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Assessment of chemical mixtures using biomarkers of combined biological activity: A screening study in human placentas

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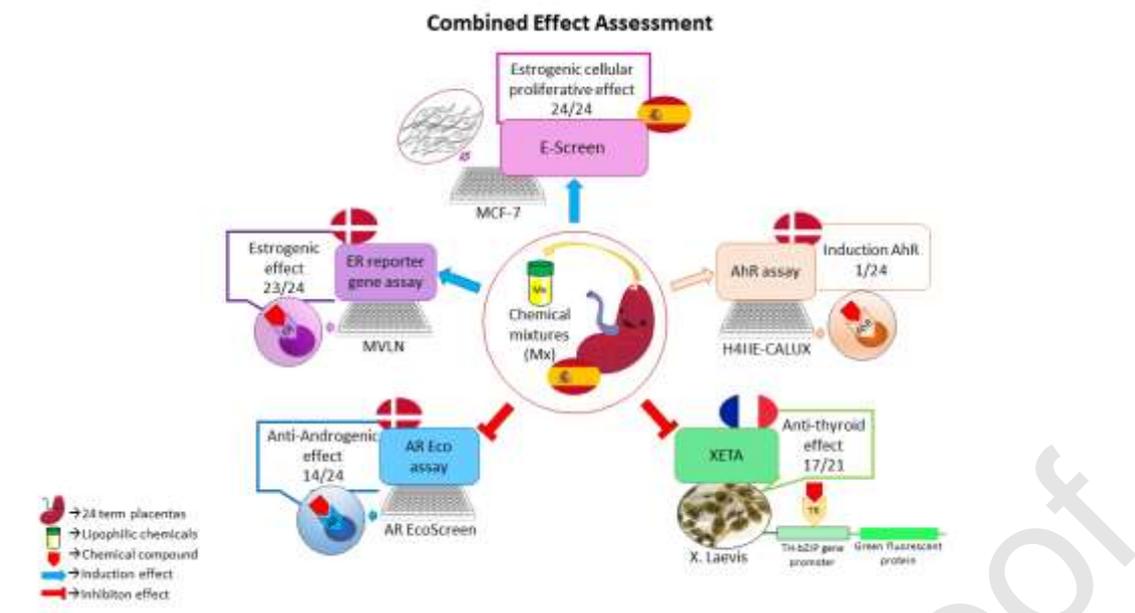
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Graphical Abstract



Highlights

- Mixtures of lipophilic chemicals were extracted from human placentas at term
- Placental extracts were tested in one *in vivo* and four *in vitro* bioassays
- The same chemical mixture affected different signaling pathways
- Most extracts increased ER-mediated cellular proliferation and ER activation
- Most extracts antagonized thyroid function and AR transactivity

Abstract

Humans are simultaneously exposed to complex mixtures of chemicals with limited knowledge on potential health effects, therefore improved tools for assessing these mixtures are needed. As part of the Human Biomonitoring for Europe (HBM4EU) Project, we aimed to examine the combined biological activity of chemical mixtures extracted from human placentas using one *in vivo* and four *in vitro* bioassays, also known as biomarkers of combined effect. Relevant endocrine activities (proliferative and/or reporter gene assays) and four endpoints were tested: the estrogen receptor (ER), androgen receptor (AR), and aryl hydrocarbon receptor (AhR) activities, as well as thyroid hormone (TH) signaling. Correlations among bioassays and their functional shapes were evaluated. Results showed that all placental extracts agonized or antagonized at least three of the abovementioned endpoints. Most placentas induced ER-mediated transactivation and ER-dependent cell proliferation, together with a strong inhibition

of TH signaling and the AR transactivity; while the induction of the AhR was found in only one placental extract. The effects in the two estrogenic bioassays were positively and significantly correlated and the AR-antagonism activity showed a positive borderline-significant correlation with both estrogenic bioassay activities. However, the *in vivo* anti-thyroid activities of placental extracts were not correlated with any of the tested *in vitro* assays. Findings highlight the importance of comprehensively mapping the biological effects of “real-world” chemical mixtures present in human samples, through a battery of *in vitro* and *in vivo* bioassays. This approach should be a complementary tool for epidemiological studies to further elucidate the combined biological fingerprint triggered by chemical mixtures.

Keywords: Endocrine disruption; Chemical mixtures; Biomarkers; Combined effect; Bioassay; Placenta; Reproduction; HBM4EU

1. Introduction

Humans are exposed to hundreds of environmental chemicals at low doses [1]. Many of these environmental pollutants have been detected in different human matrices such as urine, serum and placenta [2–5] and constitute an important part of the human exposome [6]. Appropriate risk assessment of chemical mixtures with human relevance relies on accurately characterizing the mixture composition and the available toxicological information on the constituents of the mixture. However, for many chemicals the information of both, exposure and toxicity is lacking [7]. Thus, predicting the effects of exposure to chemical mixtures in human populations is one of the main challenges of current toxicology and environmental epidemiology [8,9].

