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Antagonistic activity towards the androgen receptor independent from natural sex hormones in human milk samples from the Norwegian HUMIS cohort

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ABSTRACT

In this paper, we investigated the possible presence of endocrine disrupting chemicals (EDCs) based on measuring the total estrogenic and androgenic activity in human milk samples. We used specific bioassays for analysis of the endocrine activity of estrogens and estrogen-like EDCs and androgens and androgen-like EDCs and developed a separation method to evaluate the contribution from natural hormones in comparison to that of EDCs to total endocrine activities. We extracted ten random samples originating from the Norwegian HUMIS biobank of human milk and analyzed their agonistic or antagonistic activity using the ERα- and AR CALUX® bioassays. The study showed antagonistic activity towards the androgen receptor in 8 out of 10 of the assessed human milk samples, while 2 out of 10 samples showed agonistic activity for the ERα. Further investigations demonstrated anti-androgenic activity in the polar fraction of 9 out of 10 samples while no apolar extracts scored positive. The culprit chemicals causing the measured antagonistic activity in AR CALUX was investigated through liquid chromatography fractionation coupled to bioanalysis and non-target screening involving UHPLC-Q-TOF-MS/MS, using a pooled polar extract. The analysis revealed that the measured anti-androgenic biological activity could not be explained by the presence of endogenous hormones nor their metabolites. We have demonstrated that human milk of Norwegian mothers contained anti-androgenic activity which is most likely associated with the presence of anthropogenic polar EDCs without direct interferences from natural sex hormones. These findings warrant a larger scale investigation into endocrine biological activity in human milk, as well as exploring the chemical sources of the activity and their potential effects on health of the developing infant.

1. Introduction

Breast milk contains a complex mixture of proteins, lipids, carbohydrates as well as a high concentration of bioactive components, and is acknowledged to be important to the infant's post-natal growth and development. Beside its valuable properties, this biofluid also constitutes an important non-invasive source of information about the quality of the perinatal environment and its potential contamination (Esteban and Castaño, 2009). Evaluation of toxicant levels in breast milk is an area of major interest, revealing the presence of diverse environmental contaminants such as persistent organic pollutants (POPs) and chemicals with estrogen-like properties (xenoestrogens) (Criswell et al., 2017; Massart et al., 2005; Thomsen et al., 2010). Xenoestrogens and several POPs are endocrine active and therefore referred to as endocrine active chemicals (EACs). Usually at relatively high dosage levels, several EACs have been shown to lead to adverse effects, and these chemicals are referred to as endocrine disrupting chemicals (EDCs) (Bergman et al., 2012). EACs and EDCs have been identified in a myriad of sources like contaminated food, indoor dust and daily-life products representing a challenge in their proper assessment and management. Their dose- and time-dependent effects can be particularly strong during vulnerable windows of development, from fetal life to the post-natal period up until puberty. Androgen- and estrogen signaling has been found to be a frequent target of hormonally active agents

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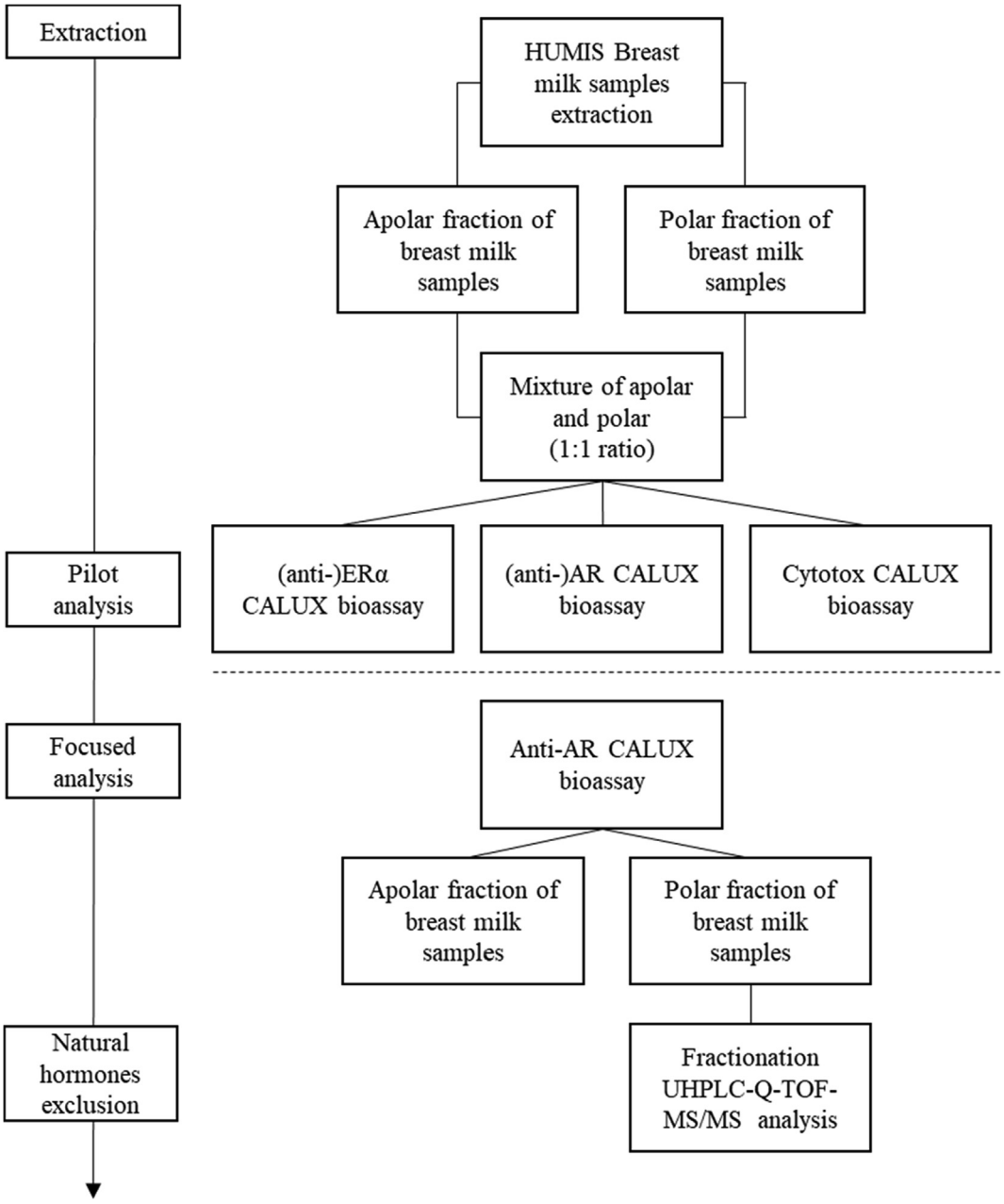

Fig. 1. Study design of the extraction procedure and pilot analysis of ten breast milk samples on the (anti-)ERα and (anti-)AR CALUX bioassays.

(Andersen et al., 2002). While linked to adversities in wildlife and supported by circumstantial evidence, the linkage between early EDC exposures and human diseases is still not firmly established (Andersson et al., 2012; Colborn et al., 1993). Many different chemicals can interact with the estrogen- and androgen receptor and it has been shown that effects of EDCs can add up in mixtures (Rajapakse et al., 2002). Various epidemiological studies focused on investigating the possible relationship between early-life exposure to EDCs and child health outcomes, such as infant growth and impaired sexual development (Andersen et al., 2008; Iszatt et al., 2016; Nørgaard et al., 2008). Exposure of Danish women workers to pesticides used in greenhouses has been associated with a rise in impaired reproductive development in their sons suggesting a link between EDC exposure and congenital deformity (Andersen et al., 2008). Although monitoring of selected chemicals provides precious information regarding biofluids' contamination, data considering the totality of these exogenous toxicants as well as the combined biological activities resulting from mixture effects are still limited.

