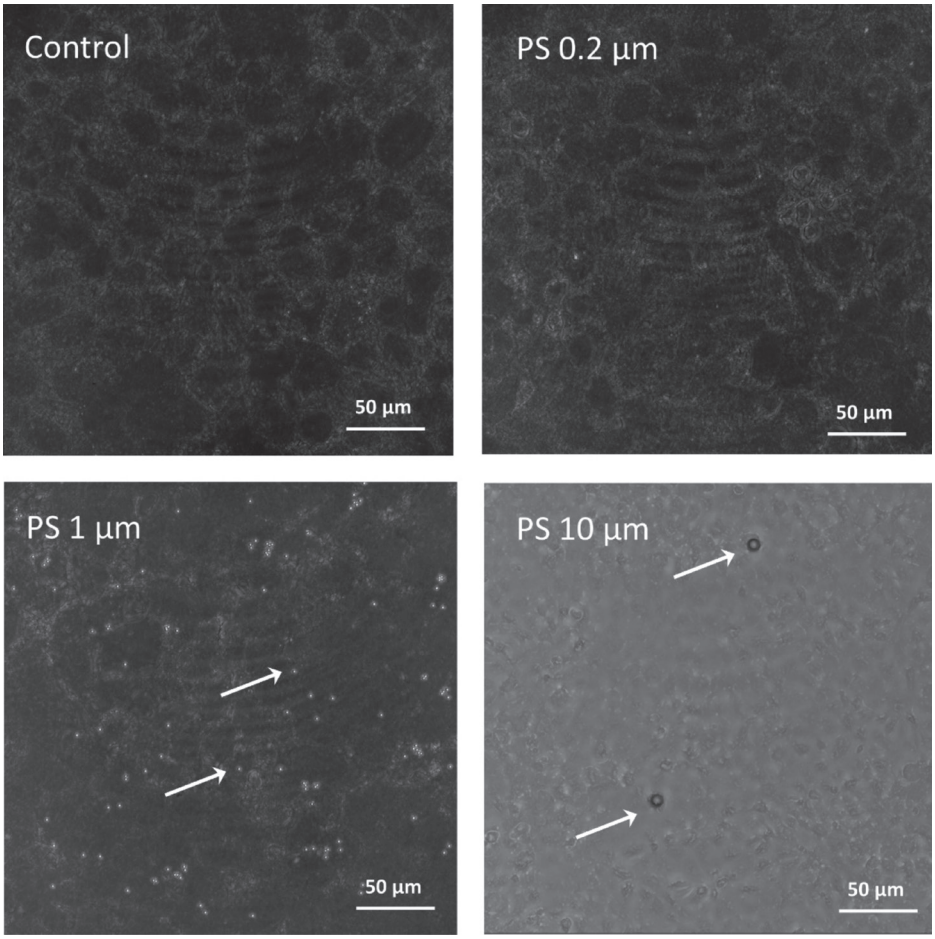


Figure 6. Nonfluorescent pristine polystyrene (PS) particles at 0.2, 1 and 10 µm imaged in transmitted light PMT with a 488-nm laser as a source, using a Leica TCS SP8 STED 3X microscope, in nonsyncytialized BeWo b30 cells. The control sample represents non-exposed cells.

The cellular uptake of 1 µm and 10 µm PS, calculated as a number of particles per area occupied by cells (mm^2), was concentration-dependent and in general lower in syncytialized than in nonsyncytialized cells regardless of the concentration, type (pristine/weathered) or size of PS (Table 1, Excel Table S4). In general, higher uptake was observed at lower exposure concentrations (e.g., for 10 µm PS, 0.83% and 0.46% of the initial mass were taken up at 0.5 and 5 ug/mL, respectively), indicating that the cell capacity for particle uptake was limited, and, might have reached saturation levels at the higher concentrations tested. Particle weathering seemed to affect the uptake of PS, resulting in a higher uptake of 1 µm but lower uptake of 10 µm, in both nonsyncytialized and syncytialized cells (Table 1, Excel Table S4).

Table 1. Comparison of the cellular uptake of pristine and weathered polystyrene (PS) particles between the nonsyncytialized and syncytialized BeWo b30 cells.

			Nonsyncytialized		Syncytialized	
Size	Condition	µg/mL	Mean (SD)	# of FOV	Mean (SD)	# of FOV
1 µm	Pristine	0.1	234 (138)	8	50 (38)	8
1 µm	Pristine	1	950 (381)	7	337 (246)	3
1 µm	Weathered	1	3143 (1917)	15	519 (253)	15
10 µm	Pristine	0.5	8 (4)	10	4 (5)	10
10 µm	Pristine	5	41 (25)	15	26 (14)	10
10 µm	Weathered	5	30 (16)	15	4 (4)	15

Note: Cellular uptake was quantified based on high-content imaging as described in the Method section. Cells were exposed to pristine (including fluorescent) and experimentally weathered PS particles at 1 µm and 10 µm in size and varying concentrations (µg/mL). The mean number of particles taken up was calculated per area occupied by the cells (mm^2), based on ≥ 3 fields of view (FOV) per sample from at least 2 independent experiments, ± SD (Excel Table S4).

Translocation of MNPs

The development and integrity of the nonsyncytialized BeWo b30 monolayer formed in transwell inserts were monitored with TEER and Na-Flu leakage assay for 12 consecutive days (Figure 7). TEER values showed a steady increase over 9 days, stabilizing at 120 $\Omega \cdot cm^2$ on day 10 post-seeding (Figure 7A). Simultaneously, the leakage of Na-Flu decreased over time, with 1.6% (0.08 µM) of the initial concentration added to the apical well measured in the basolateral compartment at day 11 post-seeding (Figure 7B). TEER values and the retardation of Na-Flu transport confirmed the completion of the monolayer formation by day 10 post-seeding.

As the transwell experiments depend on the measurement of fluorescence, the transport of MNPs was investigated only with the Fluo-PS particles. Fluo-PS particles at 0.05 µm, 0.2 µm and 1 µm were added (50 µg) to the apical well at day 11 post-seeding. Additionally, NC, P and DP Fluo-PS particles were used to investigate if the pre-coating with human plasma would influence the transport kinetics. The biggest size particles (10 µm) were excluded from the transport study due to the use of a transwell with a 3 µm pore size i.e., the biggest pore size that allowed for uniform cell growth without cell migration to the basolateral side. The transport of the particles was monitored over 48h at 6 different time points (1, 2, 3, 5, 24 and 48h). All three sizes of Fluo-PS were transported through the nonsyncytialized cell monolayer (Figure 7C-F, Table S2). The quickest transport over the monolayer was observed for 0.05 µm Fluo-PS with the fluorescent signal (> LOD, Table S2A) measured in the basolateral compartment already 1h after exposure, followed by 0.2 µm (5h) and 1µm particles (24h) (Figure 7C-F, Table S2B). After 48h of exposure, the average number of NC particles transported through the monolayer was 6.0×10^{10} (4.13 µg), 4.6×10^{7} (0.2 µg) and 1.4×10^{5} (0.08 µg) for 0.05 µm, 0.2 µm and 1 µm Fluo-PS, which represented 8%, 0.4% and 0.16 % of the initial mass (50 µg) added to the apical chamber, respectively (Figure 7C-F, Table S2C). For 0.05 µm and 0.2 µm par-

ticles, even after 48h exposure, no flattening off of the number of particles transported was observed, which suggests that longer incubation times would result in higher translocation. No significant differences in the rate of transport were observed between NC, P and HDP particles, at any of the time points measured (p>0.05).

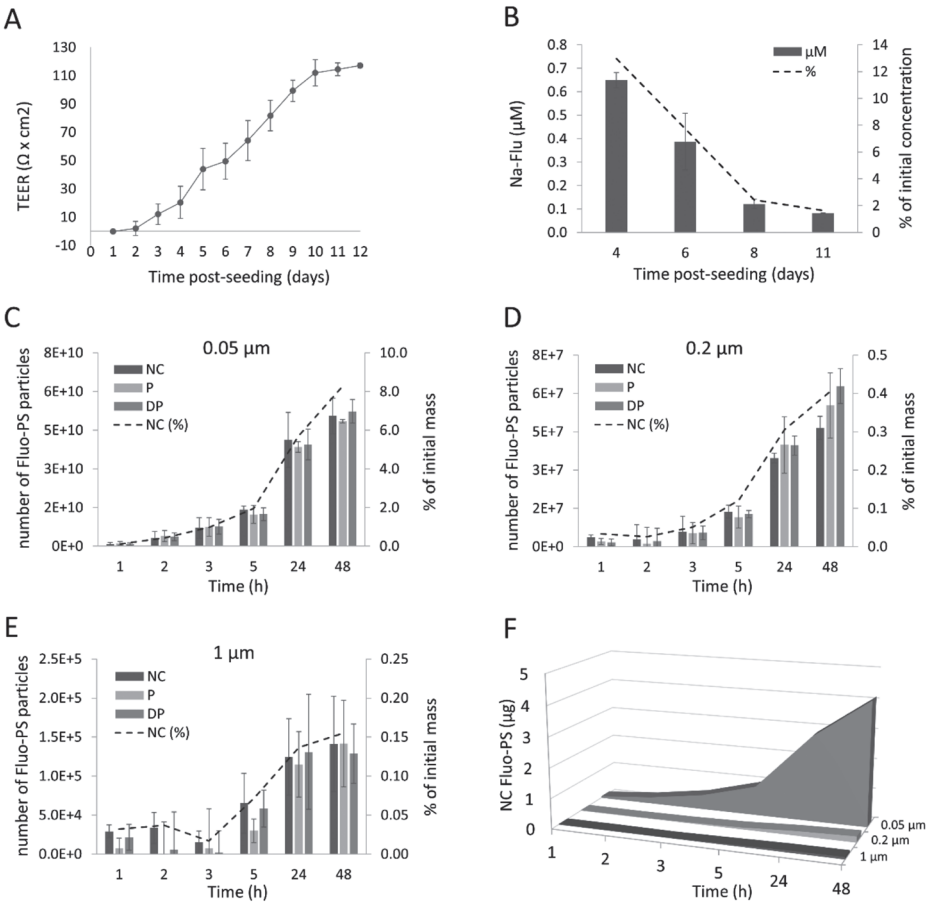


Figure 7. Translocation of fluorescent polystyrene (PS) particles through nonsyncytialized BeWo b30 cells. (A) The formation of the monolayer was measured with TEER. (B) Barrier integrity was monitored with retardation of NA-Flu leakage (from apical to basolateral chamber), reported in µM (or percentage of initial concentration) measured in the basolateral chamber. Time-dependent apical to basolateral transport of (C) 0.05 µm, (D) 0.2 µm and (E) 1 µm, of NC, P and DP Fluo-PS particles, expressed as a number of particles (or percentage of the initial mass (50 µg) transported) measured in the basolateral chamber. (F) Comparison of the time-dependent apical to basolateral translocation (in micrograms of initial mass (50 µg) transported) between different sizes of NC Fluo-PS across BeWo b30 monolayer. Translocation experiments were performed on day 11 post-seeding. Data points represent mean values ± SD (n=3, Table S2). No significant differences in the rate of transport were observed between NC, P and DP particles, at any time point measured (mixed-effects model, p>0.05). Note: DP, pre-coated with heat-deactivated human plasma; NC, noncoated; P, pre-coated human plasma; PS, polystyrene; SD, standard deviation.

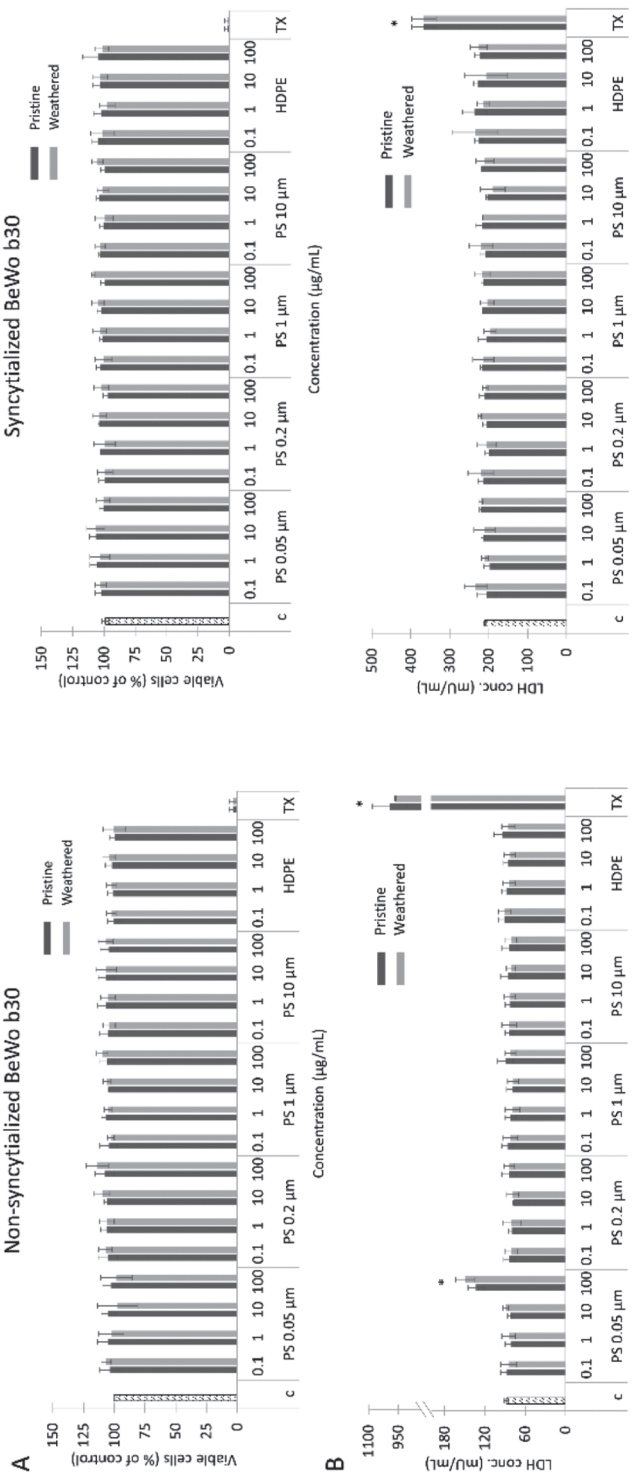


Figure 8. Cell viability (A) and plasma membrane integrity (B) of the nonsyncytialized and syncytialized BeWo b30 cells exposed for 24h to 0.1, 1, 10 and 100 µg/mL pristine and weathered HDPE, PS particles (0.05, 0.2, 1 and 10 µm) and Triton X (TX, 0.1%) as a positive control, measured with CellTiter-Glo® viability assay LDH activity assay respectively, as described in the "Material and Methods" section. Data points represent the mean percentage of ATP present ± SD (n=3, Excel Table S5) and LDH concentration (mU/mL) ± SD (n=3, Excel Table S5). Control (c) represents non-exposed cells (one-way ANOVA, * $p \leq 0.05$). Note: ANOVA, analysis of variance; ATP, adenosine triphosphate; HDPE, high-density polyethylene; LDH, lactate dehydrogenase; PS, polystyrene; SD, standard deviation.

Cytotoxicity, LDH activity and effects on steroidogenesis

The cytotoxicity of MNPs was investigated using CellTiter-Glo luminescent assay, which measures the amount of ATP present in culture as an indicator of metabolically active cells. None of the pristine or weathered PS (0.05, 0.2, 1, 10 µm) and HDPE particles showed cytotoxic effects in the nonsyncytialized or syncytialized cells, after 24 h exposure to 0.1, 1, 10 and 100 µg/mL concentration (Figure 8A, Excel Table S5). Plasma membrane integrity, quantified by measuring the release of the intracellular LDH into the cell culture medium upon damage to the plasma membrane, was also not affected by any of the conditions tested, except for 0.05 μm PS particles at the highest concentration (100 µg/ml), in the nonsyncytialized cells only (Figure 8B, Excel Table S5). In general, the LDH concentration measured in the cell culture medium was two times higher in the syncytialized cells (approx. 200 mU/mL) as compared to the nonsyncytialized cells (approx. 100 mU/mL, Excel Table S5).

We also examined the expression of five genes involved in the placental biosynthesis of steroid hormones in syncytialized BeWo b30 cells to investigate the effects of pristine and weathered MNP and associated chemicals on placental steroidogenesis. Of the five investigated genes, only hsd17b1 was downregulated in cells exposed to pristine MNPs and to a lesser degree weathered MNPs, whereas hsd3b1 was marginally downregulated only after exposure to pristine particles (Figure 9, Figure S4, Excel Table S6). This trend was observed regardless of the type of size of MNP tested, nevertheless, it was not statistically significant ($p \leq 0.05$).

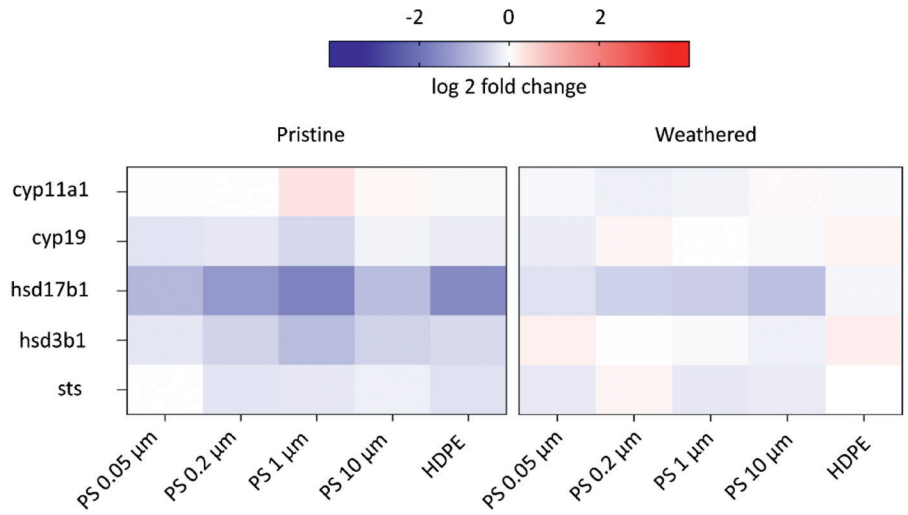


Figure 9. Heat map representing the log2 fold change values in the expression of genes involved in steroidogenesis measured in the syncytialized BeWo b30 cells after 24h exposure to 10 µg/mL of pristine and weathered HDPE and PS particles (0.05, 0.2, 1 and 10 µm). Values were normalized against the endogenous reference genes β-actin and YWHAZ. Red implies higher expression, white no difference, and blue lower expression relative to the non-exposed cells (n=3, Excel Table S6). Note: HDPE, high-density polyethylene; PS, polystyrene.

