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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 <1% estrogenic and (anti-)androgenic activity was 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.

1. Introduction

Environmental pollutants are increasingly recognized for their ability to alter fetal programming during critical windows of development (Heindel et al., 2015; Vrijheid et al., 2016). 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 (Gore et al., 2015; 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 (Rachoń, 2015), metabolic and neurobehavioral disorders and cardiovascular diseases (Bellanger et al., 2015; Heindel et al., 2017; Pinson et al., 2016), 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

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wide range of physicochemical properties, including differing polarity. Nonpolar, lipophilic EDCs are ubiquitously present in the environment with a widespread chronic, low-level exposure in the general population (Bonefeld-Jørgensen et al., 2014; Lee et al., 2006). 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 (Lehmann et al., 2014). 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 (Barr et al., 2005). The circulating low-level of persistent pollutants in the maternal compartment may result in chronic exposure to the fetus. Although many factors influence transplacental transport of environmental chemicals, in general EDCs with higher lipophilicity are thought to more readily cross the placenta than the more hydrophilic compounds (Li et al., 2013; Poulsen et al., 2009).

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 (Andersson et al., 2016; World Health Organization, 2013). 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 (Cooke, 2014; Deji et al., 2021; Kahn et al., 2020; Marie et al., 2015; Mitro et al., 2015). However, considering complex placental and *in utero* specific toxicokinetics, extrapolation of exposure from the maternal to the fetal compartment remains challenging (Prouillac and Lecoeur, 2010; Syme et al., 2004). 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 (Underwood et al., 2005). The ongoing cycle of fetal ingestion and excretion of AF may result in fetal accumulation (Burt et al., 2012; Mann et al., 1996)) and/or prolonged fetal exposure (Bradman et al., 2003). 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 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 (Dusza et al., 2019). We used an effect directed approach (EDA) in which chromatographic separation of chemicals in AF extract allowed for 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 (Dusza et al., 2019). 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 analysis 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 predict 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 (Richard et al., 2016) available through EPA CompTox Chemicals Dashboard (Williams et al., 2017)), predictive QSARs (OPERA (Mansouri et al., 2018) and Danish (Q)SARs Database (Klimenko et al., 2019)) and endpoint specific profiling models (OECD QSAR Toolbox (Schultz et al., 2018)). 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.

2. Materials and methods

2.1. 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).

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 (Dusza et al., 2019). 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 Fig. 1. Details of chemicals, reagents and bioassays used are given in Supplementary Material S1.1 and S1.2.