Over the past decades, new biological, non-targeted testing procedures have been developed to supplement the targeted chemical-analytical techniques. Interestingly, it has been shown that single specific *in vitro* tests covering only a limited set of conserved pathways can also provide very good predictions with respect to developmental and reproductive toxicity landmarks such as sex organ deformities (Van der Burg et al., 2014). These highly selective reporter gene methods to assess (anti-)estrogenic and androgenic activity, were also shown to be excellent alternatives to the traditional *in vivo* techniques in rodents and extremely suitable for measurements in complex mixtures such as body fluids (Pedersen et al., 2010; Sonneveld et al., 2006). Estrogen Receptor alpha (ERα) and Androgen Receptor (AR) mediated bioassays are based on two human U2-OS osteoblastic osteosarcoma cell lines stably transfected to endogenously express the ERα or AR, respectively. The activation of these specific receptors upon ligand stimulation triggers their binding to hormone-responsive elements (HRE), which is linked to

a luciferase ("reporter") gene, leading to luciferase expression. By measuring the subsequent light production by luciferase, this bioassay can quantify hormonal activity of any chemical or sample. To ensure the robustness of the *in vitro* method, the ERα CALUX® bioassay was validated in 2015 according to the Organization for Economic Co-operation and Development (OECD) Test No.455: Performance-Based Test Guideline (PBTG) for stably transfected transactivation *in vitro* assays to detect estrogen receptor agonists and antagonists (Besselink, 2015). This study confirmed that the (anti-)ERα CALUX reporter gene bioassay is suitable for accurately predicting estrogen-disrupting activities. In turn, the (anti-)AR CALUX assay was recently implemented in the EU-NETVAL (European Union Network of Laboratories for the Validation of Alternative Methods) validation project, following the OECD Test Guideline Androgen Receptor Transactivation Assays and is currently in the process of an inter-laboratory evaluation. Over the years CALUX bioassays have been proven to be quick, specific methods able to measure the total effect of ligands on a receptor of interest using a limited volume of samples (Houtman et al., 2009; Kraus et al., 1995; Sonneveld et al., 2005).

Being the primary source of nutrition for most infants, breast milk contamination by exogenous chemicals could affect hormone-dependent mechanisms compromising post-natal growth. In that way, it is of major interest to assess the potential presence of EDCs in human milk as well as its short- and long-term impact on the developing child. In this paper we perform a pilot study aiming to extract EDCs from breast milk samples to further evaluate their impact on the endocrine system using the ERα and AR as endpoints. As a first step we demonstrate the performance of the AR CALUX bioassay, both in agonistic and antagonistic mode, by comparing our internal database to the *in vivo* Hershberger database recently established by Browne et al. (2018), as has been done to evaluate the ToxCast/Tox 21 AR model conducted by Kleinstreuer et al. (2017). As a second part, a set of ten breast milk samples derived from the "Norwegian Human Milk Study" (HUMIS), a birth cohort of mother–child pairs, was analyzed. Due to the various biochemical properties of human milk contaminants, ensuring their total extraction from breast milk using one universal method appeared to be challenging. Therefore, we developed a two-step method capable of extracting apolar EDCs fraction and polar components, including endogenous hormones, separately. Both fractions along with a reconstituted mixture, consisting of the combination of both apolar and polar extracts, were analyzed following the workflow detailed in Fig. 1. In addition to analysing the polar and apolar extracts, we also analysed a reconstituted mixture of the two fractions derived from each sample.

2. Materials & methods

2.1. Chemicals

17α-methyltestosterone (CAS: 58-18-4), 17β-estradiol (E2) (CAS: 50-28-2), 4-nonylphenol (CAS: 104-40-5), 5α-dihydrotestosterone (DHT) (CAS: 521-18-6), amitrol (CAS: 69182-5), bis-(2-ethylhexyl) phthalate (DEHP) (CAS: 117-81-7), bisphenol A (BPA) (CAS: 80-05-7), chlorothalonil (CAS: 1897-45-6), chlorpyrifos (CAS: 2921-88-2), dibutyl-phthalate (DBP) (CAS: 84-74-2), estrone (E1) (CAS: 53-16-7), fenarimol (CAS: 60168-88-9), flutamide (CAS: 13311-84-7), flutolanil (CAS: 66332-96-5), glyphosate (CAS: 1071-83-6), linuron (CAS: 330-55-2), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) (CAS: 72-55-9), pregnenolone (CAS: 145-13-1), procymidone (CAS: 32809-16-8), progesterone (CAS: 57-83-0), tamoxifen (CAS: 10540-29-1), testosterone (CAS: 58-22-0) and vinclozolin (CAS: 50471-44-8) were obtained from Sigma-Aldrich (The Netherlands). 4-androstan-17b-ol-3-one (androstenedione) (CAS: 63-05-8) and 5-androstan-3b-ol-17-one (DHEA) (CAS: 53-43-0) were purchased from Steraloids Inc. (USA). Tributyltin acetate (CAS: 56-36-0) was obtained from Merck Chemicals B.V. (The Netherlands).

2.2. Cell lines

The AR CALUX, ERα CALUX and Cytotox CALUX bioassays (Sonneveld et al., 2005; Van der Linden et al., 2014) are based on a stably transfected human osteoblastic osteosarcoma U2-OS cell-line (American Type Culture Collection). The highly selective cell line used in the (anti-)AR CALUX contains a full-length human AR expression vector stably co-transfected with a reporter construct containing a minimal promoter element, the TATA box, coupled to a luciferase reporter construct containing three androgen responsive elements (Sonneveld et al., 2005). The (anti-)ERα CALUX contains a similar expression vector expressing human ERα and a reporter construct 3xpERE-TATA-Luc, as described earlier (Sonneveld et al., 2005). Both cell lines were also used to perform the antagonistic (anti-) AR and ERα CALUX bioassays. As a control to detect non-specific activities (e.g. cellular death), each antagonistic measurement was performed along with the Cytotox CALUX, consisting of U2-OS cells constitutively expressing the luciferase gene (Van der Linden et al., 2014). The threshold was set at cytotoxicity $\leq$ 20%. For evaluation of analysis data, concentrations surpassing this value were excluded. All CALUX cell lines were cultured as described previously (Sonneveld et al., 2005).

2.3. Reference compound data selection

The Hershberger *in vivo* androgenicity assay database established by Browne et al. (2018) was browsed to establish a set of reference chemicals for the evaluation of the performance of the (anti)AR CALUX. Chemicals with consistent effects in Hershberger study were pre-selected. Compounds present in both *in vivo* database and internal CALUX dataset were chosen for the final list. A total of sixteen reference compounds including six non-active chemicals (4-nonylphenol, amitrol, chlorothalonil, chlorpyrifos, flutolanil and glyphosate), eight AR antagonists (DBP, DEHP, fenarimol, flutamide, linuron p,p'-DDE, procymidone and vinclozolin) and two steroidal androgens (17α-methyltestosterone and testosterone) were selected on that basis. The internal (anti-)AR CALUX database gathers > 200 pure compounds individually analyzed using an automated version of the (anti-)AR CALUX.

2.4. Human milk samples

Samples were derived from the mother–child cohort study HUMIS (Human Milk Study), cooperatively conducted by the Norwegian Institute of Public Health between 2002 and 2009 (Eggesbø et al., 2004). Between the first two weeks and months after delivery, women enrolled in the study were asked to collect 25 mL of breast milk every morning for eight consecutive days, preferably by hand. Milk aliquots were collected in 250 mL natural HDPE Packaging Bottles (Cat. No.: 967-21244, Thermo Scientific Nalgene®) made from high-purity resins, a food-grade material free of plasticizers. Aliquots were sent by the mothers along with a questionnaire gathering the following information: maternal age, weight, height, residence, smoking habits, parity, nationality, education, work and dietary habits. Samples were stored upon arrival at −20 °C in a Biobank of the Norwegian Institute of Public Health. The study was approved by the Norwegian Data Inspectorate (ref. 2002/1398) and Regional Ethics Committee for Medical Research (ref. S-02122). Mothers were included after oral and written informed consent had been obtained.

2.5. Sample preparation

For this study, ten aliquots were used to extract apolar and polar components, following the methods described below.

For apolar compounds, 5 mL of homogenized milk sample was transferred to a 60 mL glass tube. The same amount of 2-propanol (CAS: 67-63-0, BioSolve) was added prior to the extraction to optimize the penetration of n-hexane into the sample material during the next stage.