Discussion

Human *in vitro* cell models of the placenta barrier are indispensable for understanding the placental uptake of MNPs and their possible adverse effects on placental functioning and consequently fetal and maternal health. Villous cytotrophoblasts and syncytiotrophoblasts are important cells of placenta villi, with the latter being the main cells responsible for maternal/fetal exchange and other specialized placental functions, such as hormone synthesis and metabolism.[28,54] Nevertheless, to our knowledge, the toxicokinetics and toxicity of MNPs in syncytiotrophoblasts *in vitro* have not been reported. This study set out to investigate the uptake, transport and toxicity of MNPs in both nonsyncytialized and syncytialized BeWo b30 cells i.e., well-established *in vitro* placenta cell models representative of villous cytotrophoblasts and syncytiotrophoblasts, respectively.

In this study, all PS particles tested, including the large 10 µm particles, were taken up by both nonsyncytialized and syncytialized cells. To our knowledge, only one other study by Cartwright et al. (2012) investigated PS uptake in nonsyncytialized BeWo cells whereas uptake in syncytialized cells has never been investigated. Overall, the nonsyncytialized cells showed higher uptake than the syncytialized cells. The smaller particles (0.05 um) were taken up more efficiently than the larger particles, confirming observation by Cartwright et al. (2012) [52], and the total uptake was higher at lower exposure concentrations, indicating that the cell capacity for particle uptake was limited, and might have reached saturation levels at the higher concentrations and/or larger sizes. This data is supported by *ex vivo* placental perfusion studies where a size-dependent maternal-to-fetal translocation of PS particles (up to 0.5 µm in size) was observed, with a lower transfer rate corresponding to a higher accumulation of larger PS particles in the placental tissue, specifically in the syncytiotrophoblast layer.[20,55] While the staining of cellular compartments together with z-stack confocal imaging and spectroscopic detection allowed for confirmation of cellular internalization of PS particles, the irregularly shaped, non-fluorescent HDPE were not distinguishable from other cellular compartments and their internalization could not be confirmed with this method. Imaging using polarized light microscopy (PLM) indicated that HDPE particles were indeed in contact with the placenta cells and possibly internalized (Figure S3). Further studies are needed to confirm HDPE uptake using other methods or labelled particles.

Transcellular transport of Flu-PS across the nonsyncytialized BeWo b30 monolayer was limited (<10%) and size-dependent, with higher transport rates observed for particles with a smaller diameter. Transcellular transport studies could only be performed with nonsyncytialized BeWo b30 cells that formed a tight, polarized monolayer on the transwell insert. Syncytialization resulted in cell fusion and loss of barrier integrity, therefore MNP translocation using syncytialized BeWo cells could not be investigated. To our knowledge, *in vitro* transplacental transport of MNPs in nonsyncytialized BeWo b30 mon-

olayers has been previously investigated in four studies, all of which used Fluo-PS particles as model MNPs. These studies reported size-dependent and limited transport within 24h of exposure.[18,19,52,56] It should be noted that there are discrepancies in the scientific literature regarding the timing of BeWo monolayer formation in the transwell set-up (ranging from 4 to 10 days post-seeding). [18,57–59] These differences might stem from different insert types, pore size, pore density, passage number, seeding density, medium type and volume used, among others.[52,60] It is, therefore, crucial to monitor the monolayer formation on a day-to-day basis, using more than one indicator, such as a combination of TEER, retardation of Na-Flu transport or/and confocal imaging.[58] Moreover, it is important to note that the transwell inserts can, to various degrees, retain particles on the apical side, which might be dependent on the pore sizes and insert type. For example, polyester insert membranes have been shown to retain particles with higher efficiency than e.g., polycarbonate inserts.[52] In this study, the translocation of MNPs through the polycarbonate insert membrane only was significantly higher than when measured with the presence of non-syncytialized BeWo cells (Table S2), which further confirmed the limited cellular transport of MNPs. In our study, the 3 µm pore size was the largest pore size that allowed BeWo b30 monolayer formation without cellular migration to the basolateral side; nevertheless, it still limits the MNP size range that can be investigated in the transwell set-up to < 3 µm. However, using imaging we showed that MNPs up to 10 µm in diameter were internalized by the nonsyncytialized and syncytialized BeWo cells.

In our study, the transport of 0.05 µm and 0.2 µm particles did not reach a plateau at 48 hours (Figure 7C and D), suggesting that longer incubation periods may result in higher transport. Different size-dependent transport mechanisms have been proposed for MNPs including passive diffusion, facilitated diffusion and energy-dependent transport such as endocytosis.[19,20] Interestingly, trophoblastic cells also have the capacity for phagocytosis. [41,61,62] Nevertheless, the extent to which these mechanisms influence MNP transplacental kinetics is still unknown. The size-dependent, limited transport of Fluo-PS observed *in vitro* is generally in agreement with *ex vivo* placenta perfusion studies.[22,55] However, in placenta perfusions, PS particles accumulated mainly in the syncytiotrophoblasts layer, suggesting that syncytiotrophoblasts and not the cytotrophoblasts play a major role in regulating transplacental particle transport.[20,35] The use of syncytialized BeWo cells may therefore provide more accurate estimations of the uptake or/and transplacental transport of MNPs. It should be noted, however, that the expression profiles of e.g., transporter proteins, such as ATP-binding cassette (ABC) transporters and solute carriers (SLC), are not fully comparable between syncytialized BeWo cells and primary human trophoblast cells, likely in part due to the different developmental stages they represent.[40] Overall, carefully validated experimental models are needed to study different aspects of trophoblast function. Furthermore, the use of placental co-cultures,

although technically challenging, may more accurately reflect complex cell-to-cell interactions and provide a better understanding of the placental toxicokinetics of MNPs.

The protein corona has been shown to play an important role in the transport of metallic nanoparticles, in *in vivo* cellular models and *ex vivo* placenta perfusion models (reviewed by Bongaerts et al. 2020)[63], however, the influence of the protein corona on transplacental transport of MNPs is still not well understood. In our study, prior incubation of Fluo-PS with human plasma or heat-deactivated human plasma did not enhance particle transport across BeWo b30 barrier. In contrast, Gruber et al. (2020)[21] found that the protein corona formed during incubation of PS with human plasma enhanced placental transfer in a human *ex vivo* placenta perfusion study. In particular, the protein corona formed by human albumin was found to significantly induce the transplacental transfer of drugs as well as PS particles.[21,64] Moreover, serum heat-deactivation has been shown to affect PS protein corona composition, resulting in enhanced nanoparticle uptake in e.g., the human lung barrier model.[65] In our study, the presence of FBS in the exposure medium might explain why we did not observe enhanced transport. It has been previously suggested that the complex interaction between particles and protein-rich media, may lead to progressive displacement of proteins with lower affinity in favour of those with higher.[66] This could change corona composition and dominate the overall properties of the particles[19,65,67], possibly neglecting the influence of prior plasma coating on cellular transport. Further studies are needed to corroborate this hypothesis.

The MNPs tested in this study showed no acute cytotoxicity. No effects on cellular ATP production were observed, whereas plasma membrane integrity was disrupted only after exposure to 50 nm PS particles at 100 µg/mL and only in the nonsyncytialized cells. At this concentration, both weathered and non-weathered particles disrupted the integrity of the plasma membrane; however, such a high concentration might not be relevant considering environmental exposures. Although, human exposure levels are still not well characterized, recently plastic particles were found in human blood at a mean concentration of 1.6 µg/ml[53], suggesting that the lower concentration range tested in this study (0.1 – 10 ug/mL) might be relevant with regard to environmental exposures. In the *in vitro* placenta cell models published to date, PS particles generally do not show cytotoxicity.[17,18,52] However, a study by Kloet et al. (2015)[19] showed that positively charged 0.05 µm PS particles induced significant cytotoxicity at concentrations ≥ 20 µg/mL, whereas negatively charged particles did not. The authors argue that the electrostatic attraction between the positively charged particle and the negatively charged cell membrane could explain the cytotoxicity observed. The protein corona formed on the surface of the particle incubated with FBS could dominate the charge of that particle making it slightly negative, which could explain why in most *in vitro* studies that used media supplemented with FBS, no cytotoxicity is generally observed. Studies with various other human cell models link MNP exposure with apoptosis, necrosis, genotoxicity and proinflammatory

responses (recently reviewed by González-Acedo et al. 2021).[11] Toxicity endpoints, other than cell viability, should therefore be considered, as well as longer exposure periods that would be more reflective of the possible long-term accumulation of MNPs in syncytiotrophoblasts *in situ*.

An important function of the placental syncytiotrophoblasts is the production/metabolism of hormones crucial for the maintenance of pregnancy and fetal development.[28,68] Accordingly, dysregulation of syncytiotrophoblasts has been shown to impact placenta functioning and fetal health.[54,68] In this study, the effects of MNPs on the expression of genes essential in steroid hormone biosynthesis were tested using syncytialized BeWo b30 cells. From the five genes tested, hsd17b1 was modestly down-regulated in cells exposed to pristine MNPs and to a lesser degree weathered MNPs, whereas hsd3b1 was marginally down-regulated only after exposure to pristine particles. The enzyme hsd17b1 catalyses the final step in estrogen biosynthesis by reducing the weak estrogen estrone to yield the potent estrogen 17β-estradiol[69], whereas hsd3b1 catalyses the conversion of pregnenolone to progesterone, amongst others.[70] Though not significantly different relative to controls, the downregulation of these genes by MNPs was observed in both PS and HPDE at all sizes tested. This finding warrants further investigation using longer exposure periods, as inhibition of hsd17b1 and hsd3b1 has been associated with a reduction of bioavailable levels of these hormones which may lead to various adverse pregnancy outcomes.[71–74]

Chemical profiling of PS particles revealed compositional differences between pristine and weathered particles, with the former showing a higher number of unique features. A recent review, based on industrial, scientific, and regulatory data sources, revealed more than 10 000 plastic monomers, additives, and processing aids associated with plastics.[50] From the plastic-related substances of potential concern (i.e., substances that met one or more of the persistence, bioaccumulation, and toxicity criteria), 58 of the substances were annotated using features detected in the pristine or weathered PS in this study (Figure 2C, Excel Table S3). The primary categories of the plastic-related compounds differing between pristine and weathered particles included 14 use categories (Figure 2C). The presence of these compounds is surprising since the PS particles were single polymer particles and a large number of impurities was not expected. In-depth chemical characterization of pristine and environmental weather plastics is warranted, which may provide insight into potential sources of impurities present in otherwise pristine particles. Nevertheless, the impurities detected in the pristine particles could be due to the leaching of residual monomers/oligomers (e.g., styrene monomers (for PS), aliphatic hydrocarbons (for HDPE), additives (plasticizers, colourants) and processing aids (lubricants, catalyst)), which could contribute to the chemical toxicity of the particles.[50,75,76] For example, certain plastic additives such as bisphenols and phthalates are known endocrine-disrupting compounds capable of inhibiting human placental hsd3b1.[70,77,78] In this

study, several plasticizers and styrene-related compounds have been tentatively identified in the pristine PS particles (Excel Table S3), however, their contribution to the observed subtle effects on gene expression is unknown. The differences in the chemical composition of pristine and weathered PS could come from leaching, hydrolysis and/or photochemical reactions that occurred during weathering, possibly explaining why weathered particles were less potent. Taken together, these results suggest weathering changes the chemical composition of MNPs, including the potential to alter the baseline composition of particles. PLS-DA analysis revealed differences in chemical characteristics between the different sizes and conditions (pristine vs weathered PS), suggesting that the surface area of pristine PS additionally influences the chemical composition. These results support the importance of considering environmental weathering processes when performing toxicological studies with MNPs.

Recent evidence demonstrates the presence of MNPs in the human placenta and meconium, which suggests that maternal exposure to MNPs may result in placental uptake, transplacental transport and fetal exposure.[23,79] However, the underlying toxicokinetics and toxicity are still not fully understood. In this study, cells central to the maternal-fetal interface showed limited uptake of pristine and weathered MNPs *in vitro*, which was associated with a lack of acute toxicity and subtle effects on placental gene expression. Although plastic-associated compounds or co-contaminants might be relevant for the effects observed in this study, more in-depth investigations are needed to better understand the potential health risks of MNPs and chemicals associated with them under real-life conditions and environmentally relevant exposure scenarios. *In vitro* placenta cell models together with the development of analytical tools and workflows for the detection and characterisation of MNPs in maternal samples and explorative cohort studies are indispensable to understanding the potential risk associated with MNPs to maternal and fetal health.

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Supplemental Material

S1. Reagents

Minimum Essential Medium (MEM, 11095-080), Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 medium (DMEM/F12) with GlutaMax (31331-028), DMEM/F12, HEPES, without phenol red (11039-021), penicillin/streptomycin (P/S, 15140-122), trypsin (25300-054) and foetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany); Dulbecco's phosphate buffered saline (PBS, D8537), dimethyl sulfoxide (DMSO, 99.9+%, D8418), collagen (type IV) from human placenta (C5533), forskolin (F3917), fluorescein sodium salt (Na-Flu, 46960), 4,6-diamidino-2-phenylindole (DAPI, 10236276001), Triton™ X-100 solution (93443), bovine serum albumin fraction V (BSA, 10735086001), Tri-reagent (T9424) and lactate dehydrogenase activity assay kit (MAK066) were purchased from Sigma Aldrich (Poole, Dorset, UK); Paraformaldehyde (PFA, 157-8) was obtained from Electron Microscopy Science (PA, USA). CellTiter-Glo® luminescent cell viability assay (G7570) was purchased from Promega (Leiden, the Netherlands). Vybrant™ CM-DiI cell-labelling solution (V22888), ZO-1 monoclonal primary antibody (ZO1-1A12, 33-9100) and goat anti-mouse secondary Alexa594 (A-11032) labelled antibodies were purchased from Invitrogen (Breda, the Netherlands) and SiR-actin Kit (SC001) from SpiroChrome AG (Stein am Rhein, Switzerland). iScript cDNA synthesis kit (170-8891) and SYBR Green universal supermix (172-5121) were purchased from BioRad (Veenendaal, the Netherlands).

Table S1. RT-qPCR primer list.

Full name	*Abbrev.*	*Key gene function*	*Strand*	*Primer sequence*	*Ref.*
Beta-actin	*β-actin*	Cytoskeletal, cell structure and integrity	Forward Reverse	TTGTTACAGGAAGTCCCTTGCC ATGCTATCACCTCCCCTGTGTG	[1]
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	Glycolysis, breakdown of glucose for energy	Forward Reverse	CTCTCTGCTCCTCCTGTTCGAC TGAGCGATGTGGCTCGGCT	[2]
14-3-3 protein zeta/delta	*ywhaz*	Regulation of signal transduction pathways	Forward Reverse	ATGCAACCAACACATCCTATC GCATTATTAGCGTGCTGTCTT	[3]
Aromatase (estrogen synthetase)	*cyp19*	Biosynthesis of estrogens	Forward Reverse	CCGGGGCCTGACAGAGCTTTCATA TTGGGCTGCAGTGCATCGGT	N/A
Cholesterol side-chain cleavage enzyme	*cyp11a1*	Conversion of cholesterol to pregnenolone	Forward Reverse	TGCATCTTCAGTCGTCTGTCCCAA AGGTGACCACTGAGAACCCATTCA	N/A
17β-Hydroxysteroid dehydrogenase 1	*hsd17b1*	Estrogen activation and androgen inactivation	Forward Reverse	ACTCCATTGGCTGTCCATGACTGA TGCACAAAGGCCCAAAGAATCTGG	N/A
3beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase1	*hsd3b1*	Production of progesterone	Forward Reverse	AGAAGAGCCTCTGGAAAACACATG TAAGGCACAAGTGTACAGGGTGC	[4]
Steroid sulfatase	*sts*	Conversion of sulphated steroid precursors to free steroid	Forward Reverse	GGAGTGAGAAGGGCATGGTA CTCCAGCAGCCTCTTTATGG	N/A

Note: Primer sequences were based on published data or designed using the NCBI primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with the following conditions: annealing temperatures of 60˚C, amplicon length between 70 and 200 bp and preferably spanning exon-exon junction. For every primer set the efficiency was determined using serial dilutions of cDNA; N/A - not applicable

Table S2. Time-dependent apical to basolateral transport of fluorescent polystyrene (Fluo-PS) particles.