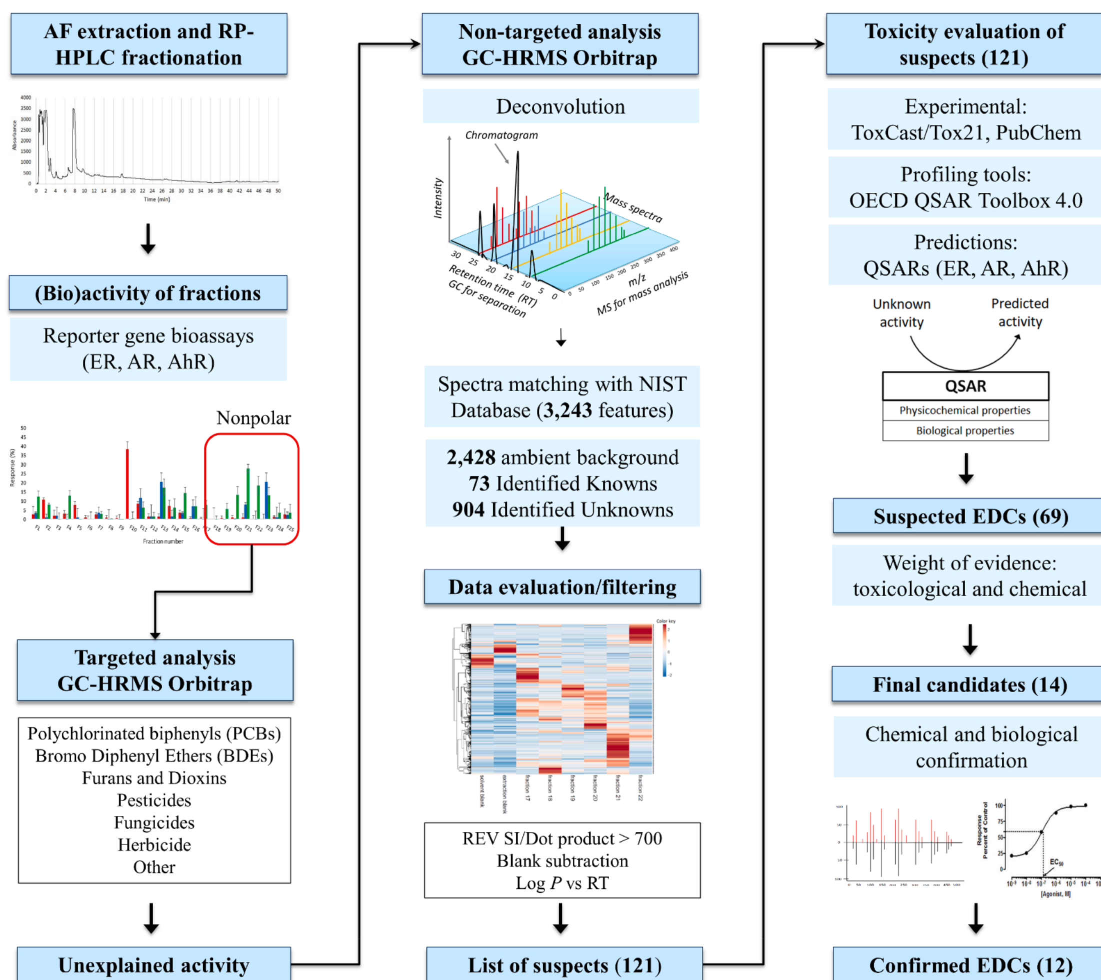

Fig. 1. Study design for identification of nonpolar endocrine disruptors (EDCs) in full-term amniotic fluid (AF), using a combination of targeted and non-targeted chemical analysis, reporter gene bioassays, toxicity profiling and prediction tools.

2.2. 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.

2.3. 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 <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%.

2.4. 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 × 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 × 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 min at 45 °C with a split gas flow rate of 100 mL/min, (2) evaporation: split mode for 1 min 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 min (the total run time was 34 min) 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 <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 splitless 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 min, 222 °C at 0.1 °C/min, and 320 °C at 7 °C/min and hold for 3 min (total run time of 49 min). Data were collected in full-scan mode between 5 and 45 min 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 (0.15 g/L) as reported in the literature (Biezenski et al., 1968; Singh and Zuspan, 1973)) and as a non-lipid adjusted molar concentration (M).

2.5. 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 >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 in 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.

2.6. Identification of candidate EDCs

The peak intensities of the Identified Unknowns found in the AF fractions were visualized with colour-coded heatmap using ClustVis web tool (Metsalu and Vilo, 2015). 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 × 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. Experimental 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) (Mansouri et al., 2018). 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.

2.6.1. Experimental data

Experimental data were retrieved from ToxCast and Tox21 high-throughput bioassays (Richard et al., 2016) 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 experimental database available through 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 (Wishart et al., 2018), to screen for known endogenous compounds.

2.6.2. 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 (O'Boyle et al., 2011),

accessed through http://www.cheminfo.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) (Mansouri et al., 2020) and CERAPP (Collaborative Estrogen Receptor Activity Prediction Project) (Mansouri et al., 2016). Second, an online-based Danish QSAR Database with estimates from>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 Fig. 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 correlation between the elution profile and the activity observed in the fractions (checked manually for each suspected EDC).

2.7. 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) were 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.

3. Results

3.1. 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 (Fig. 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 (Dusza et al., 2019), 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 (Fig. 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 (Fig. 2A). None of the nonpolar fractions showed anti-ER or anti-DR activity. However, anti-AR activity was observed, with 4 fractions (F17-F20, Fig. 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.