Tubes were shaken for 10 min on a shaker at 200 ± 20 S per minute. 14 mL of n-hexane (CAS: 110-54-3, BioSolve) was added and the tubes were shaken for an extra hour. The upper layers were transferred to clean collection tube and the procedure was repeated twice with a shaking time reduced to 30 min. The collected fractions were evaporated to dryness and reconstituted in 1 mL of n-hexane. The extracts were cleaned (including fat removal) using glass columns filled with 5 g of 2% deactivated silica, previously conditioned with 12 mL of n-hexane. The samples were eluted with 30 mL of a 3:1n-hexane and dichloromethane mixture (CAS: 75-09-2, BioSolve). The eluate obtained was evaporated to dryness and reconstituted in 30 µL of DMSO.

The efficiency of the apolar extraction was assessed by including four procedural controls extracted along the set of breast milk samples, using gas chromatograph coupled with tandem mass spectrometer (GC–MS/MS) as the detection technique. The controls consisted of 5 mL of a pooled breast milk sample spiked with 50 µL of ^{13}C-labeled internal standard solution containing PCB153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) and PCB180 (2,2′,3,4,4′,5,5′-heptachlorobiphenyl) (200 ng/mL, diluted from MBP-D7, Wellington Laboratories). Due to their apolar properties and their known presence in breast milk, PCB153 and PCB180 were suitable contaminants to evaluate the performance of the extraction method (IARC Working Group on the Evaluation of Carcinogenic Risk to Humans, 2016). Controls were extracted following the same procedure as used for samples with the exception that they were dissolved in 1 mL of isooctane (CAS: 540-84-1, BioSolve), which is a more suitable and common solvent for chemical analysis with GC–MS/MS. A recovery standard containing PCB 112 (10 ng/mL, diluted from C-112S-TP, AccuStandard) was added to each control extract just prior to the injection. This extra step was included to correct for response variations due to internal matrix effects during GC–MS/MS measurement, and is independent from the extraction performance. Internal standard recoveries were evaluated on a GC–MS/MS system, consisting of a gas chromatograph (GC-2010 Plus, Shimadzu) and a gas chromatograph mass detector (GCMS-TQ8050, Shimadzu), both controlled by the GCMS Real Time Analysis software program (available at: https://www.ssi.shimadzu.com/products/gas-chromatography-mass-spectrometry/gcmssolution-software.html). The injection was carried out using a CTC CombiPal autosampler controlled by the Cycle Composer software program (CTC Analytics AG). The system equipped with a DB-5MS column (60 m × 0.25 mm × 0.25 µm) (Cat. No.: 122-5562, Agilent Technologies), operated using the following parameters: carrier gas Helium 6.0 (BIPX10S, Air Products), constant flow rate of 1.00 mL/min (electronically controlled), injection temperature 280.0 °C, injection volume 1 µL, splitless mode, ion source temperature 230.0 °C, interface temperature 300.0 °C. The analysis was performed using the parameters and settings described in Tables 1 and 2. After PCB112 correction, recovery values were evaluated to 92 ± 5.4 and 91 ± 4.1% for PCB153 and PCB180, respectively.

QuEChERS (Quick Easy Cheap Effective Rugged and Safe) was used to extract polar compounds. It is a simplified sample extraction technique developed to assess multiple pesticide residues in food (Anastassiades et al., 2003). We used a protocol derived and adapted from the original method, as described below. Samples were homogenized and 5 mL was transferred to a 50 mL Greiner tube. 15 mL of acetonitrile (ACN) (CAS: 75-05-8, BioSolve) was added as the extraction solvent and the mixture was shaken vigorously manually for 30 s. One QuEChERS EN 15,662 extraction packet (Cat. No.: 5982-5650, Agilent) containing 4 g of magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate dehydrate and 0.5 g of sodium hydrogen citrate sesquihydrate was added directly into the tube and shaken strongly for 15 min. The tubes were centrifuged for 5 min at 4000 rpm at 4 °C and the upper layers were transfer into a clean collection tube. The same procedure, including ACN and addition of salts, was repeated once and the resulting layers were combined. Each combined extract was loaded to a 15 mL QuEChERS dispersive solid phase extraction (d-SPE) column (Cat. No.: 5982-5158, Agilent) for clean-up and vortexed for 1 min. The tubes were centrifuged for 5 min at 4000 rpm at 4 °C. The upper layer was transferred to a clean collecting tube and evaporate until dryness. The samples were reconstituted in 30 µL of DMSO and stored at −20 °C until analysis.

Reproducibility and efficiency of the polar extraction was assessed by adding four controls to the analysis series. The controls consisted of 5 mL of breast milk spiked with 100 µL of a mixture of BPA, E2 and testosterone as internal standards (100 µg of each compound/mL). Due to their polarity and their known presence in breast milk, BPA (logP = 4.0), E2 (logP = 3.7) and testosterone (logP = 3.4) were three appropriate compounds to assess the efficiency of the polar extraction method (Mendonca et al., 2014). Controls and samples were processed following the same extraction procedure, except that controls were redissolved in 30 µL ACN, a suitable and common solvent for the liquid chromatography (LC) analysis of these components. Chemical analysis was performed by LC using a modular system from Agilent consisting of 1260 Infinity High Performance Degasser (G4225-64000), a 1260 Infinity Binary pump (specified up to 600 bar - G1312B), Multisampler (G7167-64000, Agilent), Diode array detector (G1315b-64050, Agilent) and a thermostatted column compartment (G1316-64050, Agilent). The samples were analyzed on a Phenomenex Kinetex Biphenyl column (150x4.6 mm 2.6µ particle size) (00F-4622-E0, Phenomenex) using the following parameters: injection volume 20 µL; flow rate of 0.8 mL/min; detection (UV) 254, 272 nm; column temperature 40 °C. The system was controlled by Agilent OpenLAB CDS (EZ ChromEdition) software program. This program was also used to process the data. Water and ACN were used as solvents according to the scheme described in Table 3. Recovery values were calculated by means of comparison of the peak height in the control samples with a standard solution. BPA, E2 and testosterone were recovered to a rate of 36 ± 13, 50 ± 7.1 and 61 ± 2.9%, respectively. On average, 49% of spiked polar chemicals were recovered after extraction. This result was used to compensate for loss of signal during the CALUX analysis, i.e polar measurements were adjusted to apolar results using a factor of 2. Moreover,

Table 1
GCMS oven temperature program for recovery assessment.

Rate	Temperature (°C)	Hold Time (min)
–	80.0	2.0
20.00	180.0	0.0
5.00	200.0	0.0
2.00	240.0	10.0
20.00	320.0	10.0

Table 2
GCMS internal and recovery standards for recovery assessment.

Internal standard	Precursor	Product
PCB153L (*m/z*)	371.80	301.90
PCB180L (*m/z*)	405.80	335.90
Recovery Standard	Precursor	Product
PCB112 (*m/z*)	323.90	253.90

Note: *m/z*: mass-to-charge ratio.

Table 3
Liquid chromatography gradient of water/acetonitrile for recovery assessment.

Time (min)	Water (%)	ACN (%)
0.00	30.0	70.0
1.00	30.0	70.0
10.00	0.0	100.0
12.00	0.0	100.0
12.50	50.0	50.0
17.50	50.0	50.0

Note: ACN: Acetonitrile.

polar and apolar ratio to reconstitute the mixture (originally 1:1), was adapted to insure the correctness of the study (2:1). All extracts were kept at −20 °C until analysis.