A: *LOD (% initial dose transported)*

Fluo-PS size	*Coating*	1h	2h	3h	5h	24h	48h
0.05 µm	*NC*	0.02	0.02	0.02	0.16	0.16	0.26
	P	0.02	0.02	0.02	0.15	0.15	0.24
	HDP	0.02	0.02	0.02	0.15	0.15	0.24
0.2 µm	*NC*	0.01	0.02	0.01	0.10	0.10	0.17
	P	0.02	0.03	0.02	0.17	0.17	0.28
	HDP	0.01	0.02	0.01	0.10	0.10	0.17
1 µm	*NC*	0.01	0.01	0.01	0.08	0.08	0.14
	P	0.01	0.01	0.01	0.09	0.09	0.15
	HDP	0.01	0.01	0.01	0.08	0.08	0.14

B: *% of initial dose transported (± SD)*

Fluo-PS size		1h		2h		3h		5h		24h		48h	
	Coating	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
0.05 µm	*NC (no cells)*	14.65	(2.64)	19.96	(3.48)	26.92	(3.52)	33.60	(4.60)	53.61	(5.80)	65.00	(5.55)
	NC	0.11	(0.08)	0.43	(0.35)	0.98	(0.55)	1.95	(0.19)	5.67	(1.47)	8.27	(0.96)
	P	0.15	(0.10)	0.54	(0.29)	1.04	(0.50)	1.70	(0.49)	5.29	(0.29)	6.78	(0.09)
	HDP	0.12	(0.06)	0.50	(0.21)	1.04	(0.39)	1.70	(0.34)	5.42	(0.82)	8.28	(0.63)
0.2 µm	*NC (no cells)*	13.74	(0.31)	18.23	(0.54)	25.13	(0.63)	30.45	(0.50)	47.40	(0.84)	61.07	(0.23)
	NC	0.03	(0.01)	0.03	(0.05)	0.05	(0.05)	0.12	(0.02)	0.31	(0.02)	0.40	(0.04)
	P	0.02	(0.01)	0.01	(0.06)	0.05	(0.04)	0.10	(0.04)	0.35	(0.10)	0.61	(0.11)
	HDP	0.01	(0.01)	0.02	(0.04)	0.05	(0.02)	0.11	(0.01)	0.35	(0.03)	0.55	(0.06)
1 µm	*NC (no cells)*	16.98	(0.20)	25.84	(0.24)	35.12	(0.33)	46.47	(0.44)	79.91	(0.46)	91.77	(0.84)
	NC	0.03	(0.01)	0.04	(0.02)	0.02	(0.02)	0.07	(0.04)	0.14	(0.05)	0.16	(0.07)
	P	0.01	(0.01)	0.00	(0.05)	0.01	(0.06)	0.03	(0.02)	0.13	(0.05)	0.16	(0.06)
	HDP	0.02	(0.02)	0.01	(0.05)	0.00	(0.03)	0.06	(0.03)	0.14	(0.08)	0.14	(0.04)

C: *number of particles transported (± SD)*

Fluo-PS size	*Coating*	1h		2h		3h		5h		24h		48h	
		%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
0.05 µm	*NC (no cells)*	1.5E+11	(4.1E+09)	2.1E+11	(5.0E+09)	2.8E+11	(6.8E+09)	3.5E+11	(9.2E+09)	5.6E+11	(9.5E+09)	6.8E+11	(1.7E+10)
	NC	7.8E+08	(5.9E+08)	3.1E+09	(2.6E+09)	7.1E+09	(4.0E+09)	1.4E+10	(1.4E+09)	4.1E+10	(1.1E+10)	6.0E+10	(7.0E+09)
	P	1.1E+09	(7.0E+08)	3.9E+09	(2.1E+09)	7.6E+09	(3.6E+09)	1.2E+10	(3.6E+09)	3.8E+10	(2.1E+09)	4.9E+10	(6.7E+08)
	HDP	8.6E+08	(4.5E+08)	3.7E+09	(1.5E+09)	7.6E+09	(2.8E+09)	1.2E+10	(2.5E+09)	3.9E+10	(6.0E+09)	6.0E+10	(4.6E+09)
0.2 µm	*NC (no cells)*	1.2E+09	(5.5E+07)	1.6E+09	(9.6E+07)	2.2E+09	(1.1E+08)	2.7E+09	(8.9E+07)	4.2E+09	(1.5E+08)	5.4E+09	(4.1E+07)
	NC	3.8E+06	(7.7E+05)	3.0E+06	(5.6E+06)	5.8E+06	(6.1E+06)	1.4E+07	(2.6E+06)	3.5E+07	(2.0E+06)	4.6E+07	(4.5E+06)
	P	2.0E+06	(1.1E+06)	1.1E+06	(6.5E+06)	5.3E+06	(4.2E+06)	1.2E+07	(4.3E+06)	4.0E+07	(1.1E+07)	6.9E+07	(1.3E+07)
	HDP	1.7E+06	(1.3E+06)	2.2E+06	(5.0E+06)	5.4E+06	(2.7E+06)	1.3E+07	(1.4E+06)	4.0E+07	(3.7E+06)	6.3E+07	(6.8E+06)
1 µm	*NC (no cells)*	1.6E+07	(5.0E+06)	2.4E+07	(6.6E+06)	3.3E+07	(6.7E+06)	4.4E+07	(8.7E+06)	7.6E+07	(1.1E+07)	8.7E+07	(1.1E+07)
	NC	2.9E+04	(8.3E+03)	3.4E+04	(2.0E+04)	1.5E+04	(1.4E+04)	6.5E+04	(3.8E+04)	1.2E+05	(4.9E+04)	1.4E+05	(6.1E+04)
	P	7.1E+03	(1.3E+04)	2.0E+04	(2.7E+04)	7.1E+03	(5.1E+04)	3.0E+04	(1.5E+04)	1.2E+05	(4.2E+04)	1.4E+05	(5.5E+04)
	HDP	2.2E+04	(1.7E+04)	5.9E+03	(4.8E+04)	1.9E+03	(2.8E+04)	5.8E+04	(2.4E+04)	1.3E+05	(7.4E+04)	1.3E+05	(3.8E+04)

Note: Time-dependent apical to basolateral transport of non-coated (NC), pre-coated with human plasma (P) and pre-coated with heat-deactivated human plasma (DP) Fluo-PS particles (0.05 µm, 0.2 µm and 1 µm) in the nonsyncytialized BeWo b30 cells, and, time-dependent apical to basolateral transport of NC particles through transwell membrane only (no cells). A) Limit of detection (LOD) was calculated for each time point separately as 3 x standard deviation of the signal measured in the exposure medium only (n=3). The particles measured in the basolateral chamber were expressed as B) % of initial mass added (50 µg) to the apical well or C) as a number of particles transported (± standard deviation (SD), n=3), as described in the method section.

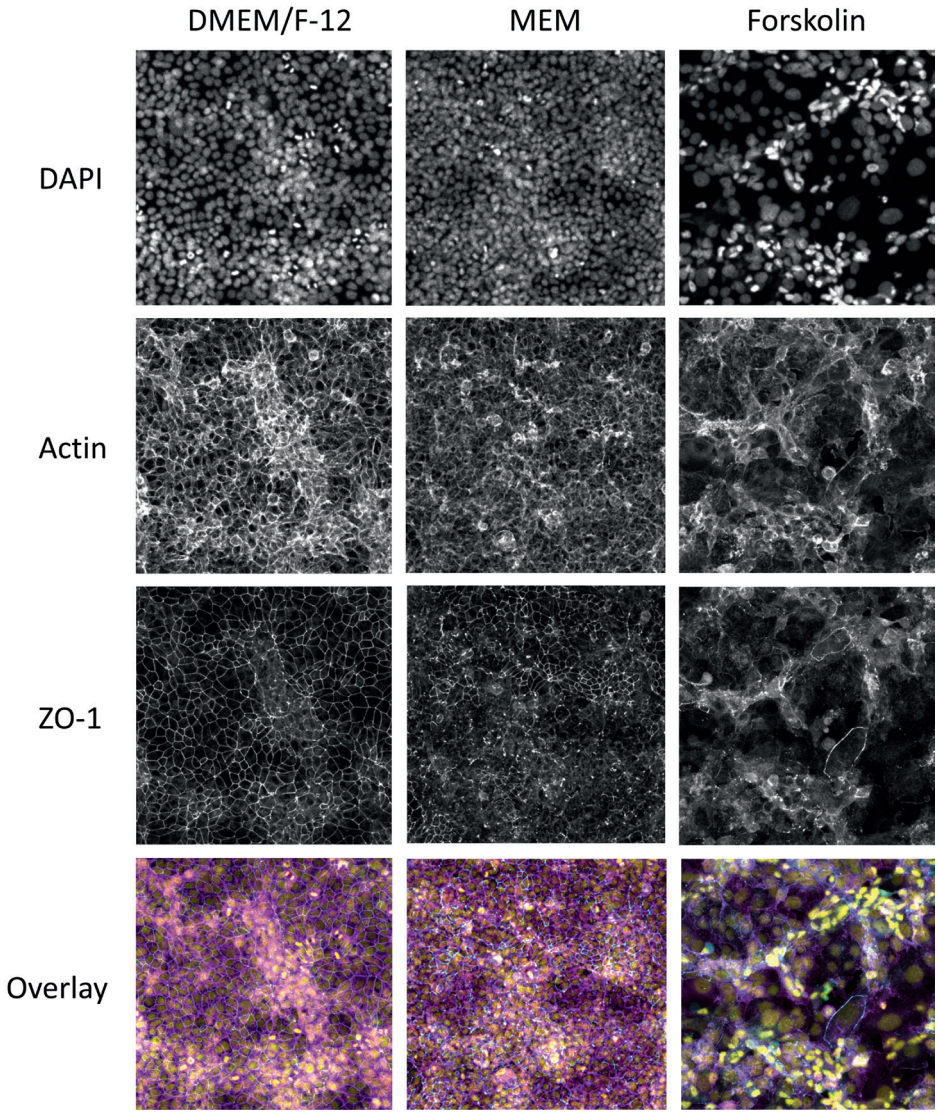

Figure S1. Nonsyncytialized BeWo b30 cells cultured with DMEM/F12 and MEM culture media, and syncytialized BeWo b30 cells (forskolin) cultured in DMEM/F12. The cellular components i.e., nucleus, β-actin and tight-junctions, were stained with DAPI, SiR-actin and ZO-1 and are presented in the overlay image as yellow, pink and blue, respectively. Images were taken with laser-scanning confocal Leica TCS SP8 STED 3X microscope using 20 x magnification (size of the field of view equal to ~ 400 x 400 μm).

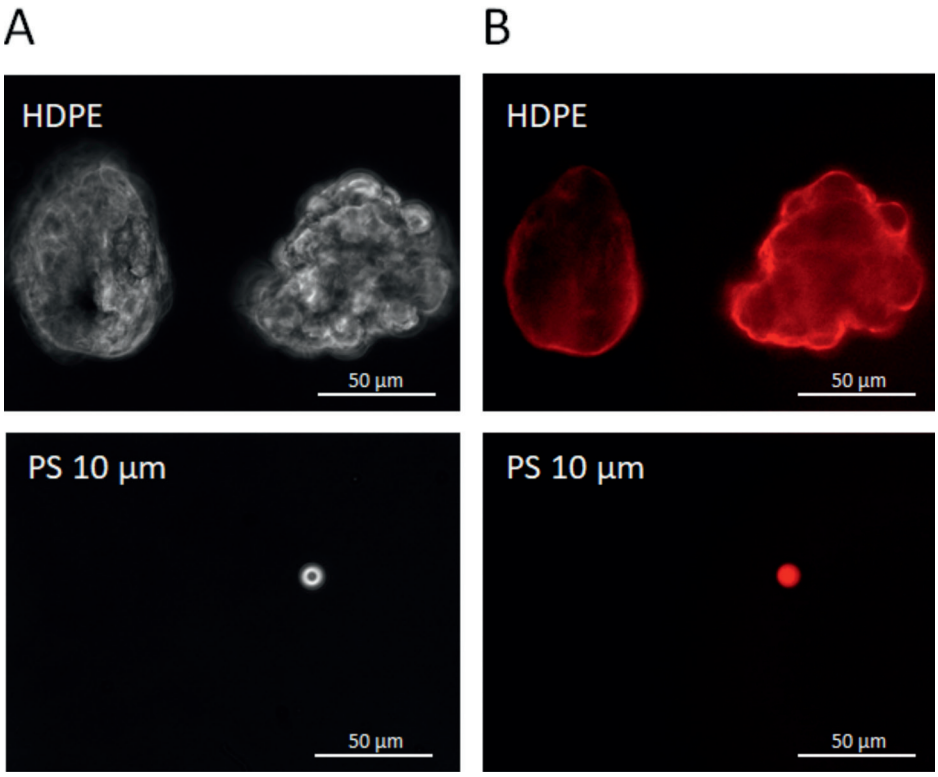

Figure S2. Pristine high-density polyethylene (HDPE) and 10 µm polystyrene (PS) particles imaged in A) phase contrast (10 ms exposure time) and B) red fluorescence (590 nm excitation/650 nm emission, 1000 ms exposure time) with Nikon Ti inverted microscope using Photometrics CoolSNAP HQ2 CCD camera.

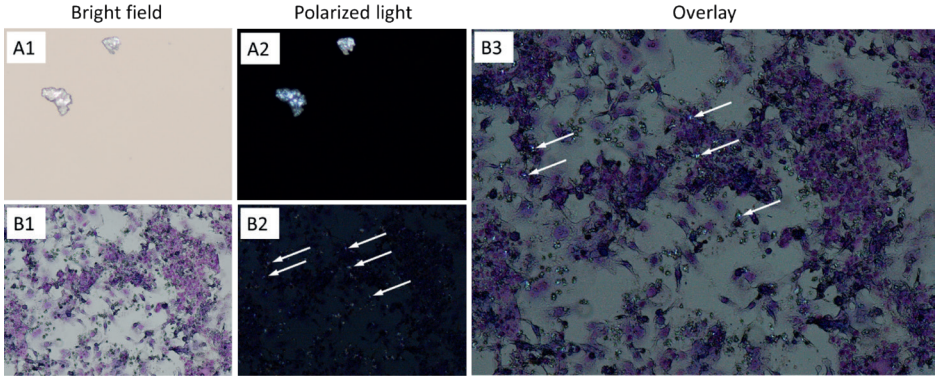

Figure S3. Pristine high-density polyethylene (HDPE) particles (A1-2) and syncytialized placenta cells after 24h exposure to HDPE particles imaged in bright field (B1), polarized light (B2), and bright field overlay with polarized light (B3), using polarized light microscopy (PLM). HDPE particles (arrows show representative particles) appear to glow blue in polarized light.

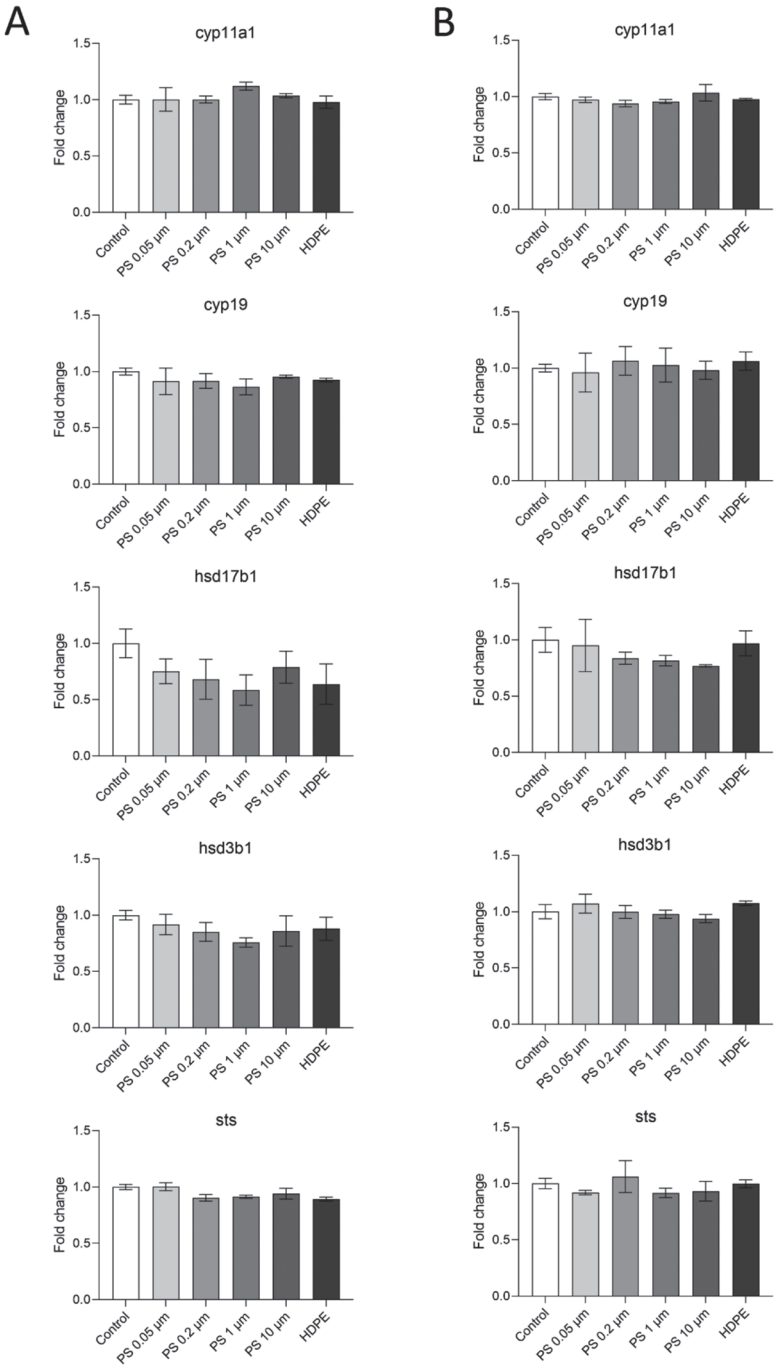

Figure S4. Expression of genes involved in steroidogenesis measured in the syncytialized BeWo b30 cells exposed to 10 µg/mL of pristine (A) and weathered (B) high-density polyethylene (HDPE) and polystyrene (PS) particles at 0.05, 0.2, 1 and 10 µm in size. Data bars represent mean fold change ± SEM, normalized against the endogenous reference genes β-actin and YWHAZ (n=3).