Epidemiologic investigations have traditionally followed a one-compound-at-a-time strategy to assess possible associations between environmental chemical exposure and adverse health effects. A disadvantage of this approach is that it ignores additive effects or synergistic and/or antagonistic interactions with other chemicals [10]. To address this challenge, complementary statistical multi-pollutant models to disentangle independent associations among several co-exposures have been applied [11,12]. However, these valuable statistical approaches also present inherent deficiencies and limitations, for example, that they only

evaluate a limited number of chemicals with pre-existing toxicological knowledge, probably underestimating the effects of exposure to real-life mixtures.

To examine the effects of “real-world” mixtures present in humans, including both known and unknown environmental chemicals, alternative approaches are needed. Chromatographic methods are of great utility since they allow the isolation of fractions containing compounds with similar physicochemical characteristics, separating them from endogenous hormones [13,14]. The isolated fractions can be tested in a battery of *in vitro* assays covering different molecular initiating events and/or molecular targets, enabling the characterization of various biological effects exerted by the chemical mixture. This approach has been successfully applied in the ecotoxicological field to monitor changes in water biological activity without the need to screen for hundreds of chemicals, and/or to identify emerging pollutants [15–17]. Likewise, human matrices can be used to evaluate the combined effect of chemical mixtures in epidemiologic studies, and identify emerging exposures. However, this research field is still immature and awaits further exploration [18].

As reported by authors of the EU-project SOLUTIONS, there is a need to balance the way to deal with mixture exposures and their combined effects, since single-chemical exposure approaches tend to ignore data gaps (i.e., missing contaminants), while effect-based approaches may lead to increased uncertainty factors such as the need to further develop suspect and non-targeted screening techniques [19]. Thus, combining both approaches may provide the best results. Previous epidemiological studies have shown that it is possible to obtain chemical fractions from human matrices in which, for example, the most persistent and lipophilic contaminants, without the presence of endogenous hormones, are isolated by various analytical techniques [20–24]. In this context, bioassays testing the combined biological effect of chemical fractions isolated from human samples are usually referred to as “biomarkers of combined activity”, “biomarkers of combined effect”, “biomarkers of *ex vivo* hormonal activity”, and/or “biomarkers of combined internal exposure” [20,25,26]. The advantage of this approach is that the biological effect is measured taking into account all bioactive chemicals as well as plausible

interactions among them that may lead to synergistic or antagonistic mixture effects. The total joint effect of human extracts is hypothesized to provide a more holistic strategy to address the true cause of the disease provided that the adverse outcome is related to a specific receptor activity or pathway.

In this regard, among the studies performed on human samples, some effect endpoints have included estrogenic and antiandrogenic activities. The so-called “total effective xenoestrogen burden” (TEXB) has been implemented in several epidemiological studies for endocrine-related diseases such as male urogenital malformations and breast cancer [27,28], by assessing the combined *in vitro* estrogenic activity of chemical mixtures extracted from different human matrices including serum, placenta and adipose tissue [13,23,29]. A higher total effective xenobiotic burden of anti-androgens (TEXB-AA), which evaluates the combined anti-androgenic activity exerted by mixtures of pollutants present in human placental extracts, was also associated with an increased risk of urogenital malformations in boys [20]. Other signaling pathways, including the aryl hydrocarbon receptor (AhR) and thyroid function, have also been explored, but to a lesser extent compared with the abovementioned endpoints [18].

There are some gaps in knowledge associated with the use of *in vitro* and *in vivo* biomarkers of combined activity that need to be addressed, such as the standardization across laboratories regarding the procedures performed for the correct execution of bioassays and comparisons among a panel of different bioassays using the same biological matrix. Therefore, the main objective of this study was to assess, characterize and compare the combined biological effect of chemical mixtures extracted from the same human placentas by quantifying the signal elicited in five bioassays, covering relevant endocrine activities (*in vitro* proliferative and reporter gene assays for estrogen, androgen and aryl-hydrocarbon, and *in vivo* thyroid function).

2. Materials and Methods

2.1 Study population

Twenty-five placenta samples were randomly selected among those kept at the biobank of the San Cecilio University Hospital (Granada, Spain) from healthy women participating in the INMA -INfancia y MedioAmbiente (Environment and Childhood)-, Granada birth cohort study, recruited from October 2000 to July 2002 [30]. Participants for which a limited quantity of placenta was available were excluded before the random selection. Characteristics of the study population were obtained from medical records and validated questionnaires [27]. Placentas were collected at time of delivery, weighed without fetal membranes/maternal decidua, and frozen at -80°C. The INMA study followed the principles of the declaration of Helsinki and was approved by the Ethics Committee of San Cecilio University Hospital. All participants signed the informed consent allowing the use of biological samples for environmental research purposes.