2.6. CALUX assay procedures

Human milk samples were extracted, and bioactivities were evaluated with the (anti-)ERα and (anti-)AR reporter gene CALUX (Chemically Activated Luciferase eXpression) bioassays at different concentrations (dilution series 1-3-10-30-100x). The (anti-)ERα and (anti-)AR CALUX bioassays were carried out typically as described by Sonneveld et al. (2005). In short, cultured U2-OS cells were re-suspended in DMEM/F12 medium without phenol red indicator (Fisher, product no.:VX1041025) with 10U/mL penicillin and 10 μg/mL streptomycin (P/S), non-essential amino acids (NEAA) (Cat No.: 11140-03, Gibco) and 5% charcoal-stripped fetal calf serum (DCC) supplemented. Cells were re-suspended in assay medium to a final concentration of $1x10^5$ cells/mL, subsequently 100 μL were seeded in clear 96-well plates. Plates were incubated for a minimum of 16 h and maintained in a humidified atmosphere at 37 °C and 5% CO_2. After a maximum of 24 h, the medium was replaced with 200 μL/well of medium including the sample to a final DMSO concentration of 0.1%. To perform the antagonistic mode, the medium was supplemented with a fixed concentration of E2 (EC50: $6.0E^{-12}$ M) or dihydrotestosterone (DHT; EC50: $3.0E^{-10}$ M), for the anti-ERα and anti-AR CALUX, respectively. After 22 ± 2-hour incubation, the medium was removed and 30 μL/well of Triton-lysis buffer was added and luciferase activity was measured using a Tristar luminometer (Berthold).

Analysis of pure compounds using the AR CALUX was essentially performed as above with the exception that the dilution (0.5 log unit increments) and the exposure was automated and performed in a 384-well plate according to a validated method as to increase the efficiency of the procedure (Van Vugt-Lussenburg et al., 2018).

All dilutions including the ones from the samples were analyzed in triplicate on the same 96-well plate. A solvent control (DMSO) was included to each plate to assess the background luciferase activity. Moreover, a full dose response curve of the reference compound E2 (ERα CALUX), DHT (AR CALUX) or tributyltin acetate (Cytotox CALUX) was included to each plate. The reference compounds tamoxifen and flutamide were used as reference for the anti-ERα and anti-AR CALUX assays, respectively. Antagonistic measurements were double-checked for non-specific interactions by assessing test compounds with a saturating excess amount of agonist i.e. 1000-times the EC50 concentration of the agonistic reference compound (Van der Burg et al., 2010). The quality and validity of the measurements were evaluated according to the following criteria: R2 of standard curve > 0.98, EC50 of the reference compound should fit between assay-specific predetermined limit values, z-factor of standard curve > 0.6 and overall SD of triplicate < 15%.

2.7. Data analysis

Luciferase activity was expressed as Relative Light Units (RLUs). The average of each triplicate was calculated and corrected for background using solvent control measurements. The maximum signal response induced by the reference compound was set to 100% for agonistic assay (complete activation of the receptors) and 0% for antagonistic mode (complete inhibition of the signal). Subsequently, all RLUs produced by the test compounds or samples were expressed as a % of this maximum response. The statistical software package GraphPad Prism 5.0 was used to fit transformed data (non-linear regression, variable slope, four parameters and robust fit). The lowest observed effect concentrations (LOECs) were determined; for agonist assays, the LOEC was defined as the PC10 value, while for antagonist assays a PC20 value was used. The PC10 concentration was defined as the concentration where the response elicited by the test substance equals 10% of the maximum response of the reference substance. PC20 values were defined as the concentration where the test substance causes 20% inhibition of the basal signal elicited by the receptor agonist. In this case, the maximum inhibition achieved by the reference antagonist was set at 100%. Compounds without a calculable PC10/PC20 value were defined as non-active (NA). For quantification of the (anti-)estrogenic and (anti-)androgenic potency of breast milk extracts, RLUs derived from CALUX analysis were plotted to the dose-response curve of the associated reference compound. A sample was reported as 'active' if its overall activity was above the limit of quantification (LOQ) value of the bioassay, i.e. lowest concentration that can be quantified with acceptable relative standard deviation. A non-active sample i.e. activity ≤ LOQ, was replaced by half of the LOQ value to give an estimation of the activity. The calculated concentrations per well (M) were converted and expressed as equivalence of the reference compound per gram of processed sample.

2.8. Instrumentation and analytical methods for fractionation

Seven natural steroid hormones, E1, E2, testosterone and their precursor androstenedione plus pregnenolone, progesterone and their product DHEA and androstenedione were targeted during an exclusion by fractionation using liquid chromatography. Androstenedione, DHEA and testosterone were selected based on their androgenic properties that could create interferences with AR, possibly counteracting anti-AR activity measured in the CALUX bioassay (Burger, 2002; Houtman et al., 2009). On the other hand, E1, E2, progesterone and pregnenolone were chosen for their estrogenic characteristics. This type of activity was earlier reported to be anti-androgenic and androgenic, depending on the concentration, which could also bias the measurements (Kelce and Wilson, 1997; Sohoni and Sumpter, 1998; Van der Burg et al., 2010).

The outlet of the high-performance liquid chromatography (HPLC) setup described earlier was connected to a Fraction collector III (WFCIII, Waters) remotely triggered by the Agilent system. In the fractionation process we used two types of columns, Phenomenex Kinetex Biphenyl 150 × 4.6 mm 2.6u particle size (00F-4622-E0, Phenomenex) and Phenomenex Kinetex Fluor 5 100 × 2.1 mm 1.7u particle size (00D-4722-AN, Phenomenex) (pentafluorophenyl stationary phase bonded to a solid silica core). We operated the described system with a combination of methanol absolute HPLC Supra-gradient (CAS: 67-56-1, Biosolve) and ultra clean water from an ELGA filtration station as solvent. For all experiments the columns were maintained at 40 °C. Data were analyzed using the Agilent OpenLAB CDS (EZ ChromEdition) software program. Parameters including column, solvents and gradients used for each fractionation are referred to as 'protocol n°1' and 'protocol n°2' (Table 4.).

2.9. Instrumental settings for chemical analysis followed by non-target screening

The active fractions along with non-active ones, collected adjacent to the active eluates, were obtained through fractionation and were analyzed in triplicate with an Nexera UHPLC system (Shimadzu, Den Bosch, the Netherlands), coupled to a maXis 4G high resolution quadrupole time-of-flight HRMS (UHPLC-Q-TOF-MS/MS) upgraded with a HD collision cell and equipped with an electrospray ionisation source (ESI) (Bruker, Leiderdorp, the Netherlands) (Appendix A.1). Liquid chromatography analysis was performed according to protocol n°2, described earlier (Table 4). 2 mM of sodium formate in 1:1 water/MeOH solution was used for automatic internal calibration of the system. Positive and negative ESI was used to acquire MS- and MS/MS data in the range of 80–1200 *m/z*. Simultaneously an UV chromatogram (254 nm) was acquired using an UV detector placed in line between the UHPLC and the Q-TOF-MS/MS. The peak retention times in the UV chromatogram were compared to those found during the fractionation

Table 4
Liquid chromatography conditions, column and gradient used for fractionation.

	Column	Time (min)	Water (%)	Methanol (%)
Protocol n°1	Phenomenex Kinetex Biphenyl	0.00	50.0	50.0
	150x4.6 mm 2.6u particle size	0.01	50.0	50.0
	0.8 mL/min	10.00	5.0	95.0
	Injection vol. 30 µL	16.00	5.0	95.0
		16.50	50.0	50.0
		25.00	50.0	50.0
Protocol n°2	Phenomenex Kinetex Fluor 5	0.00	80.0	20.0
	100x2.1 mm 1.7u particle size	0.01	80.0	20.0
	0.250 mL/min	25.00	0.0	100.0
	Injection vol. 20 µL	28.00	0.0	100.0
		28.50	80.0	20.0
		48.00	80.0	20.0

to compensate for differences between both LC systems.

The data acquired from the non-target analysis (NTA) was processed using an in-house developed R package 'patRoon' (Helmus et al., 2018) which is an open-source platform that harmonizes various commonly used software tools employed in NTA. More information is available at https://github.com/rickhelmus/patRoon (Patroon: Open Source Software Platform for Environmental Mass Spectrometry Based Non-target Screening 2020). The first step of the patRoon workflow consisted of converting the manufacturer specific data to open mzML format. Second, features (single peaks within the extracted ion chromatogram) were extracted from all analysed fractions and grouped according to retention time and *m/z* (mass to charge ratio). Third, the features were filtered by employing different constraints and thresholds. The feature data were filtered following these steps:

a. Removing any features below an intensity threshold (10000).
b. Removing features that are not at least 5 times higher in intensity compared to the blank.
c. Remove features that are not present in all triplicate injections and with intensity variations amongst triplicates of > 75% RSD.
d. Only unique features that are present in active fractions were kept, features from non-active fractions were removed.