References

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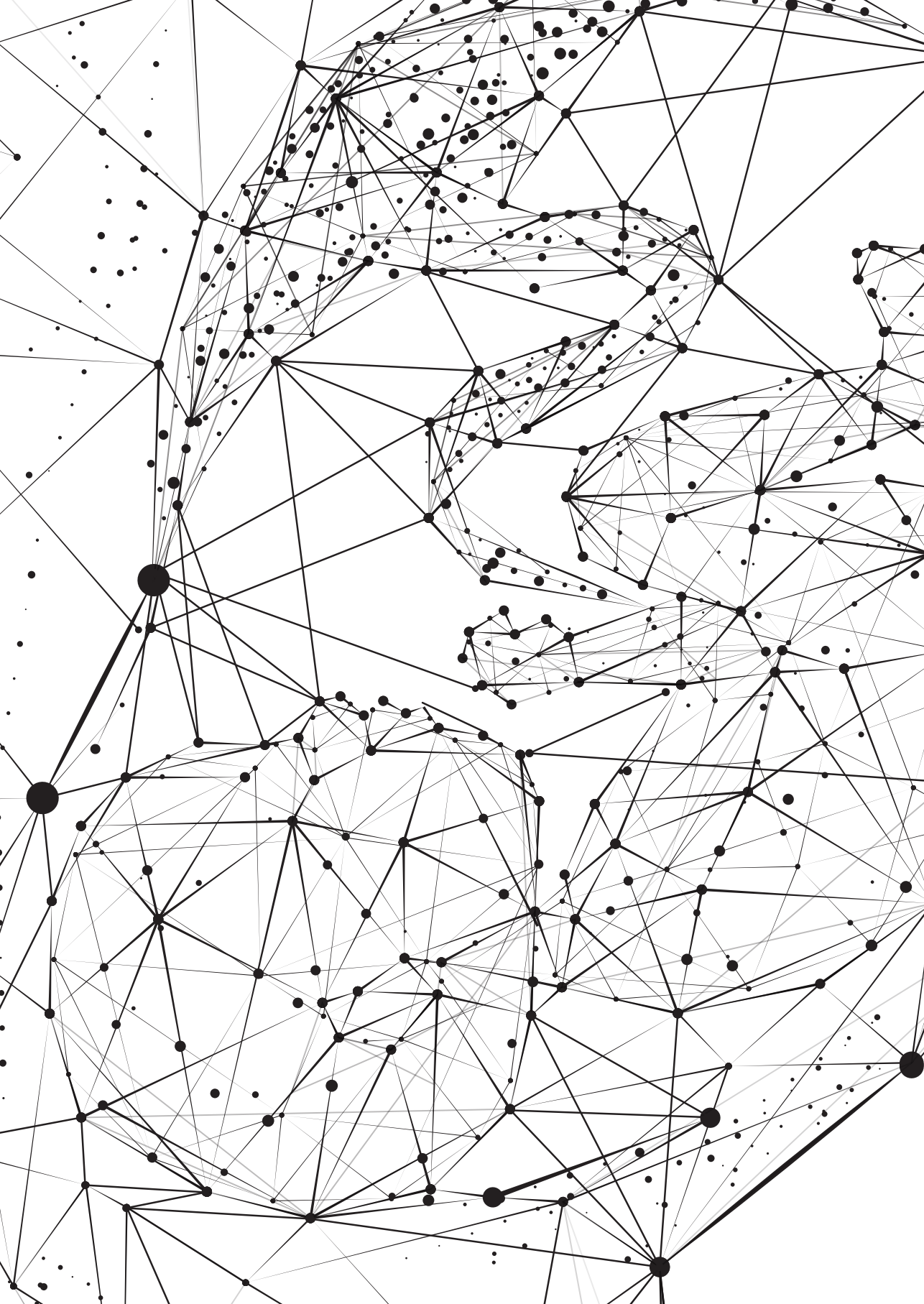
PART III

GENERAL DISCUSSION AND CONCLUSIONS



6

General discussion and conclusions

There are hundreds if not thousands of environmental contaminants to which the general population, including pregnant women, are widely and unceasingly exposed. Many have already been shown to reach the *in utero* environment, where they expose the developing fetus during its most vulnerable stages of life. Prenatal exposure to environmental contaminants may have profound effects on the way the body programmes itself for later life, influencing developmental processes and contributing to adult-onset diseases. Specifically, EDCs are increasingly recognized as important risk factors for the fetal programming of adult disease as the developing fetus is highly sensitive to hormonal perturbations. To date, only a small fraction of the hundreds of thousands of synthetic chemicals in existence have been assessed for endocrine disrupting activity. Most probably our current understanding of human exposure, especially *in utero* exposure, to these compounds is only "the tip of the iceberg". The obvious ethical limitations of studying the *in utero* environment compel researchers to use maternal matrices as surrogates of fetal exposures. Unfortunately, our limited understanding of the intricate placental and *in utero* toxicokinetics largely hinders maternal-fetal extrapolation and risk assessment efforts. Furthermore, the cumulative effect of co-occurring chemicals is of great concern, nevertheless, still rarely addressed in the studies on early life exposures as only a limited number of well-known EDCs are usually investigated. As follows, characterization of the entire spectrum of EDCs and their metabolites present *in utero* is still a major challenge and the disease risk due to *in utero* exposure to mixtures of EDCs may be significantly underestimated. Considering the growing body of evidence, from epidemiological and experimental animal studies, that associates prenatal exposures to a small number of known EDCs with a multitude of pregnancy complications, developmental dysfunctions and health issues that can manifest themselves later in life, prenatal exposure to complex mixtures and possibly novel EDCs is worrisome, to say the least.

Moreover, there are new contaminants of emerging concern such as MNPs for which human health hazards, specifically their effects on pregnancy, are virtually unknown. MNPs are ubiquitous in the environment and have recently been found in human lungs and blood demonstrating internal human exposure. MNPs that infiltrate the bloodstream may reach the placenta and expose the fetus during its most vulnerable stages of life. The placental transport and toxicity of MNPs are exceptionally understudied, but specifically important to investigate since the placenta is an intermediate between the maternal and fetal compartments which, through diverse and complex mechanisms, strictly regulates developmental processes and maternal physiology during pregnancy. Accordingly, the disruption of placental development or functioning will adversely affect fetal and maternal health. Alarmingly, the first reports on the presence of MNPs in the human placenta are emerging, but little is known about their toxicokinetics and toxicity in this crucial temporary organ. To move this field forward and provide data needed to assess the risks of MNP exposure during pregnancy, we first need to understand the challenges

and most pressing knowledge gaps and examine available methods that can be used to gather robust data.

The comprehensive assessment of *in utero* exposure to these contaminants of emerging concern is critical to our understanding of the relationship between prenatal exposure, maternal and fetal health and postnatal disease and however challenging, in the light of recent evidence, it should be prioritized in order to protect the most vulnerable. Consequently, this thesis set out to address the imminent knowledge gaps concerning the *in utero* EDC and MNP exposure discussed above. To this end the main objective of this thesis was to 1) develop a method for analysis of a wide range of EDCs including novel compounds using amniotic fluid as a direct matrix of fetal exposure; 2) identify known and novel, previously unidentified EDCs *in utero*; 3) determine appropriate methods and models to study placental MNPs and to fill the pressing knowledge gaps; 4) investigate the uptake, transport and toxicity of MNPs and associated compounds in human-relevant placental cell models.

Characterization of EDCs *in utero*

The first objective of this thesis was to develop a novel method to characterize a broad spectrum of EDCs present *in utero*. In order to address this objective, our approach required 1) an alternative matrix to maternal matrices, unique to the *in utero* environment and representative of the direct fetal exposure; 2) a new method capable of measuring the chemical "exposome" *in utero,* focusing on a wide range of known and potential novel EDCs.

Amniotic fluid as a matrix of direct fetal exposure

In this thesis, AF was used as a matrix of choice for studying the direct chemical environment of the fetus (**Chapters 2 & 3**). AF is a unique biological medium and *in utero* repository of environmental chemicals and their metabolites that the mother was exposed to during pregnancy. As compounds in AF continuously circulate through the fetal membrane, AF reflects not only maternal/placental but also fetal toxicokinetics, and overall is a better approximation of fetal body burden to environmental pollutants than maternal matrices. Van de Beek et al. (2004) showed that there is a low correlation of endogenous hormones between maternal serum and AF. Moreover, sex differences in testosterone and androstenedione were found in AF but not in maternal serum [1]. The authors argued that from all available sources (maternal serum, umbilical cord blood and AF), amniotic fluid was most suitable to investigate the effects of early fetal androgen exposure. An overall low correlation of organophosphorus flame retardants (OPs), polyfluoroalkyl compounds (PFCs), benzophenones, parabens and bisphenols have been found between paired maternal samples (serum, blood, urine) and AF [2–6]. For a lot of compounds, including endogenous molecules, maternal-placental-fetal kinetics (possible

transplacental transport mechanisms including protein binding, passive diffusion and active transport) are still not well understood, making extrapolation from maternal matrices to *in utero* environment challenging.

AF can be collected in the second or third trimesters, during amniocentesis or noninvasively during delivery. Considering that placental/fetal toxicokinetics as well as the volume of AF changes during pregnancy, the composition of full-term AF may significantly differ from second-trimester AF and can provide exposure information during specific windows of susceptibility. However, the use of AF for the characterization of fetal exposure to EDCs has so far been limited. AF is considered an aqueous matrix which is less complex in its composition than other matrices such as maternal blood, umbilical cord blood or meconium, and the reported concentrations of EDCs are generally an order of magnitude higher in these matrices than in AF. Nevertheless, with the development of sensitive analytical tools, most notably mass spectrometry, a range of inorganic and organic environmental contaminants have been measured in AF and our study further confirms the successful detection of a wide range of compounds with differing polarity in this matrix. It is important to note, that this is the first study to characterize fetal exposure to EDCs in AF by EDA analysis. A limited number of AF samples was used initially to demonstrate the feasibility of this matrix for analysis of substances that may be a health risk to the developing fetus. Further work using a larger cohort would be required to identify exposure and risks on a population level. Overall, there is a largely unexplored potential to use AF to investigate the direct chemical environment of the fetus, and more studies with this unique fetal matrix are recommended.

Sample preparation for EDA analysis

Due to the low concentrations of exogenous compounds in AF, sample preconcentration is required to increase the detection rate. However, AF contains a mixture of biological components and developmental products, such as endogenous hormones, cells, nutrients, growth factors, lipids and proteins, and thus significant preconcentration may cause the accumulation of organic constituents that may interfere with consecutive biological and/or chemical analysis. Considering that EDCs are extremely heterogeneous and include chemically and structurally diverse compounds, a nondiscriminating method for extraction of a wide range of lipophilic and hydrophilic EDCs from AF with low interference from the matrix is needed. The development and validation of an effective extraction method is a crucial part of the EDA workflow.

AF was spiked with a mixture of 18 reference EDCs, representing diverse chemical groups and polarities, and extracted using a combination of different SPE sorbents and DLLE solvents (**Chapter 2**). The mixture of DCM/acetone (extraction solvent/dispersive solvent) as a stronger nonpolar mixture performed better in extracting more hydrophobic compounds than the mixture of chloroform/acetone. However, in both cases, recov-

eries below 40% were found for some weak acids with a high number of hydroxyl groups, such as genistein and E4, that have a low affinity to partition to nonpolar organic solvents. To aid the recovery of compounds with higher polarity, two different SPE columns, MCX and HLB, have been additionally tested with HLB showing overall higher recoveries (68%) for the reference compounds than MCX (47%). The two methods were combined to maximize the extraction efficiency i.e., the nonpolar compounds that did not adsorb to HLB-SPE during loading were recovered with DLLE (DCM/acetone). Additionally, acidification of the sample increased the extraction efficiency of the weak acids, resulting in a successful and highly efficient extraction method for a largely diverse group of compounds (87 ± 12%) (**Chapter 2**).

Despite the satisfactory recovery of diverse EDCs, some considerations should be noted with our approach. Protein binding may cause low recoveries of compounds which have a high affinity to bind to proteins. Since albumin is alkaline and represents the major protein found in AF, we cannot exclude that some acidic and neutral compounds would bind to albumin during the extraction procedure and would precipitate with proteins during AF sample preconcentration [7,8]. However, the amount of total protein in AF is low (~0.4%) and significantly lower than in maternal blood (~7%) or umbilical cord blood (~5%), therefore disruption of protein binding was omitted in the pre-treatment stage. Moreover, hydrolysis of phase II conjugates is often performed before sample extraction to increase concentrations of parent compounds and their metabolites in the final extract. However, in this study deconjugation step was omitted for three important reasons: 1) deconjugation is not always complete and has to be optimized as it depends on the analyte, enzyme type, reaction time and temperature [9]; 2) use of deconjugation enzymes may lead to the formation of additional metabolites; 3) excluding the deconjugation step allows for analysis of free, unconjugated and biologically active compounds which are more physiologically relevant than their conjugated counterparts. Moreover, two reference compounds, namely FICZ and MNP, showed low recoveries at different stages of the sample preparation procedure, possibly due to their decomposition under UV light in aqueous solutions [10]. Decomposition during sample processing might, at least partially, contribute to the absence of other photosensitive compounds in the final extract/fractions and thus sample preparation under low UV exposure and storage in opaque vials should be considered.

To isolate xenobiotics from natural hormones and identify known and unknown EDCs, the separation of compounds into fractions and consequent reduction of the chemical complexity of the extract is needed before bioassay analysis. For this purpose, fractionation of the AF extract was performed on the RP-HPLC column. An RP-HPLC column was chosen as it is highly stable and can be applied for the separation of a broad range of compounds with differing polarities. However, lipids, specifically phospholipids, can interfere with RP chromatographic methods, as they are strongly retained on hydrophobic columns

and can cause significant ionization suppression during mass spectrometric detection [11]. Although the extraction method developed here showed that the combination of SPE and DLLE can be used to extract a broad spectrum of EDCs from AF with low interference from the matrix (**Chapter 2**), matrix effects cannot be fully excluded.

It should be noted that while higher resolution fractionation results in a reduction of the chemical complexity of the fractions, it may also result in loss of sensitivity during bioassay analysis as eluting compounds become divided over an increasing number of fractions. Considering the overall low concentrations of EDCs in AF reported in the literature, lower resolution fractionation (25 fractions) was applied to avoid loss in sensitivity. In general, good separation of all reference compounds was achieved and a significant correlation was observed between RT and compounds' log *P*, which aid the identification of unknowns by narrowing down candidate compounds that eluted in the biologically active fractions. Considering the high activity observed in the diluted fractions, higher resolution fractionation might be possible, and together with the use of high-throughput bioassays (e.g., 96 well-plate format), might further accelerate compound identification [12,13]. Overall, our study showed that a broad spectrum of EDCs of non-persistent and persistent nature, with a range of polarities including highly nonpolar compounds such as dioxins, furans, PBDEs and PCBs can be successfully extracted, fractionated and detected in full-term AF with low interference from the matrix (**Chapters 2 & 3**).

Endocrine activity in amniotic fluid

In this study, a battery of cell-based *in vitro* reporter gene bioassays that measure reporter gene expression under the control of important steroid hormone receptors and transcription factors were used to determine the endocrine disrupting activity profile in the AF extract and fractions (**Chapters 2 & 3**). These bioassays are cost-effective, fast, animal-free, quantitative, robust and highly sensitive and therefore invaluable in the EDA approach for trace-level detection of compounds with a diverse endocrine disrupting mode of action. We tested serial dilutions of the AF extract and the 25 fractions on six different endpoints from three different reporter gene assays, that is, the AR-Luc, ER-Luc, and DR-GFP in agonistic and antagonistic modes. The diluted AF extract showed agonistic (but not antagonistic) receptor activity in all three bioassays. Estrogenic activity was specifically high, even in the extract diluted 500 times, corresponding to 1.4 nmol (374.5 ng) EEQ/L. This is not surprising as AF contains multiple steroid hormones, including multiple estrogens that could contribute to the activity observed. The levels of natural estrogens such as 17ß-estradiol (E2), estrone (E1) and estetrol (E4) (hormone produced exclusively by the fetal liver) increase throughout pregnancy and peak at parturition, and concentrations found in full-term AF are usually considerably higher than in e.g., mid-gestation [14,15]. However, only 48% of the activity observed in the ER-Luc bioassay could be

explained by these potent hormones. After fractionation, elevated activity was observed mainly in the early polar (F2, F5, F10) and mid-polar (F11, F14, F15) fractions. Activity in the mid-polar fractions corresponded well with the retention times of reference estrogenic compounds, that is, natural hormones (E2 and E1, F14–15) and phytoestrogens (enterolactone, F11). However, activity in the polar fractions could not be explained by any of the compounds measured in this study. Interestingly, the highest estrogenic activity was observed in F10 but none of the measured potent natural estrogens eluted in this fraction. Other estrogens with higher polarity such as E3, and hydroxylated estrogen metabolites together with other weaker ER ligands (xenobiotics (e.g., perfluorinated compounds) or phytoestrogens) previously measured in AF are most likely contributors to the remaining activity [16–19]. There are also sulphated steroid hormones present in AF (usually at much higher concentrations than the unconjugated steroids) however these are known to be biologically inactive [20].