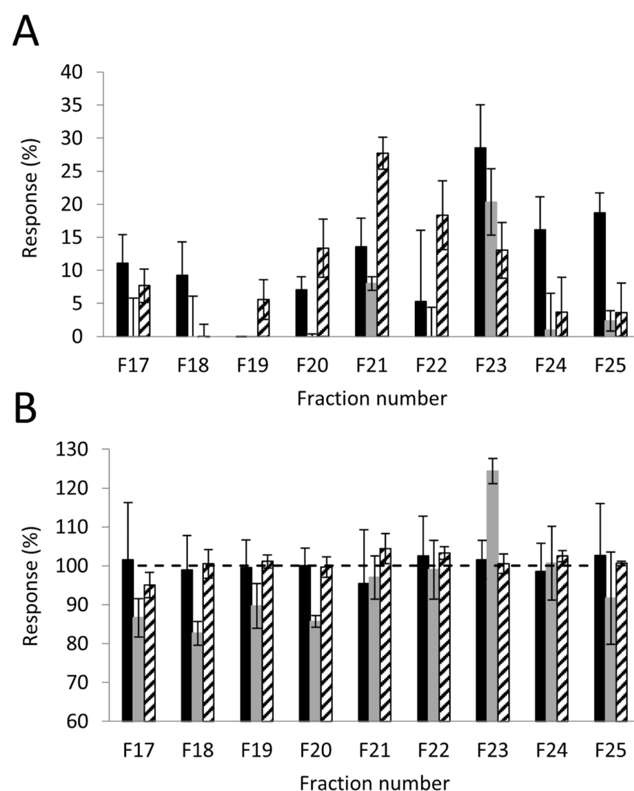

Fig. 2. Agonistic (A) and antagonistic (B) activity of nonpolar fractions of amniotic fluid (AF) in ER (black), AR (grey), and DR (striped) responsive cell lines. Agonistic response is expressed relative to the maximum induction observed in the concentration–response curve of the positive control E2 (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).

3.2. 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 in Dusza et al. (2019), were also included in Table 1. In total, 42 compounds were detected above LODs (Tables 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

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 lipid 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	$4.6 x10^{-12}$	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	–
	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
Chemical group	Compound name	MW	Log*P*^	Conc. (ng/L AF)	Conc. (ng/g lipid)	Conc. (M)	Fraction nr.
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.

phenol, 2,4,5-trichloro- and azobenzene, the concentrations found were close to LOD levels (Table S2).

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

Dioxin-like activity was found for 13 compounds with EC50 values ranging from 9.5×10^{-12} M for the most potent compounds (2,3,7,8-TCDD and 1,2,3,7,8-PCDD) to $> 1.5 \times 10^{-5}$ 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 6.4×10^{-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 2.0×10^{-6} M (o,p'-DDT) to $> 1.5 \times 10^{-5}$ 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 1.0×10^{-8} M, 3.0×10^{-7} M, 4.0×10^{-7} M and 5.0×10^{-7} M, respectively (Table 2).

3.4. 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, Fig. 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).

The androgenic activity observed in the F21 and F23 fractions was not explained by any of the compounds measured in this study, and the contribution of the only weak AR agonist, mesitylene (Tables 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 and 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 (Fig. 2B).