Next, possible human metabolites of the seven endogenous hormones were predicted using BioTransformer (http://biotransformer.ca/) and the software R. Based on all known reaction pathways from BioTransformer, a list of 248 metabolites was generated (Appendix A.1). The presence of these metabolites in the active fractions was determined by comparing their *m/z* (within a window of 0.002 Da) to those of the unique post-processed features for the background.

3. Results

3.1. Evaluation of the (anti-)AR CALUX assay performance

In order to test the performance of the (anti-)AR CALUX assays relative to reference methods we used a similar approach as has been used for the ERα CALUX assay (Vugt-Lussenburg et al., 2018). A set of sixteen reference compounds including six non-active chemicals, eight AR antagonists and two steroidal androgens were selected based on the Hershberger *in vivo* androgenicity assay database developed by Browne et al. (2018) (Table 5). In the agonistic mode, only two steroids, 17α-methyl testosterone and testosterone, had sufficient reference data. They were evaluated and found to be strong agonists with a PC10 of −9.6. This is consistent with earlier findings that this assay can predict androgenicity of chemicals, including steroids, and is predictive for androgenicity as determined in the Hershberger assay (Houtman et al., 2009; Sonneveld et al., 2006). Expectedly, none of these agonists were active in the anti-AR CALUX assay. A larger set of eight reference anti-androgens with consistent effects in the *in vivo* Hershberger assay was assessed in the anti-AR CALUX assay. All chemicals showed anti-androgenic activity, with the exception of DEHP which showed no response at the highest tested concentration (-4). This is consistent with the fact that DEHP is not a receptor antagonist but rather decreases circulating levels of androgens (Borch et al., 2006). Six chemicals (4-nonyphenol, amitrol, chlorothalonil, chlorpyrifos, flutolanil, glyphosate), which demonstrated no effect in the *in vivo* Hershberger model scored negative in both the agonist and antagonistic modes of the AR CALUX assay. Overall, the AR CALUX assay demonstrated a very satisfying predictivity suggesting that this assay is suitable to assess androgen receptor-mediated effects of chemicals.

Table 5
Summary of the comparison between (anti-)AR CALUX results and *in vivo* reference classification.

CAS no.	Compound	*in vivo* reference classification	AR CALUX PC10* (logM)	Anti-AR CALUX PC20* (log M)
58-18-4	17α-methyl testosterone	androgenic	−9.6	NA (> -4)
58-22-0	testosterone	androgenic	−9.6	NA (> -4)
84-74-2	DBP	anti-androgenic	NA (> -4)	−4.7
117-81-7	DEHP	anti-androgenic	NA (> -4)	NA (> -4)
60168-88-9	fenarimol	anti-androgenic	NA (> -4)	−6
13311-84-7	flutamide	anti-androgenic	NA (> -4)	−6.5
330-55-2	linuron	anti-androgenic	NA (> -4)	−6.3
72-55-9	*p,p'*-DDE	anti-androgenic	NA (> -4)	−7.5
32809-16-8	procymidone	anti-androgenic	NA (> -4.5)	−7.3
50471-44-8	vinclozolin	anti-androgenic	NA (> -4)	−6.7
104-40-5	4-nonylphenol	negative	NA (> -4)	NA (> -4)
61-82-5	amitrol	negative	NA (> -4)	NA (> -4)
1897-45-6	chlorothalonil	negative	NA (> -4)	NA (> -4)
2921-88-2	chlorpyrifos	negative	NA (> -6)	NA (> -6)
66332-96-5	flutolanil	negative	NA (> -4)	NA (> -4)
1071-83-6	glyphosate	negative	NA (> -4.3)	NA (> -4.3)

Note: *PC10 (agonistic mode) or PC20 (antagonistic mode), i.e., the concentration where the response elicited by the test compound equals 10 or 20% of the maximum response were calculated. Abbreviations: DHT: 5α-dihydrotestosterone; DEHP: bis-(2-ethylhexyl)phthalate (DEHP) (CAS: 117-81-7); *p,p'*-DDE: *p,p'*-dichlorodiphenyldichloroethylene. NA: no activity observed at the highest concentration tested; value in between brackets is the highest concentration tested.

3.2. Determination of hormone-related activity in breast milk samples

Next, we used the AR CALUX along with the ERα CALUX assay to assess the hormonal activity in ten breast milk samples. To optimally extract chemicals from the breast milk, apolar and polar analytes derived from each sample were extracted using two separate methods and combined subsequently to form reconstituted extracts. These mixtures were analyzed in the ERα and AR CALUX bioassays, both in the agonistic- and antagonistic mode (Table 6). All samples were first tested and corrected for cytotoxicity to exclude non-specific effects. No antagonistic activity towards ERα was observed and only two samples scored positive in the agonistic ERα CALUX bioassay. All samples demonstrated anti-AR activity except number 1 and 8 which did not reach the limit of quantification (LOQ) of the AR CALUX bioassay.

Considering that 80% of breast milk samples were positive for anti-androgenic testing, a migration experiment was performed according to European Commission Regulation No 10/2011 and EN1186, described by Kirchnawy et al. (2014) to assess possible artificial activities derived

Table 6
Hormonal activity of ten reconstituted human milk samples assessed in the (anti-)AR and (anti-)ERα CALUX bioassays.

Number	ERα CALUX (ng E2 eq./g)	Anti-ERα CALUX (μg tamoxifen eq./g)	AR CALUX (ng DHT eq./g)	Anti-AR CALUX (μg flutamide eq./g)
1	0.15	0.15 (< LOQ)	4.0	0.22 (< LOQ)
2	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.73
3	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.53
4	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.58
5	0.14	0.15 (< LOQ)	0.02 (< LOQ)	0.61
6	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.85
7	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.64
8	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.23 (< LOQ)
9	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.61
10	0.002 (< LOQ)	0.15 (< LOQ)	0.02 (< LOQ)	0.8

Note: Each human milk sample was individually reconstituted from apolar and polar extracts. Samples are arbitrarily numbered from 1 to 10. Results are expressed as equivalent per gram of material using the reference compound of the assay as reference. Abbreviations: AR: androgen receptor; ERα: estrogen receptor α; E2: 17β-estradiol; DHT: 5α-dihydrotestosterone; LOQ: Limit of quantification.

from plastic HDPE bottles. Results demonstrated that an anti-androgenic activity equals to 0.18 ± 0.04 μg flutamide eq./g of material could be attributed to the containers. These findings showed that only a small proportion of the activity present in human milk samples could be explained by storage conditions, however all active samples were well above this value suggesting that they contain biologically active components with anti-androgenic activity. As a first step towards understanding the nature of the anti-AR activities, the same ten human milk samples were analyzed as two distinct fractions.

In all apolar fractions the antagonistic activities detected were below LOQ (Table 7). All polar fractions scored positive ranging from 1.8 to 9.0 μg flutamide eq./g with the exception of sample n°1 which was classified as < LOQ due to its extremely low signal. Anti-androgenic activity in polar fractions were found to be significantly higher than the average background levels from leachable components. All activities measured in the polar extracts were considerably higher when assessed alone rather than in combination with the apolar fraction as a reconstituted mixture. These results suggest an accumulation of polar compounds, possibly EACs with AR antagonistic properties in breast milk of the HUMIS cohort. However further investigation is required to determine whether the anti-AR activity is caused by endogenous compounds in human milk i.e., natural hormones, or is of anthropogenic nature.

Table 7
AR antagonistic activities of breast milk sample extracts measured using the antagonistic mode of the AR CALUX bioassay.