The AR and DR activity of the AF extract was significantly lower than ER activity, corresponding to 76.6 pmol (22.2 ng) DHT-EQ/L and 10.1 pmol (3.3 ng) TEQ/L, respectively. In this study, three mid-polar and two nonpolar fractions showed elevated androgenic activity. It is possible that the activity in the mid-polar fractions, at least to some extent, is caused by endogenous AR ligands, such as testosterone (log *P* 3.32) and weaker androsterone (log *P* 3.7) commonly detected in AF [14,15,21]. The relatively high androgenic activity found in F20 and F21 was noteworthy and could be of xenobiotic origin, as natural androgens are not expected to elute in these highly nonpolar fractions. The antiandrogenic activity was observed for multiple fractions without observed effects on cell viability (**Chapter 2**). Interestingly, antiandrogenic activity was not observed in the unfractionated extract. Indeveri et al. (2014) noted a similar effect with lower total antiandrogenic activity in placental extracts than in the sum activities in the fractions. The androgenic compounds in the unfractionated extract may mask the antiandrogenic effects, highlighting the importance of fractionation in the EDA approach and the subsequent reduction of chemical complexity. The dioxin-like activity was prominent, specifically in the nonpolar fraction (F19 to F23). This is not surprising as AhR ligands typically consist of highly lipophilic environmental contaminants, including polychlorinated dibenzo-p-dioxins, polychlorinated/polybrominated biphenyls, polychlorinated dibenzofurans, and polycyclic aromatic hydrocarbons [22]. Curiously, weaker dioxin-like activity was also found in earlier fractions (F13–F17), possibly indicating the presence of dioxin-like compounds with intermediate polarity or the presence of hydroxylated metabolites [23]. The dioxin-like compounds are commonly measured in maternal matrices and significant dioxin-like activity was previously measured in maternal plasma, cord plasma [24] maternal serum [25] and placenta [26]. Nevertheless, data on direct *in utero* exposure to these compounds is still surprisingly scarce.

To our knowledge, one other study looked at estrogenic, androgenic, and dioxin-like activity in samples of AF, focusing mainly on endogenous hormones, PFAS and metals [21]. The values for ER and AR activity obtained in this study fall within the range reported by Long et al. (2019). However, the dioxin-like activity was approximately one and a half times higher than the highest value reported by Long et al. (2019). It is important to note that Long et al. (2019) used samples of second-trimester AF whereas our study used full-term AF. The more lipophilic dioxin-like compounds may gradually accumulate in AF throughout pregnancy, resulting in the higher activity observed in full-term AF compared to samples from mid-gestation. Overall, due to changes in both hormone levels and the volume of AF during pregnancy, chemical composition and concentration may differ significantly between second and third-trimester AF, thus direct comparison between these samples is not warranted.

Also, two studies applied the EDA approach for profiling a mixture of EDCs during pregnancy, using placenta rather than AF samples. Lopez-Espinosa et al. (2009) used EDA to assess the "total effective xenoestrogen burden" (TEXB). Two fractions containing lipophilic xenoestrogens (alpha) and endogenous hormones (beta) were assessed for their estrogenicity in MCF-7 breast cancer cell-based E-Screen and Yeast Oestrogen Screen (YES) bioassays. In both assays, alpha and beta fractions showed significant estrogenic activity [27]. Whereas, Indiveri et al. (2014) used EDA and extracts of placental samples to profile chemicals with receptor-mediated (anti)androgenic activities using an *in vitro* yeast-based human androgen receptor transcription screen. The study identified several anti-androgenic compounds, including antimicrobials, insecticides and components of plastic, surfactants and cosmetics as well as new EDCs [28]. These are examples of the broad potential of the EDA approach for the prenatal screening of EDCs. However, the placenta is a mixed-origin sample complex in its morphology. The two sides of the placental membrane i.e., the maternal-facing plasma membrane and the basal membrane facing foetal circulation, are structurally distinct. It has been shown that some compounds (e.g. metals) are not uniformly distributed within the placenta, which may be attributed to the heterogeneity of the matrix [29]. Moreover, it has been shown that the placenta may or may not act as a barrier and accumulate certain pollutants [26]. Considering the above, analysis of placental tissue alone may not be fully representative of the fetal chemical burden.

Identification of known and novel EDCs in AF

The second objective of this thesis was to identify known and novel, previously unidentified EDCs responsible for the observed endocrine activity in AF. Although endocrine activity in AF was observed across fractions of differing polarity, we prioritized analysis of the nonpolar fractions (**Chapter 3**), for four main reasons: 1) many known EDCs are nonpolar and lipophilic; 2) nonpolar EDCs have long elimination half-lives, tend to accu-

mulate over time in the maternal body lipids and can be mobilized during gestation and reach the fetus via placental transport; 3) EDCs with higher lipophilicity are thought to more readily cross the placenta than the more hydrophilic compounds; 4) the high activity in the nonpolar AF fractions is noteworthy and is most likely of xenobiotic origin, as natural hormones are not expected to elute in the highly nonpolar fractions.

A high-resolution GC-MS was used for the targeted and non-targeted analysis of chemicals present in the DR, (anti-)AR, and (anti-)ER active nonpolar AF fractions. GC separation coupled with high-resolution MS (HRMS) and EI ionization provides an excellent tool for the analysis of volatile nonpolar compounds, which usually remain undetected with softer ionization methods like ESI [30]. In this study, GC-HRMS was operated at a high mass spectral resolution (60,000) and provided a trace level (pg/mL) detection of nonpolar compounds with high mass accuracy (<1 ppm).

Targeted analysis of the active nonpolar AF fractions

A total of 48 compounds including well-known EDCs were measured in the active nonpolar AF fractions with targeted GC-HRMS analysis (**Chapter 3**). Furthermore, fractions were screened for the presence of additional environmental contaminants by comparing features present in the fractions to a single calibration point of a mixture of 350 environmental pollutants. Such an approach provided fast, accurate and broader chemical profiling of the active fractions and identification of additional (known) contaminants that could be further screened for their potential endocrine disrupting activity using curated online databases e.g., ToxCast/Tox21. From the 350 compounds, 73 were detected in the nonpolar AF fractions, 62 were present in the ToxCast/Tox21 database, but only 14 showed endocrine activity and thus were further quantified using a full calibration curve. Although the remaining 48 environmental contaminants were not relevant for the endocrine endpoints, they might be important for other endpoints relevant to developmental toxicity not investigated in this study (e.g., neurotoxicity, genotoxicity, mutagenicity).

In total, 67 environmental contaminants were quantified during the targeted analysis in the nonpolar AF fractions from which 42 were detected above LODs, at the pg-ng/L AF concentration range. These compounds included a wide range of EDCs from multiple chemical classes (4 dioxins and furans, 10 PBDEs, 12 pesticides, fungicides and herbicides, 6 PCBs, and 10 other compounds, such as plasticizers and PAHs) many of which had never before been measured in AF samples. The lipophilic compounds are usually detected at higher concentrations in more lipid-rich matrices such as fat tissue, breast milk or blood serum (approx. 95%, 4% and 0.5% lipid content, respectively) [26,31–33]. However, they can be eliminated from the maternal compartment via the bloodstream through the placenta and into the fetal blood supply. At the same time, they are released from the maternal lipophilic storage back into the blood to remain the equilibrium [31]. Hence, the circulating low-level of lipophilic contaminants in the maternal blood may result in chronic

exposure to the foetus. It is however difficult to evaluate the distribution of lipophilic compounds between maternal and fetal compartments, specifically AF. The lipid-adjusted concentrations normally used for such comparison, may not yield meaningful results since the AF lipid profile differs significantly from the lipid profile of maternal blood or plasma [34].

It has been suggested that the relative bromination (for BDEs), the extent of chlorination (for PCBs) and the length of a chain and active group (for PFCs) may affect the degree of placental transfer and *in utero* accumulation, perhaps as a result in the difference in molecular weight [35,36]. Consequently, compounds with higher molecular weight are thought to retain in the maternal compartment i.e., can be found in a higher concentration in maternal blood rather than e.g., cord blood. In this study, the higher brominated BDEs (e.g., BDE 209) were detected in AF at a higher concentration than some of the more common lower brominated BDEs (e.g., BDE 47). The higher concentration of BDE 209 detected in AF indicates effective passage through the placenta barrier, lower placental and fetal clearance and/or *in utero* accumulation. One other study measured BDEs in AF and also there the dominant congeners were the high-brominated compounds e.g., BDE-208, 209, 203, 206 [37]. However, this hypothesis is still largely unexplored since data on *in utero* BDE exposure is lacking. Although only 5 out of the 14 PCB congeners analysed were detected above LODs similarly, the presence of the higher chlorinated PCBs in our samples (e.g., PCB 206, 138) confirmed their transplacental transport and *in utero* exposure. These PCBs were previously measured in AF samples [38,39]. The most abundant compounds detected in AF were organochlorine pesticides (OCPs) namely, o,p'-DDD, p,p'-DDD and δ-HCH. Usually, DDE is the most abundant metabolite of DDT measured in maternal samples as well as placenta and cord blood, and thus the higher concentrations of DDD metabolites in AF could point out to specific fetal metabolism and/or toxicokinetics [40,41]. However, this is largely unexplored since data on OCPs in AF is also limited. Multiple PAHs such as IPyr, B[a]P and B[b]F were detected in AF and their for transplacental transport has been previously demonstrated [42,43]. Overall, the partitions of nonpolar compounds between maternal and fetal samples are not uniform and our data suggest that the underlying *in utero* metabolism and toxicokinetics are still not fully understood.

To our knowledge, we are the first to report the most potent, highly toxic dioxins i.e., 2,3,7,8-TCDD, 1,2,3,7,8- PCDD and 1,2,3,4,7,8-HCDF in human AF samples. These, as well as many other compounds detected in AF, are classified as POPs and are recognised for their slow degradation, bioaccumulation, long biological half-lives and high toxicity. Although their production and use have been banned or severely restricted (under The Stockholm Convention on Persistent Organic Pollutants, 2001), their legacy continues and their presence in AF is worrisome. Low-level exposure to these compounds during critical windows of development has been associated with hormone-sensitive cancers, metabolic disorders, neurobehavioral disorders and cardiovascular diseases manifest-

ing themselves later in life [44–47]. The presence of these compounds in AF indisputably demonstrates placental transfer and fetal exposure and further validates the use of AF for the *in utero* analysis of nonpolar compounds.

REP values and contribution of Identified Knowns to AF activity

The contribution of detected known EDCs to the observed activity in the nonpolar AF fractions was determined based on their relative potencies (REP) values. Analytical EQs are calculated using REP values, and, for the same chemical and the same endpoint, a high variation in REP values can be found when different species or cell bioassays are used, which can lead to inaccurate estimation of compound contribution to the observed activity. Here, REP values were derived from reporter gene bioassays identical or similar to the bioassays used in this study and with comparable responses to the reference compounds, which ensures a reliable estimation of the respective analytical EQs (**Chapter 3**). Approximately 30% of the dioxin-like activity and only a small fraction of the estrogenic and (anti-)androgenic activity observed in the nonpolar fractions was explained by the 42 quantified compounds, which indicated the presence of potential novel, potent EDCs in the AF.

Non-targeted analysis of the active nonpolar AF fractions

To investigate the remaining unexplained activity further, we developed an innovative, weight-of-evidence approach to prioritize novel EDCs from the multitude of *m/z* features identified through the non-targeted screening. While targeted methods rely on the availability of reference standards for compound identification, non-targeted screening relies on chromatographic data, high-resolution mass data, curated online resources and prediction tools to narrow down the list of candidate compounds from the thousands of features usually identified in human matrices. From the 14,110 features detected in the active nonpolar AF fractions, compounds were tentatively identified through RI and spectra matching to the NIST and in-house spectral library (with over 250,000 compounds) resulting in 3,243 match-identified compounds from which 904 were Identified Unknowns and the rest fell into the ambient background noise (**Chapter 3**). It is important to note that the chemical profiling of the AF fractions was therefore based on a suspect screening approach for the "known unknowns" present in the spectral libraries, rather than a classical non-targeted screening. Consequently, the tentatively Identified Unknowns presented here do not include the true unknowns i.e., unregistered compounds, by-products or specific *in utero* metabolites not present in these databases. These true unknowns might be responsible for some activity observed in the fractions but are not easily identifiable by the currently available methods [48]. Moreover, such screening techniques provide a qualitative list of possible biomarkers, not quantitative results, and thus further targeted analyses are required for the quantification of candidate compounds. However,

the levels of compounds measured in AF represent their residual concentrations and until we gain a comprehensive understanding of the *in utero* toxicokinetics (e.g., degree of accumulation in fetal tissue, excretion in AF and placental clearance) they cannot be used as a proxy for the changing exposure levels experienced by the fetus throughout pregnancy. The goal of our approach was thus to characterise (detect and identify) EDCs present *in utero*, rather than to provide a quantitative measure of fetal exposure.

Matching spectral information with the compound's RT provides much improved tentative identifications during GC-HRMS analysis. In this study, also the correlation between the compound's log*P* value and elution profile during RP-LC fractionation greatly facilitated compound identification. It is important to note that this approach relies heavily on log*P* prediction tools which are not always accurate. To ensure that no compound was eliminated from further analysis based on inaccurate log*P* values, two different log*P* prediction tools were used (Kowwin and OPERA) and any inconsistencies in the predictions were checked against online data sources (e.g., PubChem) (**Chapter 3**). Also, heatmaps can be used to visualize compounds' peak intensities across the different fractions, inspect the chemical complexity of each fraction and further corroborate the correlation between compound elution and bioassay activity. Furthermore, adding multiple procedural blanks (solvent and extraction blanks) is extremely helpful in the additional reduction of potential suspects, specifically when sample processing is long and includes multiple steps, as in the case of the EDA approach. The filtering and prioritization strategy further narrowed down the list of suspects from 904 to 121 Identified Unknowns. The remaining compounds were evaluated for their potential (anti-)ER, (anti-)AR and DR activity using a weight-of-evidence approach that relied on a battery of publicly available experimental data and *in silico* prediction and profiling tools.

The use of the appropriate chemical identifier/descriptor during data mining and *in silico* predictions is extremely important. Although CAS numbers are most commonly used as a compound's identifier, they are proprietary, do not include structural information and are not unique per compound. There are many other common formats used in cheminformatics for storing and representing chemical structures (e.g., SMILES, InChI, MOL, SDF, CML). It is therefore often needed to interconvert formats used for different applications so that they are compatible with the software used without losing important structural information. This is specifically important for *in silico* prediction and profiling tools (e.g., QSARs) that heavily rely on molecular descriptors. InChI identifiers are reliable descriptors that are unique for each compound, include structural information that helps identify isomers, are open-source and freely available and therefore avoid the interoperability issue that other chemical identifiers may have. In our study, the use of InChI returned more hits during data mining than e.g., proprietary CAS numbers. InChI is the only descriptor suitable for the development of a standard canonical SMILES string that can easily be supported by many different software libraries [49]. As follows, InChI strings

converted to canonical SMILES (using Open Babel software) were the best suitable formats for log*P* and QSAR predictions, as they express more information than the simpler SMILES notation and take stereochemistry into account [49,50]. Considerations of molecular descriptors are extremely important in the EDA approach for accurate predictions of physicochemical properties as well as biological activity of compounds, and consequently identification of possible suspects.

A battery of publicly available experimental data and *in silico* prediction tools used for (anti-)ER, (anti-)AR and DR activity-profiling of the 121 Identified Unknowns included experimental data obtained from multiple high-throughput bioassays (ToxCast and Tox21 databases), predictive QSARs (OPERA and Danish (Q)SAR Database) endpoint specific profiling models (OECD QSAR Toolbox) and online databases (CompTox Chemicals Dashboard). These sources, especially in combination, provide a wealth of information that can significantly improve the prioritization of suspected EDCs from the list of thousands of tentatively identified compounds [48,51]. The increase in the availability and use of high-throughput data continuously improves modelling algorithms and predictive performances of QSARs ensuring higher sensitivity, specificity and accuracy in profiling of novel EDCs. The experimental, profiling and QSAR data and the correlation between the elution profile and the activity observed in the fractions proved to be a powerful driver for the selection and prioritization of possible novel EDCs for further testing.

Only 8 out of the 121 Identified Unknowns were previously experimentally tested for their endocrine activity and only 2 compounds (squalene and heptadecane) were identified as endogenous metabolites. The lack of endogenous metabolites in nonpolar AF fractions is not surprising as the majority of endogenous compounds in the human body (e.g., hormones, fatty acids, amino acids, carbohydrates, cyclic amines) are polar or semi-polar. Further evaluation using *in silico* prediction and profiling tools resulted in a total of 69 additional suspected EDCs. To our knowledge, none of these compounds has been previously measured in AF samples, and for only a few of them, limited information on production and use was found. For example, diphenyl isophthalate has been discovered in AF as a novel potent ER ligand. It is used as a plasticizer and a flame retardant but its presence in human samples has never been reported before. Similarly, for other identified industrial chemicals e.g., p,p'-ditolylamine or benzoin isobutyl, no information on production/use or human exposure could be found. The identities of many of the 350 000 chemicals registered globally for production and use remain publicly unknown because they are often claimed as confidential or are ambiguously described[52]. Furthermore, the 69 compounds identified include only the nonpolar compounds, it is unknown how many more (polar and mid-polar) EDCs are present *in utero*. A good indication is a recent study by Wang et al. (2021) where non-targeted screening of paired maternal and cord serum samples identified 55 polar environmental chemicals, likewise, with little to no information on their production and use. Further analysis of the polar fractions is thus

recommended. Overall, the results presented here are alarming and show that so far prenatal exposure to EDCs has not been adequately characterized. There is a vast knowledge gap regarding *in utero* exposure to EDCs and the compounds identified here have not been assessed by scientific or regulatory bodies. This calls for an urgent investigation into their origins and possible health effects.

MNPs and human placenta

Recent evidence demonstrated the internalization of MNPs in the human body, with the possibility of translocation to the placenta and *in utero* exposure [53,54]. The placenta is especially important to investigate as it serves as a gatekeeper for the transport of exogenous agents at the feto-maternal interface. Nevertheless, so far it has been largely overlooked in the MNP research. The third objective of this thesis was to determine the knowledge gaps and appropriate methods and models for studying MNPs in the fetal environment, focusing specifically on the human placenta. In order to do so, we provided a critical review of the available human-relevant *in vitro* and *ex vivo* placental models, the current state of the science on placental MNP research, and, highlighted the most important areas that require urgent scrutiny (**Chapter 4**).