3.5. 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, 904 Identified

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	1.1×10^{-11a}	–	–	–	$1.0 \times 10^{-8\ h}$	23
1,2,3,7,8-PCDD	9.5×10^{-12a}	NA	NA	NA	NA	19–20
1,2,3,4,7,8-HCDF	9.6×10^{-11a}	NA	NA	NA	NA	21–22
OCDF	4.0×10^{-8b}	NA	NA	NA	NA	17
BDE 3	–	–	$5.9 \times 10^{-5\ \hat{}}$	–	$7.5 \times 10^{-5\hat{}}$	21–22
BDE 28	–	–	3.1×10^{-6c}	$>1.5 \times 10^{-5c}$	–	17–20
BDE 47	–	–	1.0×10^{-6c}	1.2×10^{-5c}	–	21–22
BDE 99	$>1.5 \times 10^{-5c\hat{}}$	–	7.8×10^{-6c}	–	–	22
BDE 100	–	–	1.0×10^{-7c}	7.0×10^{-6c}	–	20
BDE 153	6.0×10^{-7c}	–	1.3×10^{-5c}	–	–	17–18
BDE 183	$2.0 \times 10^{-6c\hat{}}$	–	$>1.5 \times 10^{-5c}$	–	6.4×10^{-6c}	20
BDE 209	–	–	–	–	–	22–23
6-OH-BDE 47	1.3×10^{-6c}	–	2.8×10^{-6c}	–	5.0×10^{-7c}	19–20
3-OH-BDE 157	NA	NA	NA	NA	NA	19–20
p,p'-DDT	–	–	1.0×10^{-6d}	$5.2 \times 10^{-6\hat{}}$	–	17
o,p'-DDT	–	–	3.3×10^{-6e}	2.0×10^{-6}	–	19–22
p,p'-DDD	–	–	7.0×10^{-7d}	$2.8 \times 10^{-5\hat{}}$	–	17–22
o,p'-DDD	–	–	$9.9 \times 10^{-6\hat{}}$	$6.3 \times 10^{-6\hat{}}$	–	17–22
Endrin	–	–	$3.2 \times 10^{-5\hat{}}$	$2.3 \times 10^{-5\hat{}}$	–	21–22
Fonofos	–	–	$2.9 \times 10^{-5\hat{}}$	–	–	17
Heptachlor	–	–	–	–	–	20
γ-HCH	–	–	4.0×10^{-6d}	NA	–	22
β-HCH	–	–	–	$2.8 \times 10^{-6\hat{}}$	–	17–22
δ-HCH	NA	–	$5.1 \times 10^{-5\hat{}}$	NA	–	17–20
Triclosan	–	–	1.3×10^{-5}	$2.0 \times 10^{-6\hat{}}$	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^{-7b}	NA	NA	NA	NA	21–22
PCB 114	4.0×10^{-7b}	NA	NA	NA	NA	22–23
PCB 138	NA	NA	1.0×10^{-6f}	NA	$1.6 \times 10^{-5f\hat{}}$	18–22
PCB 206	NA	NA	NA	NA	NA	21
TPP	–	–	$7.7 \times 10^{-6\hat{}}$	$7.3 \times 10^{-6\hat{}}$	–	21
EDP	–	–	–	–	–	23
Pyrene	–	–	$2.5 \times 10^{-6\hat{}}$	–	–	17–20
Fluorene	–	–	–	–	–	19–23

(*continued on next page*)

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)	
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 were 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.

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.

The strategy for filtering and prioritization of Identified Unknowns is shown in Fig. S1. The heatmap (Fig. 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* $\geq$ 4.0 observed in $\geq$ F17, Dusza et al., 2019), only compounds with log*P* $\geq$ 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 (Fig. S2). For a few compounds, such as BDE 209 and B(b)F, Kowwin overestimated, whereas OPERA underestimated the log*P* values (Fig. S2). To

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).

	REPs*	Analytical TEQs (ng TCDD-EQs/L)							∑ TEQ per compound	% contribution to ∑ analytical TEQs
		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.

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	7.5×10^4	365.7	1
F21	2.5×10^4	53.4	<1
F22	1.9×10^4	21.8	<1
F23	–	–	ND
∑	$\mathbf{33.6 \times 10^4}$	**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.

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.

The final list of Identified Unknowns consisted of 121 unique compounds (Supplementary Excel File), 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 $\times 10^{-5}$ 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.

3.6. *Chemical and biological confirmation of candidates*

The 14 prioritized candidates were tested for both agonistic and antagonistic potency (Fig. 3). Nine compounds showed anti-androgenic activity, with IC50 values ranging from 1.4×10^{-5} M (CAB) to 5.2×10^{-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 (Fig. 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 (Fig. S4).

Estrogenic activity was observed for 6 compounds: p,p'-

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.

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 8.2×10^{-7} M and 3.9×10^{-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, Fig. 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).

4. 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 (Barr et al., 2005). 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 (Dusza et al., 2019). 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.

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.

	AR-antagonism			ER-agonism			DR-agonism		
Compound (fraction nr.)	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[a] (F22)	–	–	–	–	–	–	–	–	–
BFBT[a] (F21)	1.0×10^{-5}	3.1×10^{-5}	72	1.3×10^{-6}	2.2×10^{-6}	113	–	–	–
TTCP[a] (F18)	–	–	–	–	–	–	1.8×10^{-5}	–	46
BMD[a] (F21)	3.3×10^{-6}	5.2×10^{-6}	100	–	–	–	–	–	–
CAB[a] (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-ethoxy-benzylthio)-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; *a*, compounds not confirmed chemically.