Number	Apolar (μg flutamide eq./g)	Polar (μg flutamide eq./g)	Combined extract (μg flutamide eq./g)
1	0.22 (< LOQ)	0.05 (< LOQ)	0.22 (< LOQ)
2	0.15 (< LOQ)	2.82	0.73
3	0.13 (< LOQ)	1.8	0.53
4	0.18 (< LOQ)	3.0	0.58
5	0.20 (< LOQ)	9.0	0.61
6	0.22 (< LOQ)	4.8	0.85
7	0.22 (< LOQ)	5.7	0.64
8	0.23 (< LOQ)	6.3	0.23 (< LOQ)
9	0.17 (< LOQ)	1.9	0.61
10	0.22 (< LOQ)	4.2	0.8
Average	< LOQ	4.0	0.58

Note: Apolar and polar endocrine active compounds derived from nine human milk sample were extracted and analyzed as two distinct fractions on the anti-AR CALUX. Both extracts were mixed and analyzed together to evaluate the effects of the combined fractions. Results are expressed as flutamide equivalent per gram of milk. Abbreviations: AR: androgen receptor; LOQ: Limit of quantification.

3.3. Chemical evaluation of the anti-androgenic activity in human milk samples

Human naturally occurring steroid hormones (e.g. DHEA, E2, E1, pregnenolone, progesterone and testosterone) could potentially interact with the androgen receptor. Using a pooled breast milk sample as a basis, we evaluated if this set of hormones is related to the AR antagonistic activity as measured in the polar fraction. For this, we used effect-directed analysis to see if the biological effect (AR antagonism) coelutes with these steroids using a chromatographic separation step based on a Biphenyl column. Four distinct fractions, A.1 to A.4, were collected using HPLC following a five-minute time-interval scheme (Fig. 2). Clearly, the steroids co-eluted with the bioactivity as an activity of 0.88 μg flutamide eq./g was measured in fraction number 3 (A.3) while no activity was present in the other fractions. Further fractionation was required to eliminate them from the extract.

A.3 was further fractionated using a Fluor 5 column. With the exception of pregnenolone and progesterone, all the hormones co-eluted within fraction B.2, while only fraction B.3 showed an AR antagonistic activity (1.7 μg flutamide eq./g) (Fig. 3). Due to their co-elution with the active fraction B.3, pregnenolone and progesterone could still play a role in the observed anti-androgenic activity in human milk samples.

The Fluor 5 chromatographic separation procedure was repeated, using smaller time window collection points. As the anti-AR activity was positioned in fraction B.3 (20 to 30 min), this 20–30 min time frame was further divided in five two-minute fractions, labelled C.1 to C.5, which were collected and analyzed separately in the anti-AR CALUX (Fig. 4). Fractions C.3 and C.4 were found to contain anti-androgenic activity with a total activity of the combined fractions equivalent to 1.1 μg flutamide eq./g. Pregnenolone and progesterone co-eluted with fractions C.1 and C.2 implying that none of the studied endogenous hormones were in the extract containing the anti-AR activity.

Subsequently, a suspect list of 248 human metabolites was generated based on the seven endogenous hormones, set as parent compounds, using a combination of the BioTransformer website and the software R (Appendix A.2). This list was used for the identification of metabolites in C.3 and C.4 fractions after UHPLC-Q-TOF-MS/MS-based non-targeted analysis. After comparison between the predicted masses and the analyzed unique features, none of the metabolites were detected in the fractions of interest showing that neither these compounds nor their parent hormones (androstenedione, DHEA, E1, E2, pregnenolone, progesterone, testosterone) can explain the anti-androgenic activity found in breast milk samples.

4. Discussion

In this manuscript, we identified anti-androgenic activity in human milk samples derived from the Norwegian HUMIS cohort. Through a

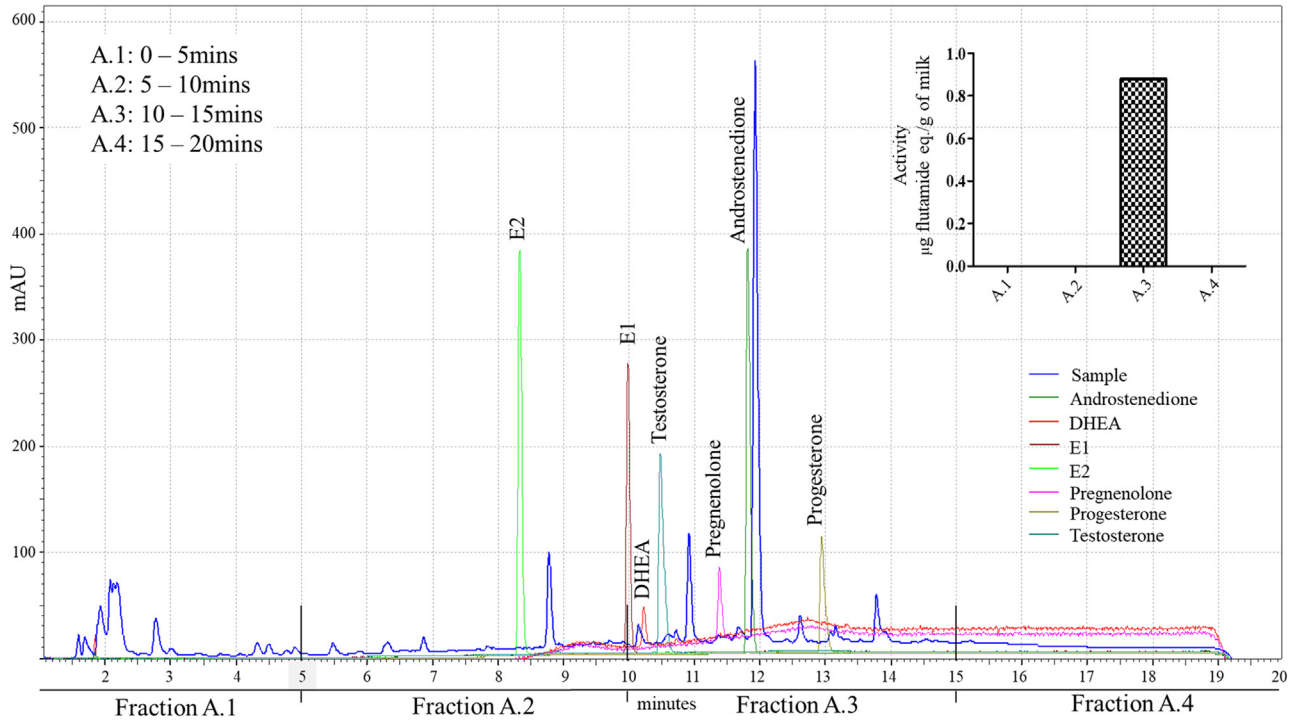

Fig. 2. Chromatographic overlay of elution patterns of the seven endogenous hormones with the pooled human milk extract. *Note:* Chromatogram derived from each individual internal standard was overlapped to the chromatogram of the polar fraction of a pooled breast milk sample (blue). All endogenous hormones except for E2 co-eluted with fraction A.3. Abbreviations: E1: estrone; E2: 17β-estradiol; DHEA: 5-androstan-3b-ol-17-one. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