Importance of human placental models for MNP research

The placenta is a crucial temporary organ that functions as a lung, kidney, endocrine and immune organ during pregnancy enabling fetal existence and supporting its development throughout gestation. Ironically, it is also one of the most underappreciated and understudied human organs. Nevertheless, its importance for maternal and fetal health is well established with placental dysfunction thought to underlie numerous pregnancy disorders. All exogenous agents present in the maternal blood have to come in contact with the placenta first before reaching the fetus. It is astonishing that at full-term as much as 30% of maternal blood flows through the placenta at any given time, bathing chorionic villi with a total surface area of 12-14 m^2. This extensive surface area is an important aspect of the placenta as it provides ample space for the exchange of nutrients, gases and waste products between maternal and fetal blood, but also for the interaction of any exogenous compounds (including particles) present in maternal blood with the placental trophoblastic “barrier”. The direct developmental toxicity of MNPs may arise from the transplacental transfer of particles to the *in utero* environment, where they can damage the fetal tissues. However, developmental toxicity may also arise from the MNPs being retained by the placenta itself, which may damage the tissue and indirectly affect not only fetal but also maternal health during pregnancy.

The increasing concern regarding hazards associated with MNP exposure stems mainly from experimental animal studies that indicate uptake and transport of MNPs across cellular barriers (including placental), systemic exposure, accumulation in inter-

nal organs and the brain; and associated particle toxicity mainly oxidative stress, local or systemic immune responses and neurotoxicity [55–58]. Although animal models have provided an invaluable contribution to science in general, they are often criticized as models for pregnancy. The placenta is the most species-specific organ with outstanding evolutionary diversity and marked morphological differences between species, including the number and type of cell layers that separate the bloodstreams of the mother and fetus and determine the placental permeability to nutrients, chemicals and particles. Consequently, extrapolation from animal models to humans is extremely challenging [59,60]. The difficulty with data extrapolation is not restricted to evolutionary distant species like rodents, but profound differences in placenta biology exist even between humans and nonhuman primates [61,62]. Although the practical and ethical considerations limit research options in humans, there are diverse *in vitro* and *ex vivo* human placental models that can be used to advance this research area.

The *in vitro* and *ex vivo* models of the human placenta have been successfully used to explore diverse aspects of placental biology, such as metabolism, endocrine and immune functions as well as, the transfer of drugs, and endo-/exogenous compounds and nanoparticles. These models reflect different levels of placental organisation and complexity, from isolated plasma membrane vesicles to simple mono-cultures and co-culture models and the more complex 3D models, placenta-on-a-chip, explant cultures and *ex vivo* human placental perfusion models (**Chapter 4**). All these models have their advantages and disadvantages. For example, on one hand, simple trophoblastic monocultures such as BeWo cells are easy to culture, provide robust data and are extremely useful for low-cost and high-throughput investigation of different aspects of placental physiology under controlled and simplified conditions. Nevertheless, they lack the physiological complexity of the multi-layered cell structures of placenta villi and do not account for the complex placental cell-cell interactions. On the other hand, placental perfusion is considered the gold standard for the evaluation of placental clearance and transport of various agents including MNPs, as it represents the functional unit of the placenta and thus takes advantage of the full complexity of the intact tissue [63]. However, usually, only the term placentas can be used, placental viability must be closely monitored, perfusion conditions may significantly affect the transport function and there are marked interindividual variations in transplacental kinetics making placental perfusion more laborious and less robust than the simpler placental mono or co-cultures. Nowadays, novel, 3D placental spheroid and organoid models are being developed providing attractive alternatives and a missing link between the “robust and simple” and the “more complex”, physiologically relevant models [64]. Placental organoids are promising models for microphysiological recapitulation of the human placenta as they show spontaneous differentiation, maintain the expression of important cell-surface molecules and retain other specific *in vivo* characteristics [65]. Although organoids are being labelled as the most

suitable models to study trophoblast development, function and pathology, we need to be careful with such statements. Specifically, necrotic damage and the size and exact composition of the cellular components of spheroids are difficult to control, and therefore robustness and replicability of these models must be carefully evaluated. Overall, we argue that there is no one gold standard and the choice of the model needs to be carefully considered depending on the aims and questions asked, and only after throughout evaluations of all the advantages and disadvantages. Nevertheless, considering the plethora of available human-relevant models, and the advancements in the 3D techniques and microfluidics resulting in increased physiological relevance, these human models can be used to pursue diverse aspects of placental functioning and facilitate MNP testing across the maternal-fetal interface while, at the same time, realising the 3R principles to replace, reduce and refine the use of animal models.

Human placental MNP research: what do we know so far?

So far, very little attention has been paid to placental MNP exposure and transplacental MNP transport. Only recently MNPs have been detected, for the first time, in human placental tissue [54,66]. To date, only ten *in vitro* and five *ex vivo* human placental models have investigated transplacental MNP uptake and transport and to lesser extent toxicity. This data is based solely on one polymer type and shape (commercially available and fluorescently labelled spherical polystyrene particles, PS), tested under acute exposures (up to 24h) and with a limited number of conditions (size range from 0.02 to 10 µm; concentration range from 0.1 to 1000 ug/mL). Nevertheless, the results from both *in vitro* and *ex vivo* studies indicate that (**Chapter 4**):

- PS particles are taken up by placental epithelial and endothelial cells *in vitro* in a size-dependent manner
- PS particles are transported through the placental *in vitro* and *ex vivo* barrier in a size- and dose-dependent manner
- The lower transfer rate *ex vivo* corresponded to a higher accumulation of PS particles in the placental tissue
- The syncytiotrophoblast layer seems to be a major barrier for the transplacental particle transport
- Next to particle size, surface charge, surface functionalization and protein corona possibly influence placental transport
- PS particles generally do not show acute cytotoxicity (the effects on cell viability and barrier integrity observed mainly at “unrealistically” high concentrations)

Although placental MNP research is still in its infancy, the grounds for concern are present. The particle toxicity to the placenta and consequent adverse pregnancy outcomes have already been demonstrated in the fields of particulate air pollution and nano-

toxicology, which should serve as cautionary examples. For example, (ultra)fine particles (e.g., black carbon particles) have been shown to translocate from the respiratory tract to the placenta, and have been associated with various pregnancy complications (e.g., pre-eclampsia, pre-term birth, low birth weight), fetal malformations (e.g., congenital heart defects) and diseases later in life [67–72]. MNPs, including fibres and traffic-generated particles, are increasingly found in ambient air pollution further adding to the atmospheric particle burden. Yet, the extent of placental exposure to MNPs after inhalation or ingestion is still unknown, and, if these MNPs (next to the (ultra)fine particles) pose accumulative or novel risks for the developing child and maternal health during pregnancy. Although the evidence so far is scarce, the initial results with PS particles call for a comprehensive investigation of placental toxicokinetics and toxicity of other environmentally relevant MNPs, and the consequent effects of such exposure on fetal and maternal health during pregnancy.

Knowledge gaps

It is necessary to provide empirical data to better understand the risks associated with placental MNPs exposure to inform risk-based policies to protect fetal and maternal health. Unfortunately, there are still many important knowledge gaps that hinder human health risk assessment [73]. MNP toxicity is complex. There are many possible drives of toxicity and toxicokinetics such as polymer type, particle size and shape, surface charge, presence of eco- or bio-corona, as well as various bacterial pathogens and chemicals that MNPs may carry [74]. The ability of microplastics to be vectors for chemicals or bacterial pathogens needs to be carefully considered as part of the overall assessment of microplastic safety. Given the huge diversity of plastic formulations in use, we need to move from using one polymer type and shape to screening a multitude of relevant particles, including environmentally weathered/degraded MNPs. Identification of the most toxic components of environmentally relevant MNPs although challenging will be critical to our understanding of the drivers of (placental) toxicity. The syncytiotrophoblast (ST) layer seems to be the main barrier to transplacental PS transport (as demonstrated by a limited amount of *ex vivo* studies) [75,76]. Considering that STs are involved in important transport, exchange, immune and endocrine functions of the placenta, the possible accumulation of MNPs in STs presents a possible hazard. However, so far STs received scant attention and no *in vitro* studies examined MNP uptake and toxicity in this cell type. Moreover, the exact mechanisms involved in MNP uptake and transport are yet to be elucidated. Furthermore, to date, mostly generic acute endpoints such as cell viability, cytotoxicity and barrier integrity have been investigated whereas the physiological and pathological placental perturbations, including more subtle but equally important endocrine or immune-related effects of particle and chemical toxicity of MNPs, have received scant attention. Specifically, oxidative stress and acute and chronic inflammation, as a result

of the persistent nature of MNPs, seem to be a universal mechanism of MNP toxicity [58,77]. This is especially important since placental inflammation is linked to placental damage with loss of function, induction of premature labour, and the release of inflammatory mediators leading to fetal organ damage and transplacental infection of the fetus, consequently leading to fetal morbidity and mortality [78]. It is not the acute but chronic exposure to MNPs, with the possibility of accumulation in placental tissue, and build-up of the subtle effects over time that should be investigated to reflect more closely on environmental exposure throughout gestation. Lastly, it is important to remember that there is intra- and inter-individual variability in the expression and function of placental drug transporters, which may affect the internalization of MNPs rendering some populations or individuals more sensitive.

Addressing the knowledge gaps

To address some of the defined research gaps the fourth and last objective of this thesis was to investigate the uptake, transport and toxicity of MNPs in human-relevant placenta cell models, including *in vitro* model representative of placental syncytiotrophoblasts. To mimic the natural environmental weathering processes, MNPs were experimentally weathered and the compositional differences between pristine and weathered particles were investigated. Also, the toxicity of the pristine and weathered MNPs on several endpoints, including the more subtle effects on important placental functions such as steroidogenesis, was investigated (**Chapter 5**).

Pristine vs weathered MNPs

Weathering processes including photo- and thermal oxidation, hydrolytic, mechanistic and biodegradation can lead to changes in polymer's surface charge potential, mass/surface ratio, chemical leaching or adsorption, free radical formation, hetero-aggregation and biofilm formation, which in turn may affect both toxicity and toxicokinetics of the aged MNPs [79,80]. Despite the importance of the weathering processes research to date, using pristine particles only did not address this important aspect. In our study to stimulate the ageing processes, MNPs of different sizes (PS (0.1-10 µm) and HDPE (0-80 µm)) were exposed for 4 weeks to surface water, sunlight and constant shaking to imitate the hydrolytic/bio-/photo- and mechanistic degradation, respectively. A non-targeted, small molecule characterization of the pristine and weathered MNPs using LC-HRMS allowed us to test for the presence of inherent or co-transported contaminants and to evaluate compositional differences following particle weathering. The results showed that weathering changed the chemical composition of MNPs with weathered particles showing a significantly lower number of unique features. This indicates that leaching, hydrolysis and/or photochemical reactions may deplete chemicals present in the polymer during weathering. Interestingly, 58 diverse substances were annotated in the pristine PS for-

mulation, including residual monomers, additives (plasticizers, colourants) and processing aids (lubricants, catalyst), showing impurities in otherwise pristine particles that could contribute to the chemical toxicity of these particles. Considering that there are more than 10 000 plastic monomers, additives, and processing aids associated with plastics products [81], including known toxicants such as phthalates and bisphenols, the possibility of compounds leaching during weathering might affect significantly the chemical vs particle toxicity of pristine vs aged MNPs [82]. However, due to the lipophilic nature of MNPs, literature to date focused largely on the adsorption of environmental contaminants on the MNP surface [83]. The complex dynamics between the leaching of plastic-associated compounds and the adsorption of co-occurring contaminants in the MNPs under environmental conditions need to be further examined. Although the weathering rate of MNPs is speculated to be lower in water than on land [80], the degree to which chemical leaching occurs in a non-aqueous environment or the human body (gastric fluids, lung lining fluids, blood, or in extra- or intracellular environment) has so far not been investigated.

MNP uptake, transport and toxicity in placental trophoblasts *in vitro*

Although syncytiotrophoblasts are the first placental cells in contact with maternal blood and are crucial for fetomaternal exchange and specialized placental endocrine and immune functions, we found no published *in vitro* data on the uptake or toxicity of MNPs in this important cell type. In our study, we used the well-established BeWo b30 choriocarcinoma cell line that morphologically and functionally resembles *in situ* cytotrophoblasts, which serves as an excellent model to study differentiation into syncytiotrophoblast [84,85]. Stimulation of differentiation results in the formation of large multinucleated and endocrine-active syncytiotrophoblasts, as reflected by elevated HCG synthesis and secretion [86–88]. Furthermore, BeWo b30 forms a confluent, polarized monolayer that provides a good *in vitro* model system to study transplacental MNP transport, in a simplified but robust manner. We investigated the uptake, transport and toxicity of MNPs in both nonsyncytialized and syncytialized BeWo b30 cells i.e., representative of cytotrophoblasts and syncytiotrophoblasts, respectively (**Chapter 5**).

Our data showed that pristine and weathered MNPs were taken up by both cell types in a size-dependent manner, and that particle weathering seemed to increase the uptake of PS, but only for the smaller particle size (**Chapter 5**). The mechanisms behind the increased cellular uptake of weathered MNPs are currently unknown. The use of fluorescent cellular dyes and confocal 3D imaging of cellular uptake confirmed that MNPs are not only present on top of the cell boundary but are truly internalized by the cells, and such 3D rendering techniques are recommended for future studies. Human exposure through ingestion and inhalation is inevitable and MNPs have already been found in lung tissue, human faeces and blood [53,89–91]. Still, the size cut-off for MNPs entering human circulation through the gut or lungs has not been established. Recently, polypropylene par-

ticles between 5 and 10 µm have been imaged and identified (by Raman microspectroscopy) in human placental tissue [54]. Moreover, our study is the first to show that even large 10 µm particles were taken up by both placental cell types *in vitro.* This finding indicates that if particles up to 10 µm in size cross the gastrointestinal epithelium and end up in systemic circulation, they can be taken up by the placental cells. However, further qualitative and quantitative detection and analysis of MNPs in human blood and placenta samples should be performed to confirm these results.

The transcellular transport of MNPs in the nonsyncytialized BeWo b30 monolayer was size and time-dependent. Although transport even for the smaller 50 nm particles was limited (<10% of initial dose transported after 48h) it did not reach a plateau, suggesting that longer incubation periods may result in higher transport. The size-dependent, limited transport of PS particles observed here is generally in agreement with other *in vitro* and *ex vivo* studies and demonstrates that MNPs cross the placenta barrier and reach the fetus. Although, in placenta perfusions, PS particles accumulated mainly in the syncytiotrophoblasts layer, suggesting that syncytiotrophoblasts and not the cytotrophoblasts play a major role in regulating transplacental MNP transport. It is important to note that placental perfusion studies are short (6h) and performed only in term placentas. However, the toxicokinetics of MNPs can vary between different stages of gestation considering that placental physiology and morphology, including the thickness of the different cell layers, change drastically throughout pregnancy [92]. These complex time-dependent toxicokinetics remain difficult to investigate. Furthermore, so far, adding differentiated syncytiotrophoblasts to *in vitro* co-cultures (with cytotrophoblasts, venous endothelial cells or/and villous mesenchymal cells) is technically challenging as syncytialization results in loss of barrier integrity. Nevertheless, such a co-culture model could provide more accurate data on placental MNP toxicokinetics as it would more closely resemble the multilayered structures of chorionic villi. Although MNPs have not yet been analysed in human foetuses, a limited number of studies using a rodent model of pregnancy demonstrated that MNPs can pass the placenta after intravenous administration and end up in various organs of the fetus [56,57,93]. Considering the complexity and high species divergence of the placenta, quantitative results of transport have to be interpreted with care. Nevertheless, taken together, this data indicates that MNPs are likely translocated over the human placental barrier and expose the fetus during sensitive windows of development. The effects of prenatal MNP exposure are currently unknown, but lessons learned from the field of nanomaterials and nanotechnology suggest inflammation, fetal malformations, retarded neonatal growth and neurotoxicity as potential endpoints [94].

The pristine or weathered MNPs tested in this study generally showed no acute cytotoxicity i.e., no effects on cellular ATP production or plasma membrane integrity, which corroborates the results of other studies with *in vitro* placental models (**Chapters 4 & 5**). The concentration used in our study (0.1-100 µg/mL) seems to fall within the environ-

mentally relevant concentration range i.e., the sum quantifiable concentration of plastic particles recently reported in human blood was 1.6 µg/ml [53]. An important function of the placenta is the production/metabolism of hormones crucial for pregnancy establishment and maintenance (e.g., placental development, angiogenesis, embryo implantation, immunotolerance and fetal development) [95]. Placental hormones are primarily secreted by the syncytiotrophoblast in a tightly regulated way. In this study, we demonstrated that 24 hr exposure to pristine and to a lesser degree weathered MNPs modestly downregulated genes responsible for the final step in estrogen biosynthesis (hsd17b1) and conversion of pregnenolone to progesterone (hsd3b1) (**Chapter 5**). So far, no other study exists that looked at the effect of MNPs on placental steroidogenesis. This finding, although not statistically significant, warrants further investigation, as inhibition of hsd17b1 and hsd3b1 has been associated with a reduction of bioavailable levels of these hormones which may lead to various adverse pregnancy outcomes, such as preeclampsia, miscarriages and fetal growth restriction [96–98]. In this study, several plasticizers and styrene-related compounds (known endocrine disruptors), have been tentatively identified in the pristine PS particles, however, their contribution to the observed subtle effects on gene expression is unknown. As noted above, we showed that even pristine MNPs are chemically complex and may leach chemicals of toxicological relevance, consequently we emphasize the importance of considering environmental weathering processes and chemical leaching when performing toxicological studies with MNPs.