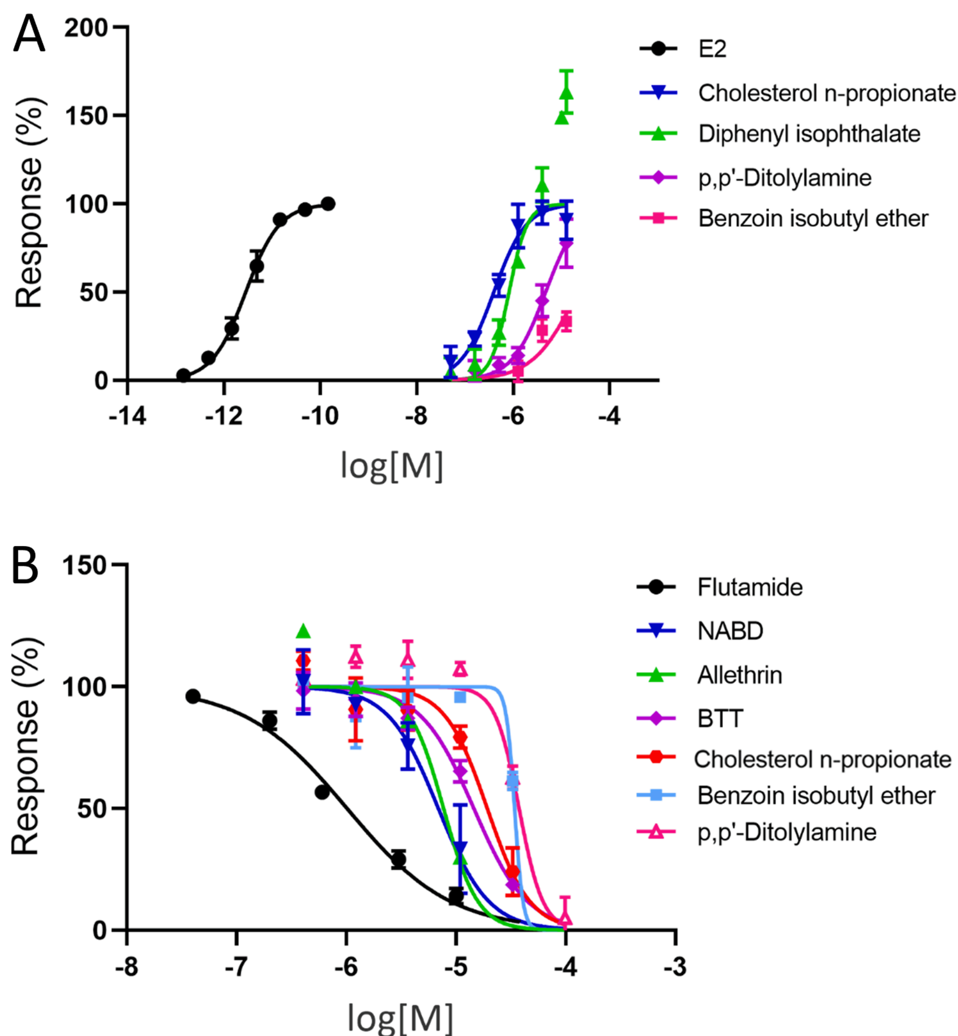

Fig. 3. Estrogenic (A) and anti-androgenic (B) activity of novel identified compounds in AF fractions, measured in ER-Luc and AR-Luc reporter gene assay, respectively. In the ER-Luc assay, the % response is expressed relative to the maximum induction by 50 pM E2. In the AR-Luc assay, cells were co-exposed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

4.1. 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 never before 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 (The Stockholm Convention on Persistent Organic Pollutants, 2001), they are continuously and frequently detected in maternal and fetal tissues and thus their legacy persists (Al-Saleh et al., 2012; Barmpas et al., 2020; Jaraczewska et al., 2006; Luzardo et al., 2009).