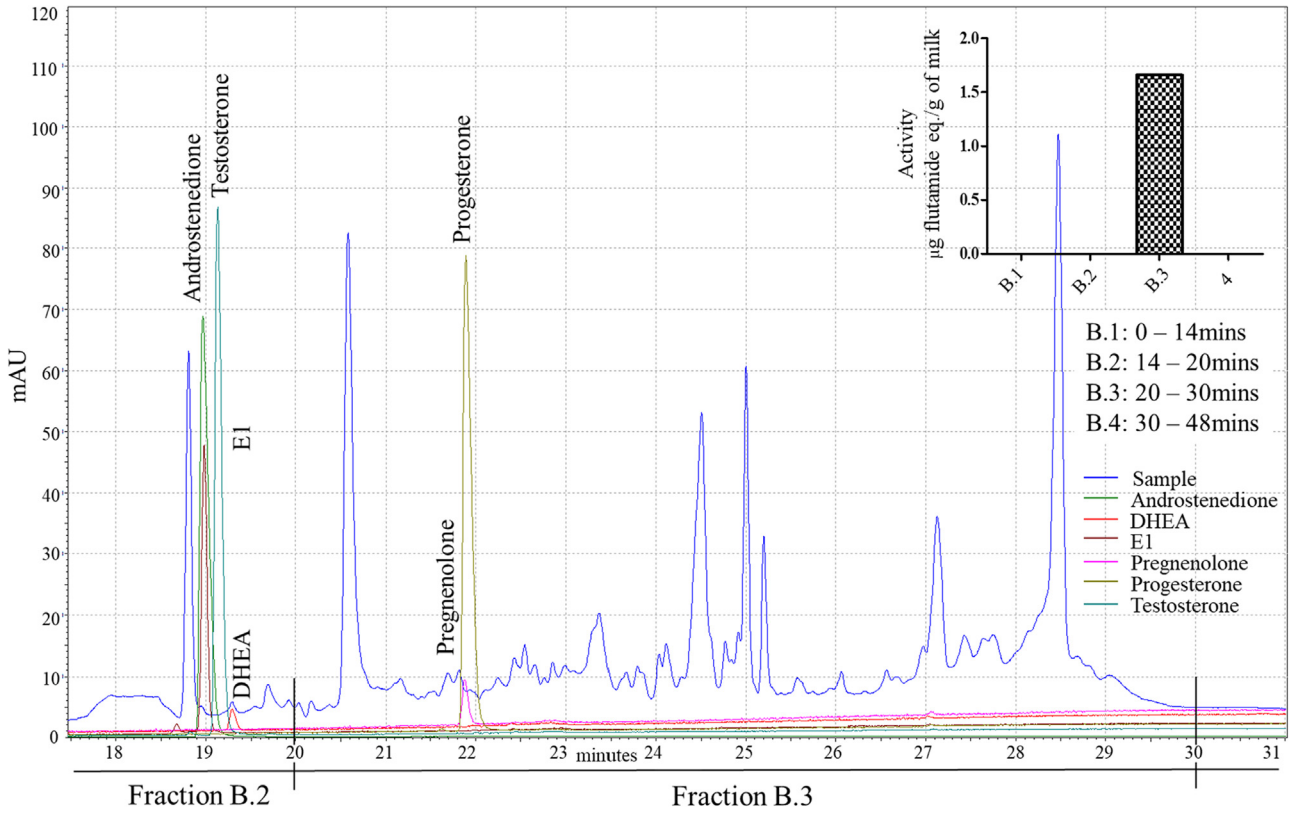

Fig. 3. Chromatographic overlay of elution profiles of endogenous hormones and anti-AR bioactivity in human milk fraction A.3 on a Phenomenex Kinetex Fluor 5 column. *Note:* Chromatogram derived from each individual internal standard was overlapped to the chromatogram of the polar fraction of a pooled breast milk sample (blue). All endogenous hormones except for pregnenolone and progesterone (b.3) co-eluted with fraction B.2. Abbreviations: E1: estrone; DHEA: 5-androstan-3b-ol-17-one. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

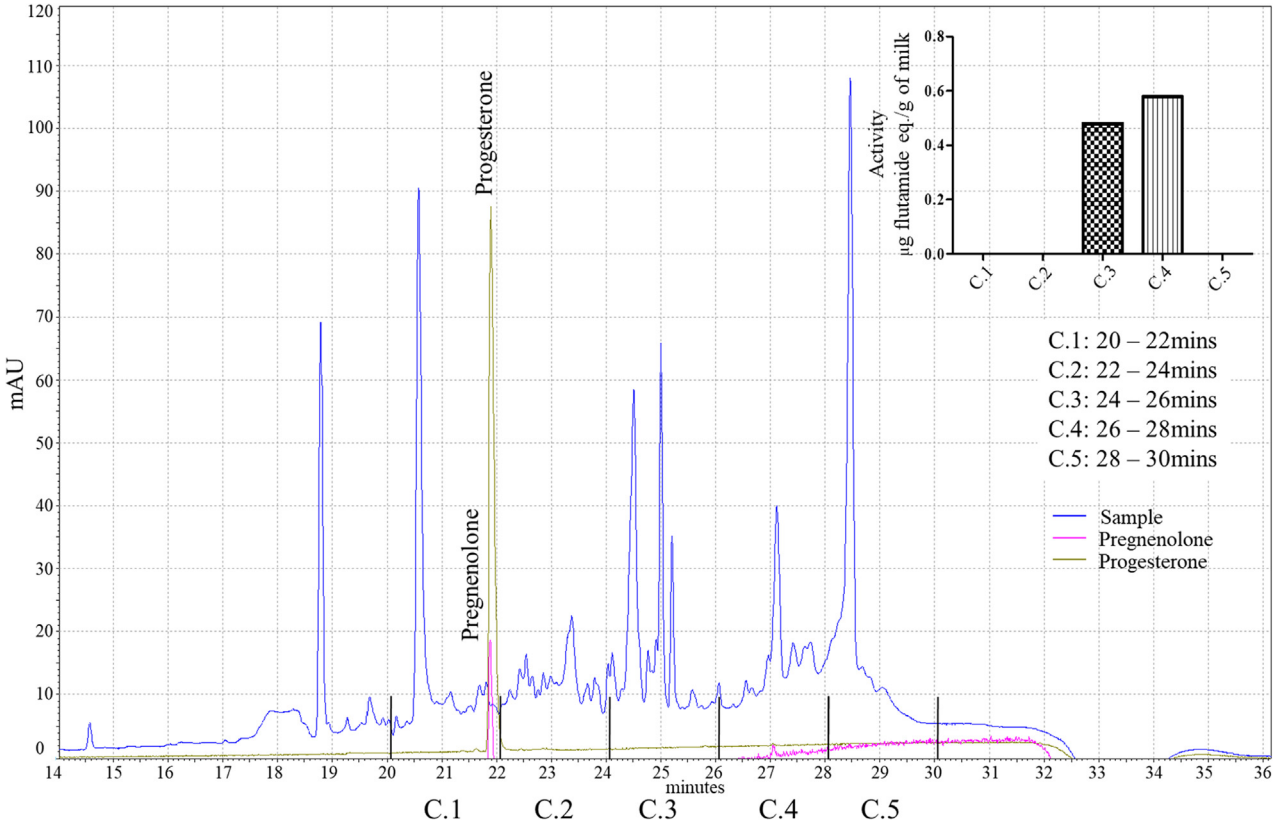

Fig. 4. Further refined sub-fractionation of the anti-AR activity containing B.3 fraction on the Phenomenex Kinetex Fluor 5 column, with elution pattern overlay of pregnenolone and progesterone.

chemico-biological analysis, we could exclude that androstenedione, DHEA, E1, E2, pregnenolone, progesterone, testosterone and their metabolites were responsible for the observed anti-androgenic activity measured using the AR CALUX bioassay. While it is not excluded that endogenous hormones might contribute to the anti-androgenic activity observed by the as yet unidentified EDCs, the findings suggest that breastfeeding might constitute a potential route of early human exposure to mixtures of anthropogenic contaminants with anti-androgenic properties.