Concluding remarks and future directions

This thesis aimed at addressing some of the most pressing knowledge gaps regarding *in utero* exposures to environmental contaminants of emerging concern. EDCs are of particular concern due to their ubiquitous presence, hormone-like mechanism of action and low-dose activity. Despite the growing concern, the characterization of fetal exposure to a full spectrum of EDCs with diverse physicochemical properties has been challenging and considerably hampered by our lack of understanding of the complex maternal-fetal toxicokinetics. To address this, in this study, effect-directed analysis of AF has been used for the first time to identify a wide range of known and novel EDCs present in the direct *in utero* environment (**Chapters 2 & 3**). The combination of off-line SPE and DLLE extraction, sample preconcentration, HPLC fractionation and the use of sensitive cell-based reporter gene assays, provided a robust method for successful extraction and detection of a range of endocrine disrupting (ER, AR and dioxin-like) activities in AF extract, and, set the foundation for further qualitative and quantitative identification of known and novel EDCs responsible for the observed activity. The agonistic and antagonistic activity spanned polar and highly nonpolar fractions revealing, for the first time, a great diversity of potent endogenous and exogenous compounds present in AF (**Chapter 2**). State-of-the-art targeted LC-HRMS and GC-HRMS approaches and relative potency

(REP) values were used to estimate the contribution of the known EDCs detected in AF to the activity observed in the nonpolar fraction. Many detected EDCs were never before analysed in AF, demonstrating their transplacental transport and direct fetal exposure. This analysis further revealed numerous fractions for which activity could not be explained by the detected known EDCs or endogenous hormones, and for which alternative suspect screening approaches needed to be applied. The innovative, weight-of-evidence approach developed in this study took advantage of curated and freely available data and *in silico* tools to prioritize possible novel endocrine disruptors from the multitude of features identified through the non-targeted screening (**Chapter 3**). Here, the application of a battery of *in vitro* experimental data (ToxCast and Tox21 databases), *in silico* prediction tools (OPERA, Danish QSARs) and endpoint-specific profiling models (OECD QSAR Toolbox) provided a powerful platform that allowed us to further identify known and novel EDCs in AF. Only a small subset (14 from 69) of the identified suspect EDCs could be commercially purchased and tested. Of the 14 prioritized suspects, 12 were chemically and biologically confirmed, validating the applicability of the weight-of-evidence approach used in this study for profiling novel EDCs. To our knowledge, none of the identified suspect EDCs, including the confirmed ones, were previously measured in AF samples, and for only a few of them, limited information on their production and use was found. This is alarming and shows that the fetus is exposed to many more EDCs than previously thought, including a large number of known and novel EDCs that have not yet been assessed by regulatory bodies. Further cause for concern is there, considering the low dose effects, non-monotonic dose responses and mixture effects of co-existing EDCs. Moreover, we need to keep in mind that the ongoing cycle of fetal ingestion and excretion of AF could result in a continuous, prolonged re-exposure of the fetus to the circulating levels of these compounds, possibly increasing the associated health risks. Overall, the results of this study revealed a vast knowledge gap, showing that the risks of EDC exposure *in utero* have so far not been adequately characterized, and are most probably underestimated.

The data presented in this thesis underscores our need to better understand the source and extent of exposure to these compounds and their possible effects on fetal development and long-term health. To do so, we need to move away from the chemical-by-chemical approach to risk assessment and direct our efforts on evaluating the complex mixtures. One way of moving forward is to consider the regulation of different classes of EDCs, grouped by common mechanisms and/or effects [99]. Here, well-defined grouping criteria for regulatory purposes will need to be established. Which compounds to consider, from the hundreds of thousands of chemicals out there with limited to no data on endocrine disruption, is the big challenge. The lack of comprehensive *in utero* exposure assessment represents, possibly, the most serious knowledge gap which hinders the assessment of health risks. The combination of EDA and AF provides a unique opportunity to address this knowledge gap. It can be used for effect-based determination of the

total EDC exposure experienced directly by the fetus during pregnancy. The most potent or relevant constituents of such a mixture can be identified and targeted first for prevention strategies. Here, the source and the extent of maternal exposure would need to be identified. Integration of *in vitro*, *in vivo*, and *in silico* data together with the development of AOPs and QIVIVE, and read-across approaches can further support risk assessment efforts. Moreover, surplus samples from amniocentesis present an additional opportunity to investigate chemical burden during earlier stages of development [18,100,101]. Since EDCs can permanently reprogramme physiological processes during the critical periods of development and are likely risk factors for developing diseases and disorders later in life, there is a largely unexplored potential to use this approach, and a combination of diagnostic and prognostic biomarkers, to better understand the exposure and impact of EDCs on developmental aetiology of disease [21,102,103]. Lastly, this approach, with the use of a wide array of sensitive cell-based bioassays, could be extended to other environmental contaminants and functional endpoints (e.g., neuro-, immune- or genotoxicity).

In addition to EDCs, this thesis studied MNPs as a new class of contaminants of emerging concern (**Chapter 4**). Recent evidence shows that MNPs are internalized in the human body and reach the placenta, suggesting that they contribute to *in utero* exposure. Given the current and projected growth in MNP pollution and the central role of the placenta in fetal and maternal wellbeing during pregnancy, these recent discoveries are alarming. Despite the growing public health concern, data on the placental toxicokinetics and toxicity of MNPs is still surprisingly limited, which significantly hampers risk assessment. Considering obvious ethical limitations, and high placental divergence between species, there is a great need for use of human placental models to advance our understanding of the potential hazards of MNPs to the placenta and consequently maternal and fetal health. However, no one gold standard exists and the choice of the model needs to be carefully considered depending on the aims and questions asked, keeping in mind that increasing model complexity usually results in increased variability and reduced reproducibility. Nonetheless, new advancements in 3D techniques and microfluidics continue to increase the physiological relevance of human models with increased reproducibility and now, together with simple mono- and co-cultures models, placental explants and placental perfusion models, they can be used to pursue diverse aspects of placental functioning at different levels of the placental organisation, facilitating MNP testing across the complex maternal-fetal interface **(Chapter 4)**.

The research on placental MNP exposure and toxicity is in its infancy and many pressing knowledge gaps remain that significantly hamper human health risk assessment (**Chapter 4**). Data on transplacental transfer and toxicity of MNPs to date is scarce and rely solely on reference polystyrene particles. The few experimental *in vivo, ex vivo* and *in vitro* studies in existence, including our study (**Chapter 5**), suggest no acute effects on placental cell viability, and limited transplacental uptake and transport. Limited uptake

and transport are not necessarily insignificant considering the long 9-month gestation period and the possibility of accumulation in the placental tissue. So far, mainly general toxicity endpoints after acute exposure have been investigated. However, the effects of chronic exposure and accumulation of MNPs over time should be investigated to reflect more closely the exposure scenarios during pregnancy. Specifically, syncytiotrophoblasts, cells central to the maternal-fetal interface with crucial endocrine and immune functions, should be examined as they appear to be the main barrier to transplacental PS transport. So far, STs received scant attention and our study was the first to investigate MNP uptake and toxicity in this cell type. The subtle effects of MNPs on steroidogenesis in STs *in vitro*, possibly mitigated by plastic-associated compounds, observed in our study warrant further examination (**Chapter 5**). Furthermore, important aspects of placental development and functioning such as placentation, angiogenesis, and immune and endocrine functions, should be examined. Particularly, placental inflammation, one of the most common perturbations linked to a wide variety of adverse pregnancy outcomes should be prioritized, considering that oxidative stress and chronic inflammation are the universal mechanisms of MNP toxicity.

MNPs represent a highly diverse class of contaminants with many possible drivers of toxicity (polymer type, particle size and shape, chemical composition, surface charge, presence of eco- or bio-corona). They are chemically very complex, possibly containing hundreds of plastic-associated compounds or co-contaminants which might be of importance when investigating chemical vs particle toxicity (Wiesinger et al. 2021). These characteristics, including their ability to act as bacterial or chemical vectors, need to be carefully considered as part of the overall assessment of microplastic safety. Logically, there is a pressing need to move from using one polymer type and shape to screening a multitude of environmental relevant particles, including weathered MNPs. Environmental weathering may cause increased chemical leaching, significantly altering the chemical composition of MNPs with a possible influence on their toxicity (**Chapter 5**). However, the potential health risks of MNPs and the chemicals associated with them under real-life conditions and environmentally relevant exposure scenarios are not yet established.

We need to better understand maternal and fetal health risks of MNP exposure. To do so, we need to be able to translate the experimental toxicological data to real-life exposure scenarios, by detecting and measuring trace amounts of plastic particles, including in the nanoscale, in environmental and human samples. For now, measurements are biased towards larger particles, most probably underestimating human exposure. Sensitive, high-resolution analytical methods for MNP detection, standardized protocols, quality control measures and certified reference materials for toxicity studies are currently lacking but urgently needed. The evaluation of the ability of microplastics to cross the lung, gastrointestinal, and skin barriers, as well as, information on absorption, distribution, metabolism and excretion (ADME), is needed to translate external exposure to

systemic exposure and facilitate risk assessment and exposure mitigation procedures [73]. Specifically, more studies on inhalation exposure including toxicokinetics and toxicity of fibres should be undertaken since it is believed that this is one of the primary routes of environmental exposure to MNPs. Furthermore, longitudinal human biomonitoring and epidemiological studies with birth cohorts are required in which maternal and fetal exposure and health outcomes are characterized. The fastest way to assess the possible risks of MNPs in the fetal environment is therefore to direct our efforts towards the development of sensitive analytical tools and experimental and epidemiological research to address the knowledge gaps regarding placental and fetal exposure, mechanisms of toxicity and biological effects. Furthermore, so far, it is unknown if MNPs exacerbate the effects of EDCs or the other way around. The combined effects of multiple stressors on placental functioning and fetal health need to be considered and investigated.

The research described in this thesis contributes toward a comprehensive characterization of the extent of *in utero* exposure to contaminants of emerging concern, which is crucial to our understanding of their impact on pregnancy, fetal development and long-term health. Such knowledge is essential to guide future exposure prevention strategies and safeguard the health of future generations.

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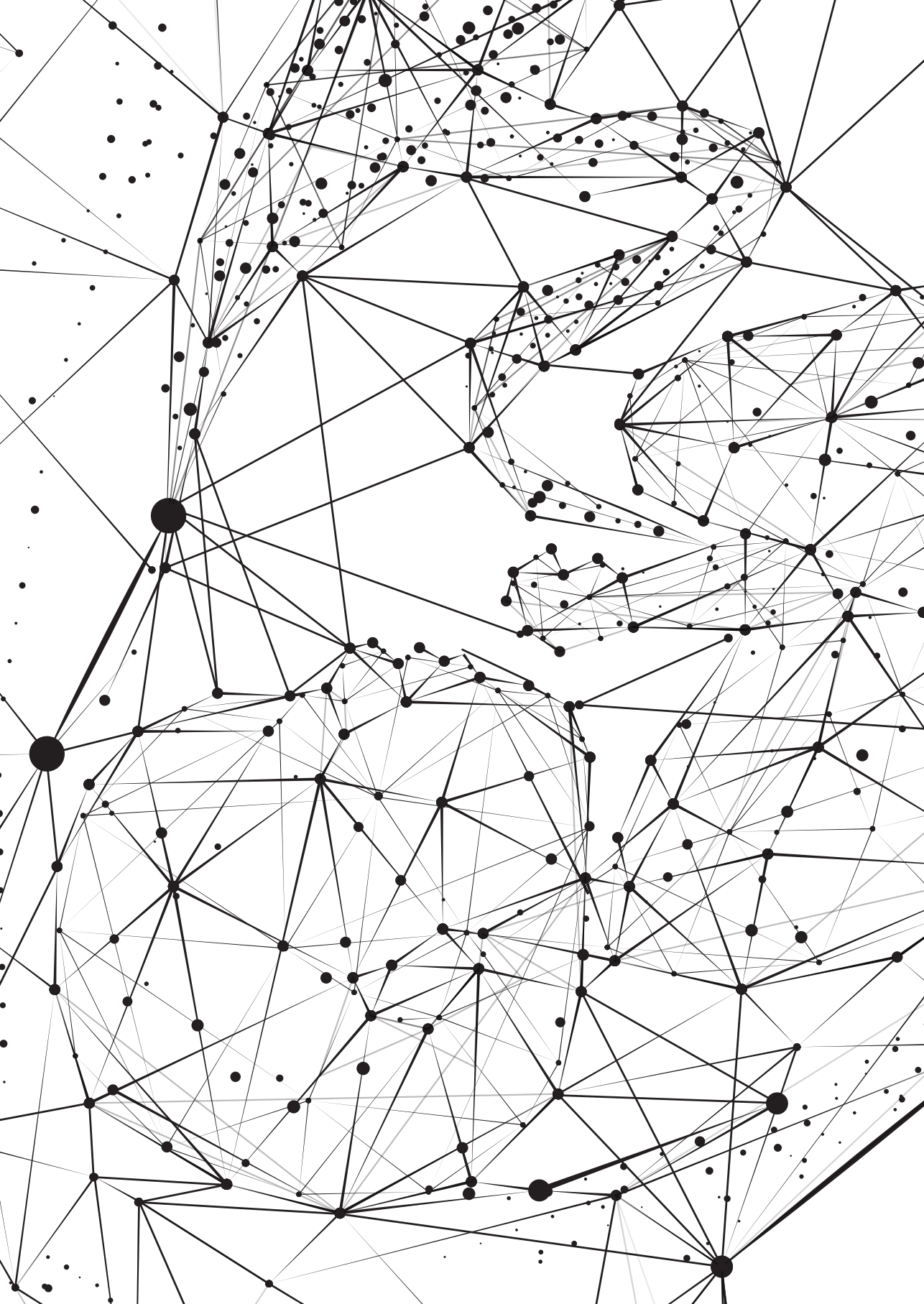

Appendices

Summary/Samenvatting

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English summary

During pregnancy, the human fetus is exposed to a broad range of environmental contaminants which reach the *in utero* environment with maternal blood. Prenatal environmental exposures may contribute to a broad spectrum of postnatal diseases or/and dysfunctions later in life. The fetus is specifically susceptible to exposure to endocrine disrupting compounds (EDCs), a diverse group of exogenous chemicals that can interfere with hormonally driven developmental processes and contribute to metabolic, reproductive, and neurobehavioral disorders. Due to the great diversity of environmental contaminants with potential endocrine activity and their complex maternal/fetal toxicokinetics, the characterization of the whole spectrum of EDCs and their metabolites present *in utero* remains exceptionally challenging. Moreover, there are other contaminants of emerging concern, such as micro- and nanoplastics (MNPs) and chemicals associated with them, that could reach the placenta and contribute to fetal exposure. The placenta is a key organ that ensures fetal and maternal well-being during pregnancy. Although plastic pollution has received worldwide attention, the effects of MNPs on placenta functioning and fetal development are still largely unexplored.

Considering the paucity of data on *in utero* exposure to these contaminants of emerging concern, the aim of our research was two-fold: to develop a method for analysis of EDCs including novel compounds in human amniotic fluid (AF), as a matrix of direct fetal exposure; and to investigate possible uptake, transport and toxicity of MNPs and associated compounds in human placenta cells *in vitro*. **Chapter 1** of this thesis introduced the state of the science in this field, including background information on EDCs, MNPs, AF, the placenta, and the concept of fetal windows of vulnerability; and discussed the current challenges in characterizing *in utero* exposures. **Part I** (**Chapters 2** & **3**) of this thesis focused on the characterization of *in utero* exposure to EDC. Here, a novel method for effect-directed analysis (EDA) of a wide range of compounds with endocrine disrupting activities was developed and applied to identify known and novel EDCs present in full-term human AF. **Part II** (**Chapters 4** & **5**) focused on MNPs and the human placenta. Here, the current state of the science on placental MNP research was reviewed, placental *in vitro* and *ex vivo* models were recapitulated and knowledge gaps were identified. The defined research gaps were then addressed by investigating the uptake, transport and toxicity of pristine and environmentally weathered MNPs in human placental trophoblasts cells *in vitro*.

Chapter 2 focused on the development and validation of the EDA approach for the comprehensive characterization of EDCs in human AF. Emphasis was put on the development of a robust and nondiscriminating extraction and fractionation method needed for the extraction of a broad spectrum of polar and nonpolar EDCs from AF and the reduction of the complexity of the sample for consequent bioassay analysis. The combination of solid phase extraction and dispersive liquid/liquid extraction allowed for maximal

recoveries of reference compounds with minimal interference from the matrix. Fractionation of a complex mixture of chemicals extracted from AF was performed with reversed-phase high-performance liquid chromatography (RP-HPLC) and consequent bioassay analysis using cell-based reporter gene assays revealed significant estrogenic (ER), androgenic (AR), and dioxin-like (DR) activity in AF fractions of differing polarity, providing the first indication of the diversity of endogenous and exogenous EDCs present in AF. Targeted liquid chromatography/mass spectrometry (LC/MS) analysis revealed 13 xenobiotics, phytoestrogens, and endogenous hormones in the AF extract that only partly contributed to the biological activity observed, indicating for the first time the presence of other diverse (unidentified) EDCs in human AF.

In **Chapter 3,** further qualitative and quantitative analyses of known and unknown EDCs present in the nonpolar AF fractions were performed using targeted and non-targeted gas chromatography high-resolution mass spectrometry (GC-HRMS). The targeted analysis revealed 42 known nonpolar EDCs in AF including dioxins and furans, polybrominated diphenyl ethers, pesticides, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. Only a small fraction of DR, (anti-)AR and (anti-)ER activity observed was explained by the detected compounds, as determined by their relative potency values (REP). Further non-targeted analysis revealed a multitude of chemical features in the nonpolar fractions, from which more than three thousand were tentatively identified through spectra matching, and further investigated using a battery of curated and freely available sources to predict their endocrine activity. In total 69 novel suspected EDCs were tentatively identified, 14 were purchased for confirmation, of which 12 were biologically active and 9 were chemically confirmed, including novel EDCs such as plasticizer diphenyl isophthalate and industrial chemical p,p'-ditolylamine. This study revealed for the first time a large number of known and novel EDCs in full-term human AF.