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 (Jayaraj et al., 2016; Peng et al., 2021; Sharma et al., 2014). Typically, DDE is the most abundant metabolite of DDT measured in maternal serum, placenta or cord blood (Barmpas et al., 2020; Shen et al., 2007; Zhao et al., 2007). Considering that AF is largely a product of 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 (Barmpas et al., 2020). 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 (Alaee et al., 2003). The presence of these compounds in maternal blood, cord blood and placenta, has been previously demonstrated (Frederiksen et al., 2009; Mazdai et al., 2003; Vizcaino et al., 2014). It has been suggested that the higher relative bromination of BDEs may decrease transplacental transport due to increased molecular weight, resulting in a higher concentration found in e.g., maternal blood rather than cord blood (Aylward et al., 2014; Frederiksen et al., 2010). 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 through 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 (Alcorn and McNamara, 2003; Vizcaino et al., 2014). To our knowledge, only one other study measured BDEs in AF, and the congeners and concentrations reported were similar to the ones reported here (Miller et al., 2012).

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 (Barmpas et al., 2020; Daglioglu et al., 2013; Foster et al., 2000; Luzardo et al., 2009; van der Ven et al., 1992) and in general, was much lower than that reported in maternal serum (Jaraczewska et al., 2006; Park et al., 2008). The presence of the higher chlorinated PCBs in our samples (e.g., PCB 206, 138), corroborated by few other studies (Correia Carreira et al., 2011; Luzardo et al., 2009; van der Ven et al., 1992; Vizcaino et al., 2014), 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 (Machado et al., 2014; Myers et al., 2008, 2003). Maternal exposure to PAHs and their ability to cross the placenta and reach cord blood has already been demonstrated (Gladen et al., 2000; Singh et al., 2008; Zhang et al., 2017). 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 (Tables 1 & 2). Although the past decades have seen a steady decrease in exposure to these compounds in the general population (Aylward and Hays, 2002), 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 (Araki et al., 2018; Berghuis et al., 2015; Bhatia et al., 2005; Burns et al., 2013; Herbstman et al., 2010; Mocarelli et al., 2008).

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 (Tables 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 (Psychogios et al., 2011) and therefore would not elute in the non-polar 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 were 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 in 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 (Wang et al., 2021).

From the 69 compounds with potential endocrine activity, 14 were chosen for further evaluation, based on the toxicity profile and commercial availability (Table 9, Supplementary Excel File). 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 (Fig. 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 (www.mainchem.com). For the other active compounds confirmed in AF, limited to no information about their source or use could be found.

4.2. Strengths and limitations in 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) (Hollender et al., 2017). It provides trace level detection of highly nonpolar compounds (BDE 209, log*P* 9.97, Table 1), and allows for non-targeted detection of thousands of volatile substances. Matching spectral information with the compound's retention time provides much improved tentative identifications (Hollender et al., 2017). 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 (Dusza et al., 2019). 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 (Fig. 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 contain a wealth of information for thousands of chemicals improved the identification and prioritization of suspected EDCs from the list of tentatively identified compounds (McEachran et al., 2017; Williams et al., 2017). The ongoing advances in *in vitro* technologies, and 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 (Singh and Zuspan, 1973). Lipid adjustment is a common practice in order to compare the inter and intra-individual differences in lipophilic toxicant concentration (Bernert et al., 2007; O`Brien et al., 2017); 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 (Elskus et al., 2005; Kammann et al., 1990). The AF lipid profile is highly specific and differs significantly from the maternal plasma (Biezenski et al., 1968). 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 (Underwood et al., 2005). In particular, as binding to plasma protein is a competitive process between exogenous compounds and endogenous ligands, specifically free fatty acids (Syme et al., 2004), 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 (Tables 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 glucocorticoid receptor (GR) (Wilson et al., 2002). 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 (Weiss et al., 2009), or unknown mixture effects resulting in significantly lower or higher bioassay activity than calculated based on the individual REP values (Orton et al., 2014), as shown with diphenyl isophthalate (Fig. 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.

5. 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, 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.

CRediT authorship contribution statement

Hanna M. Dusza: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing,

Visualization. **Katherine E. Manz:** Investigation, Validation, Data curation, Writing – review & editing. **Kurt D. Pennell:** Resources, Writing – review & editing, Supervision. **Rakesh Kanda:** Resources, Writing – review & editing, Supervision. **Juliette Legler:** Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

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