Excellent correlations were observed between the *in vivo* Hershberger classifications and the AR CALUX results for the sixteen reference compounds selected. Similar to the highly selective ERα CALUX bioassay, the (anti-)AR CALUX reporter gene bioassay may provide a reliable alternative test method to the traditional *in vivo* methods in rodents (Sonneveld et al., 2006). Indeed, the six chemicals, reported as non-active in the Hershberger database established by Browne et al. (2018) scored negative on both AR-based CALUX assays. Testosterone and 17α-methyl testosterone, two natural androgens, shown strong agonistic properties in the AR CALUX bioassay, matching our expectations. These results are consistent with Sonneveld et al. (2006) demonstrating the abilities of the AR CALUX in detecting agonistic activities using a wide array of mainly steroidal compounds, also showing a parallel between *in vitro* and *in vivo* predictions. Moreover, previous studies showed that the assay also reponds to all bioactive androgens present on the prohibited list of the World Anti Doping Authority (Houtman et al., 2009). The AR CALUX in its antagonistic mode was, however, never compared to *in vivo* studies. Consequently, a larger set of antagonists was selected. Predictivity of the anti-AR CALUX bioassays was very satisfying and in line with earlier work by Van der Burg et al. (2010). However, one compound, DEHP, scored differently than reported in the Hershberger database. This phthalate was inactive in the *in vitro* anti-AR CALUX assay, which is in line with the results from the ToxCast AR model described by Kleinstreuer et al. (2018) also scoring DEHP negative. In the ToxCast study the highest concentration tested was equal to 100 µM, similar to the concentration used in the anti-AR CALUX. By extrapolating the estimated administered dose, Kleinstreuer et al., showed that this concentration was similar to the lowest observed effect level (LOEL) values derived from the Hershberger method, ranging from 100 to 200 mg/kg body weight per day, suggesting that the discrepancy in classification between the methods could not be explained by a difference in concentration (Kleinstreuer et al., 2018). The mismatch is very likely caused by the fact that DEHP antagonizes steroid production, which will be measured *in vivo* as an antagonistic effect, while this is not picked up *in vitro* in a receptor-based assay, but rather in a steroidogenesis assay as present in an EATS panel of assays (Borch et al., 2006). Both ERα- and AR- *in vitro* bioassays showed a good performance and applicability in detecting developmental and reproductive toxicity of chemicals and were found to be powerful tools in predicting the effects of EDCs on the endocrine system (Piersma et al., 2013; Van der Burg et al., 2014).

The pilot epidemiological study including ten breast milk samples revealed an activation of the estrogen receptor α in two extracts only. However, an antagonistic activity towards the androgen receptor was measured in most of the assessed samples. The findings regarding ERα activation are crucial to understand the origins of the measured anti-androgenic activity. Indeed, diverse estrogens such as E1 and E2 were earlier reported to be both androgenic and anti-androgenic, albeit at much higher dosages (> 1000-fold), levels not reached in normal physiology. The absence of estrogenic activity in most of breast milk extracts could be explained by the typical drop in estrogens levels immediately in the postpartum period, programmed to induce and maintain lactation during the first months after delivery (Lu et al., 2017). This hypothesis is in line with the sampling time of the present samples, ranging from two weeks to two months after birth. Conversely, high levels of estrogens can lead to a reduced milk production resulting in difficulties to breastfeed, which is obviously not relevant for our

study group. Further investigations on the anti-AR CALUX showed that the antagonistic activity could be fully attributed to the polar fraction with an average of 4.0 μg flutamide eq./g. On the contrary, all measurements of the apolar fraction were found to be below the limit of quantification. No indication of synergy between the two fractions was noticed. However, it appears that the presence of the apolar fraction, although having a limited response, induces the repression of the activity of the polar extracts when assessed as a mixture (0.58 μg flutamide eq./g on average). Interestingly, this response contrasts with the additive or synergistic effects usually expected from EDCs mixing cocktails (Bergman et al., 2012; Kortenkamp 2014). While the presence of polar anti-androgenic EDCs could be linked to the activity measured on the anti-AR CALUX bioassay, we cannot exclude the fact that a mixture effect could lead to an enhanced anti-AR activity in the samples. Indeed, studies showed that chemicals with similar anti-androgenic properties may induce strong adverse outcome in male rats, in comparison, only showing small effects were noticed during individual chemical analysis (Hass et al., 2007). Therefore, it is unlikely that the activity measured in breast milk samples could be solely attributed to the presence of certain highly anti-androgenic EDCs whereas, a more complex combination of different type of chemicals is more plausible. Total activity measurements such as the CALUX method gives an overview of the activity considering all chemicals present in the sample however, further analysis is required to identify a specific chemical to be avoided.

In our samples, we expected natural estrogens and androgens to be extracted along with polar chemicals, thus their potential presence and activity had to be assessed. Using a pooled human milk sample as a basis, we investigated the possible contribution of seven relevant steroids to the measured anti-androgenic activity (Barreiro et al., 2015; Burger, 2002). E1, E2, androstenedione, DHEA and testosterone were eluted together as may be expected due to their structural similarities and comparable physico-chemical properties. Elimination of the natural AR agonists, androstenedione, DHEA and testosterone from the extract might explain the observed increase in antagonistic activity, from 0.88 to 1.7 μg flutamide eq./g (Houtman et al., 2009). On the contrary, pregnenolone and progesterone's removal coincided with a drop of about 30% of the activity. Overall an antagonistic activity of 1.1 μg flutamide eq./g was still noticeable after exclusion of the seven endogenous steroid hormones. The remaining anti-AR activity in human milk samples after removal of the sex hormones indicates that other compounds than these natural sex hormones are causative for the observed anti-androgenic activity. It is important to note that, steroid metabolites might also exert an influence on the anti-AR activity, although this seems unlikely since steroidal metabolites do hardly show any remaining receptor-based bioactivities (Houtman et al., 2009). In addition, UHPLC-Q-TOF-MS/MS-based screening of a pooled breast milk sample indicated the absence of 248 potential metabolites derived from our initial set of hormones, which further excluded the hypothesis of involvement of natural hormones or their metabolites in the observed anti-androgenic activity in human milk. Thus, it is more likely that the observed anti-AR activity in human milk samples is caused by yet unidentified anthropogenic, exogenous chemicals which may be present as contaminants. Over the past decades, many studies reported breast milk contamination by various EDC classes. In 1998, Sonawane (1995) established an extensive list of these groups of contaminants describing pesticides (DDT, DDE, dieldrin, aldrin and endrin), polychlorinated biphenyls (PCBs) and dioxins as the most preeminent compounds. The physico-chemical properties of aldrin, dieldrin and endrin imply that these particular pesticides might be present in the polar fraction of breast milk. For *o,p'*-DDT and *p,p'*-DDE which turned out to be potent anti-androgens in the anti-AR CALUX, it is unlikely that they may have contributed to the anti-AR activity in human milk, due to their apolar nature, and therefore they would not likely elute in the polar fraction of our samples where the anti-AR activity was observed (Van der Burg et al., 2010). The same argument goes for dioxins, dioxin-like PCBs and PCBs which can be expected in the apolar extract rather than the polar fractions. While these pollutants are not in the fraction of current interest, their role in the activity measured in the human milk samples should not be excluded. As demonstrated in the previous section, the apolar fraction, while remaining quite low in anti-AR activity, might still play a role in diminishing the overall activity when measured as a reconstituted sample. Over the years, studies focusing on human milk have identified brominated flame retardants (polybrominated diphenyl ethers (PBDEs) and hexabrominated cyclododecane (HBCD)), dibenzo-p-dioxins (PCDDs), phthalates esters along with other organochlorine pesticides (aldrin, chlordane, α-endosulfan, methoxychlor, etcetera), constituting an non-exhaustive list of potential actors in the measured anti-AR activity (Damgaard et al., 2006; Main et al., 2006, 2007; Norén and Meironyté 2000).

5. Conclusion

The present manuscript describes a first attempt in understanding the observed anti-androgenic activity in breast milk samples. We found that the anti-AR activity could be distinguished from the major natural steroid hormones, and their metabolites, present in human milk and therefore suggests that the observed anti-AR activity might be associated with yet unidentified contaminants of anthropogenic origin. In future studies, further targeted and untargeted analysis are required to precisely identify their chemical nature. In addition, the possible role of this anti-AR activity in the observed increase in adverse health outcome e.g., frequency of cryptorchidism in offspring from the Norwegian HUMIS cohort will be studied in more detail as well.

CRediT authorship contribution statement

Bérénice Collet: Conceptualization, Methodology, Investigation, Writing - original draft. **Barbara M.A. van Vugt-Lussenburg:** Writing - original draft, Supervision. **Kees Swart:** Methodology, Validation, Investigation, Writing - original draft. **Rick Helmus:** Methodology, Software, Validation, Investigation, Writing - original draft. **Matthijs Naderman:** Investigation. **Eva de Rijke:** Methodology, Supervision. **Merete Eggesbø:** Conceptualization, Resources. **Abraham Brouwer:** Conceptualization, Writing - review & editing, Supervision. **Bart van der Burg:** Conceptualization, 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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