In **Chapter 4,** MNPs were studied as a new class of contaminants of emerging concern for fetal and maternal health. Here, the current state of the science on placental MNP research was reviewed, the most important areas that require further scrutiny were highlighted and the available human-relevant *in vitro* and *ex vivo* placental models were recapitulated. Although the limited number of *in vitro* studies published up to now strongly suggests that specific MNPs are taken up and transported through the placenta barrier, and could affect placental function, the transplacental transport and toxicity of the wide range of MNPs that humans are exposed to are still largely understudied. Further toxicity studies with MNPs in human-relevant placental models are urgently needed. In **Chapter 4,** suitable models are described (e.g., plasma membrane vesicles, mono-culture and co-culture of placental cells, placenta-on-a-chip, villous tissue explants, and placental perfusion) that could be utilized now to address the most pressing knowledge gaps such as physiological and pathological placental perturbations, including endocrine or immune-related effects of particle and chemical toxicity of environmentally relevant MNPs.

In **Chapter 5**, the research gaps defined in the previous chapter were addressed by investigating the uptake, transport and toxicity of pristine and experimentally weathered polystyrene (PS) and high-density polyethylene (HDPE) MNPs in human nonsyncytialized and syncytialized BeWo b30 choriocarcinoma cells representative of placental cytotrophoblasts and syncytiotrophoblasts, respectively. Our results showed a limited, size-dependent internalization and translocation of pristine and weathered MNPs in placental cells. Although acute exposure did not affect cell viability or cause damage to the plasma membrane, subtle effects on the expression of genes involved in steroidogenesis were observed, but only for the pristine particles. A non-targeted chemical characterization of pristine and weathered MNPs using LC-MS analysis evaluated the compositional differences following particle weathering and showed a significantly higher number of unique features in pristine particles compared to weathered particles. These results support the importance of considering environmental weathering processes when performing toxicological studies with MNPs. More in-depth investigations are needed to better understand the potential health risks of MNPs and the chemicals associated with them under environmentally relevant exposure scenarios.

Finally, in **Chapter 6** the main findings of this thesis, the overall conclusions and the perspective for future research are discussed. Briefly, risk assessment of EDCs and MNPs is currently hampered by the lack of data on direct *in utero* exposure. In addition, the effects of these contaminants of emerging concern on placental functioning and fetal health are rarely considered. The characterization of such exposures is challenging but crucial in our understanding of the relationship between prenatal exposure and long-term health. The research described in this thesis contributes toward a comprehensive characterization of the extent of *in utero* exposure to these contaminants of emerging concern, as well as their potential hazards. Further assessment of the effects of these novel contaminants on pregnancy and fetal development under environmentally relevant exposure scenarios is urgently needed to support health policies and mitigation strategies, and protect the health of future generations.

Nederlandse samenvatting

Tijdens de zwangerschap wordt de menselijke foetus blootgesteld aan een breed scala van milieuverontreinigende stoffen die via het bloed van de moeder in de baarmoeder terecht kunnen komen. Prenatale milieublootstelling kan bijdragen aan een breed spectrum van postnatale ziekten en/of stoornissen op latere leeftijd. De foetus is in het bijzonder gevoelig voor blootstelling aan hormoonverstorende stoffen (EDCs), een diverse groep exogene chemische stoffen die hormonaal gestuurde ontwikkelingsprocessen kunnen verstoren en daardoor kunnen bijdragen tot stofwisselings-, voortplantings- en neurogedragsstoornissen later in het leven. Door de grote diversiteit aan milieuverontreinigende stoffen met potentiële endocriene activiteit en hun complexe maternale/foetale toxicokinetiek blijft de karakterisering van het hele spectrum van EDCs en hun metabolieten, die *in utero* aanwezig zijn, een buitengewone uitdaging. Bovendien zijn er ook andere opkomende stoffen, zoals micro- en nanoplastics (MNPs) en daarmee geassocieerde chemicaliën, die de placenta kunnen bereiken en kunnen bijdragen aan foetale blootstelling. De placenta is een belangrijk orgaan dat zorgt voor het welzijn van de foetus en de moeder tijdens de zwangerschap. Hoewel plasticvervuiling wereldwijd aandacht krijgt, zijn de effecten van MNPs op de werking van de placenta en de ontwikkeling van de foetus nog grotendeels onbekend.

Gezien de schaarste aan gegevens over blootstelling *in utero* aan deze opkomende stoffen was het doel van ons onderzoek tweeledig: het ontwikkelen van een methode voor de analyse van EDCs inclusief nieuwe stoffen in menselijk vruchtwater (AF), welke als een matrix dient voor directe foetale blootstelling; en het onderzoeken van mogelijke opname, transport en toxiciteit van MNPs en geassocieerde stoffen in menselijke placentacellen *in vitro.* In **Hoofdstuk 1** van dit proefschrift wordt de stand van de wetenschap op dit gebied, inclusief achtergrondinformatie over EDCs, MNPs, AF, de placenta, en het concept van foetale vensters van kwetsbaarheid geïntroduceerd. Tevens worden de huidige uitdagingen bij het karakteriseren van blootstelling *in utero* beschreven. **Deel I** (**hoofdstuk 2** & **3**) van dit proefschrift richtte zich op de karakterisering van blootstelling aan EDCs *in utero*. Hiervoor werd een nieuwe methode voor effectgerichte analyse (EDA) van een breed scala van chemische stoffen met hormoonverstorende werking ontwikkeld en toegepast om bekende en nieuwe EDCs te identificeren, die aanwezig zijn in volgroeid menselijk AF. **Deel II** (**hoofdstuk 4** & **5**) richtte zich op MNPs en de menselijke placenta. Hierin werd de huidige stand van de wetenschap inzake MNP-onderzoek in de placenta bekeken en werden ook de huidige *in vitro* en *ex vivo* placentamodellen beschreven en hiaten in de kennis vastgesteld. Het gebrek aan kennis op bepaalde onderzoeksvragen in voorgaand onderzoek werd vervolgens aangepakt door *in vitro* de opname, het transport en de toxiciteit van ongerepte en door het milieu verweerde MNPs in menselijke placenta trofoblastencellen te onderzoeken.

Hoofdstuk 2 richtte zich op de ontwikkeling en validatie van de EDA-methode voor de uitgebreide karakterisering van EDCs in menselijk AF. De nadruk werd gelegd op de ontwikkeling van een robuuste en niet-discriminerende extractie- en fractioneringsmethode die nodig is voor de extractie van een breed spectrum van polaire en niet-polaire EDCs uit AF. Daarnaast moest de complexiteit van monsters voor de daaropvolgende bioassay-analyse worden verminderd. De combinatie van vaste-fase-extractie en dispersieve vloeistof/vloeistofextractie maakte maximale terugvinding van referentieverbindingen mogelijk met minimale interferentie van de matrix. Fractionering van een complex mengsel van uit AF geëxtraheerde chemicaliën werd uitgevoerd met reversed-phase hogedrukvloeistofchromatografie (RP-HPLC). De daaropvolgende bioassay-analyse met behulp van reportergen-testen op celniveau bracht significante oestrogene (ER), androgene (AR) en dioxineachtige (DR) activiteit aan het licht in bepaalde AF-fracties met verschillende polariteit, waarmee de eerste indicatie werd gegeven van de diversiteit van endogene en exogene EDCs in AF. Gerichte vloeistofchromatografie/massaspectrometrie (LC/MS) analyse onthulde 13 xenobiotica, fyto-oestrogenen, en endogene hormonen in het AF extract die slechts gedeeltelijk bijdroegen aan de waargenomen biologische activiteit, wat voor het eerst wijst op de aanwezigheid van andere diverse en niet eerder geïdentificeerde EDCs in menselijk AF.

In **hoofdstuk 3** werden verdere kwalitatieve en kwantitatieve analyses van bekende en onbekende EDC's in de niet-polaire AF fracties uitgevoerd met behulp van gerichte en niet-gerichte gaschromatografie hoge-resolutie massaspectrometrie (GC-HRMS). De gerichte analyse bracht 42 bekende niet-polaire EDC's in AF aan het licht, waaronder dioxinen en furanen, polybroomdifenylethers, pesticiden, polychloorbifenylen en polycyclische aromatische koolwaterstoffen. Slechts een klein deel van de waargenomen DR-, (anti-)AR- en (anti-)ER-activiteit werd verklaard door de gedetecteerde verbindingen, zoals bepaald door hun relatieve potentiewaarden (REP). Verdere niet-gerichte analyse bracht een veelheid van chemische kenmerken in de niet-polaire fracties aan het licht, waarvan er meer dan drieduizend chemische stoffen voorlopig werden geïdentificeerd door middel van overeenkomende spectra, en verder werden onderzocht met behulp van een batterij aan gecureerde en vrij beschikbare bronnen om hun endocriene activiteit te voorspellen. In totaal werden 69 vermoedelijke nieuwe EDCs voorlopig geïdentificeerd, 14 werden aangekocht ter bevestiging, waarvan er 12 biologisch actief waren en 9 chemisch werden bevestigd, waaronder nieuwe EDCs zoals de weekmaker difenylisoftalaat en de industriële chemische stof p,p'-ditolylamine. In deze studie konden we voor het eerst een groot aantal bekende maar ook nieuwe EDCs in menselijk AF aantonen.

In **hoofdstuk 4** werden MNPs bestudeerd als een nieuwe klasse van verontreinigende stoffen die een nieuw probleem vormen voor de gezondheid van foetussen en moeders. Hier werd de huidige stand van de wetenschap inzake MNP-onderzoek in de placenta geëvalueerd, werden de belangrijkste gebieden die nader onderzoek behoeven belicht

en werden de beschikbare voor de mens relevante *in vitro* en *ex vivo* placentamodellen gerecapituleerd. Hoewel het beperkte aantal tot dusver gepubliceerde *in vitro* studies sterk doet vermoeden dat specifieke MNPs worden opgenomen en door de placentabarrière worden getransporteerd, en daardoor ook de placentafunctie kunnen beïnvloeden, zijn het transplacentale transport en de toxiciteit van het brede scala van MNPs waaraan de mens wordt blootgesteld nog grotendeels onderbelicht. Verder onderzoek naar de toxiciteit van MNPs in voor de mens relevante placentamodellen is dringend noodzakelijk. In **hoofdstuk 4** worden geschikte modellen beschreven (bv. plasmamembraanblaasjes, mono- en co-cultuur van placentacellen, placenta-op-een-chip, darmweefselexplanten en placentaperfusie) die nu kunnen worden gebruikt om de meest dringende leemten in de kennis op te vullen, zoals fysiologische en pathologische verstoringen van de placenta, met inbegrip van endocriene of immuungerelateerde effecten van deeltjes- en chemische toxiciteit van milieurelevante MNPs.

In **hoofdstuk 5** werden de in het vorige hoofdstuk gedefinieerde onderzoekshiaten aangepakt door de opname, het transport en de toxiciteit van ongerepte en experimenteel verweerde polystyreen (PS) en hogedichtheidspolyethyleen (HDPE) MNPs te onderzoeken in menselijke niet-gesyncytialiseerde en gesyncytialiseerde BeWo b30 choriocarcinomacellen die representatief zijn voor respectievelijk placentale cytotrofoblasten en syncytiotrofoblasten. Onze resultaten toonden een beperkte, grootte-afhankelijke internalisatie en translocatie van ongerepte en verweerde MNPs in placentacellen aan. Hoewel acute blootstelling de levensvatbaarheid van de cellen niet aantastte en geen schade aan het plasmamembraan veroorzaakte, werden subtiele effecten op de expressie van genen waargenomen die betrokken zijn bij steroidogenesemaar dit vonden we alleen na blootstelling aan de ongerepte deeltjes. Een niet-gerichte chemische karakterisering van ongerepte en verweerde MNPs met behulp van LC-MS analyse liet compositieverschillen zien na verwering van de deeltjes en toonde een significant hoger aantal unieke kenmerken in ongerepte deeltjes in vergelijking met verweerde deeltjes. Deze resultaten ondersteunen het belang om bij toxicologisch onderzoek met MNPs rekening te houden met verweringsprocessen in het milieu. Diepgaander onderzoek is nodig om de potentiële gezondheidsrisico's van MNPs en de daarmee geassocieerde chemische stoffen in ecologisch relevante blootstellingsscenario's beter te begrijpen.

Ten slotte worden in **hoofdstuk 6** de belangrijkste bevindingen van dit proefschrift, de algemene conclusies en het perspectief voor toekomstig onderzoek besproken. Kort gezegd wordt de risicobeoordeling van EDCs en MNPs momenteel belemmerd door het gebrek aan gegevens over directe blootstelling *in utero*. Bovendien worden de effecten van deze opkomende stoffen op het functioneren van de placenta en de gezondheid van de foetus zelden in acht genomen. De karakterisering van dergelijke blootstellingen is een uitdaging, maar cruciaal voor ons begrip van het verband tussen prenatale blootstelling en gezondheid op lange termijn. Het in dit proefschrift beschreven onderzoek draagt

bij tot een uitgebreide karakterisering, en de omvang van de blootstelling *in utero* aan deze opkomende stoffen zorgt voor toenemende bezorgheid over hun potentiële gevaren. Verdere beoordeling van de effecten van deze nieuwe verontreinigingen op zwangerschap en foetale ontwikkeling bij milieurelevante blootstellingsscenario's is dringend nodig om gezondheidsbeleid en mitigatiestrategieën te ondersteunen en de gezondheid van toekomstige generaties te beschermen.

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About the author

Hanna Dusza was born on February 19th, 1986 in Słupsk, Poland, where she also grew up. After graduating from Stanisław Staszic Economy School in Słupsk, she started her bachelor's degree in Environmental Biology at the University of Warmia and Mazury (Olsztyn, Poland). Hanna obtained her bachelor's degree in 2008 and, in the same year, started her master's degree in Environmental Biology at the University of Gdansk (Gdansk, Poland). During her master's Hanna volunteered as a research assistant at the Waterbird Research Group "Kuling". In the second year of her master's, Hanna got accepted to an Erasmus exchange programme at the University of the West of Scotland (Paisley, Scotland) where she continued her education in Environmental Sciences. Upon obtaining her master's degree in 2010, Hanna moved to Tenerife (Canary Islands, Spain) where she worked as a volunteer at the Atlantic Whale and Dolphin Foundation. Hanna moved to the Netherlands in 2011, and after living and working in Amsterdam, she started her second master's degree in Environmental Chemistry and Toxicology at the Vrije Universiteit Amsterdam (VU). During this programme, she performed two research internships at the Amsterdam Institute for Life and Environment and participated in the "SENSE MSc Honours Programme". She obtained her master's degree in 2015 and worked briefly as an analyst before starting her PhD. Hanna started her PhD in 2016 at Brunel University London (London, England) under the supervision of Prof. dr. ir. Juliette Legler and Prof. dr. Rakesh Kanda, where she worked on the research presented in this thesis. While at Brunel University she also worked as a research assistant on the development of an Adverse Outcome Pathway for cardiotoxicity. In 2018, Hanna together with Prof. Legler moved back to the Netherlands and, until October 2020, continued her PhD research at the Institute for Risk Assessment Sciences (IRAS) at Utrecht University. During her PhD, she completed several postgraduate courses of the postgraduate education in toxicology (PET) to obtain her registration as a European Registered Toxicologist (ERT). Hanna is currently working as a postdoctoral researcher at Utrecht University in the research group of Prof. Legler, where she will continue to study the impact of environmental pollutants on pregnancy and early life health.

List of publications, awards and oral presentations

List of publications

Dusza HM, Cenijn PH, Kamstra JH, Westerink RHS, Leonards PEG, Hamers T. Effects of environmental pollutants on calcium release and uptake by rat cortical microsomes. *NeuroToxicology*. 2018; 69. doi: 10.1016/j.neuro.2018.07.015

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Dusza HM[*], van Boxel J[*], van Duursen MB, Forsberg MM, Legler J, Vähäkangas KH. Experimental human placental models for studying uptake, transport and toxicity of micro- and nanoplastics. *Sci Total Environ.* 2022 Accepted.

[*]both authors contributed equally

Honours & awards

Best poster presentation award at the annual meeting of The British Toxicology Society (BTS), April 2017, Liverpool, England

Best PhD platform presentation award at the annual meeting of The Dutch Society of Toxicology (NVT), June 2019, Ede, the Netherlands

On the cover of Environmental Science & Technology (ES&T), December 2019, Vol. 53, issue 24, publisher: American Chemical Society, United States

Environmental Health Perspectives (EHP) Science Selection “Protecting the fetus: placental uptake of micro- and nanoplastic particles”, November 2022, publisher: National Institute of Environmental Health Sciences, United States

Oral presentations

Early life exposure to endocrine disrupting chemicals: unravelling relevant exposures in the foetal environment. The Dutch Society of Toxicology (NVT), June 2019, Ede, the Netherlands

Microplastics and human placenta. Plastic Health Summit, October 2021, Amsterdam, the Netherlands. https://www.youtube.com/watch?v=wSYiP2C255g

Potential Endocrine Disrupting Effects of Microplastics in Human Placenta and Aquatic Life. Healthy Environment and Endocrine Disruptor Strategies (HEEDS) and Young Endocrine disruptor researcher virtual Seminar Series (YESS), December 2021, the Netherlands, https://www.youtube.com/watch?v=zHaQb_3KTno

Studying the early life human health impacts of MNPs. International Congress of Toxicology (ICT XVI), invited speaker, September 2022, Maastricht, the Netherlands.

Utrecht University

INVITATION
to attend the public defence
of my doctoral thesis

Contaminants of emerging concern in the fetal environment

unravelling the exposure and effects
of endocrine disrupting compounds
and micro(nano)plastics in utero

which will take place on
the **25th of November 2022** at **14:15**
in the **Senate Room**
Academy Building, Domplein 29
Utrecht, or online

After the ceremony
please join us at a reception
in the Masquerade Hall (Room 1636)
or outside in the Pandhof
(if the weather permits)

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