2.2 Study design

This work aimed to address the combined effects of “real-world” chemical mixtures of persistent and lipophilic chemicals present in human placenta samples using several bioassays, taking into account all bioactive chemicals as well as plausible interactions among them. Placental homogenates were extracted using a validated semi-preparative chromatographic separation protocol [24, 29] at the facilities of the University of Granada (UGR), Spain. Afterwards, dried high-performance liquid chromatography (HPLC) fractions were sent on dry ice to the participating institutions in order to conduct specific bioassays. Five bioassays were performed: the E-Screen, conducted at the University of Granada (UGR) Spain; the estrogen receptor (ER) reporter gene assay, performed at the Aarhus University (AU), Denmark; the androgen receptor (AR) and aryl hydrocarbon receptor (AhR) reporter gene assays, tested at the Technical University of Denmark (DTU); and the *Xenopus* eleutheroembryonic thyroid assay (XETA), tested at the National Center of Scientific Research (CNRS) in France (Table 1).

Table 1. Bioassays and institutions in which they were performed.

Laboratory	Biological material	Bioassays (Biomarkers of combined effect)
UGR (Spain)	24 α -Fractions	E-Screen. Estrogenic proliferative effect.
AU (Denmark)	24 α -Fractions	ER reporter gene assay. ER induction effect.
DTU (Denmark)	24 α -Fractions	AR reporter gene assay. Anti-androgenic effect. AhR reporter gene assay. Aryl Hydrocarbon receptor induction.
CNRS (France)	24 α -Fractions	Xenopus leutheroembryonic thyroid assay. Thyroid hormone disruption.

AU: Aarhus University; CNRS: National Center of Scientific Research; DTU: Technical University of Denmark; UGR: University of Granada.

2.3 Placenta extraction protocol

To ensure the representativity of the whole placenta tissue, half of the placenta was cut, defrosted, placed in the glass container of a mixer (Büchi Mixer B-400 Büchi Laboratories AG, Flawil, Switzerland) and homogenized (Figure 1.A). Placental homogenates were extracted following a previously validated semipreparative HPLC protocol [27,29] to efficiently separate organo-halogenated lipophilic chemicals from endogenous hormones and more polar compounds, using a normal-phase column and a gradient with two mobile phases. Of the initial 25 placentas, one was excluded due to technical issues during extraction. The placental homogenization and the semi-preparative HPLC extraction protocol are graphically summarized in Figure 1.

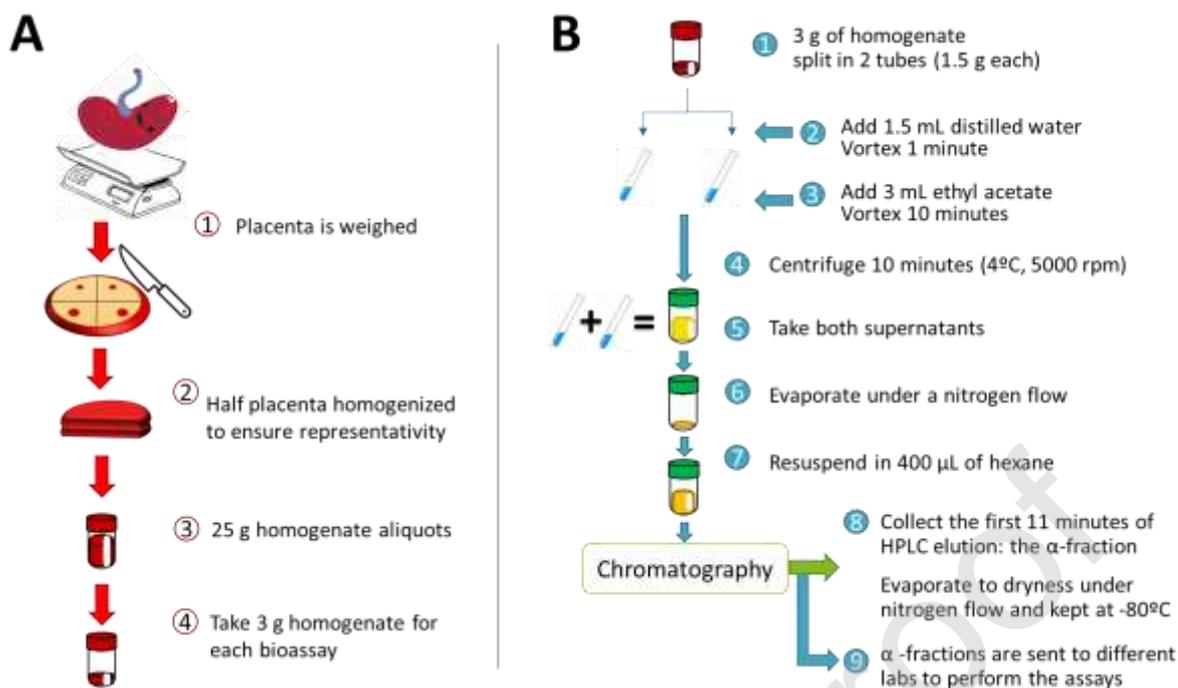


Figure 1. A) Placental homogenization. After weighing the placenta (1), it was cut in half by using a template (2). One half was homogenized and the rest was kept at -80°C . The homogenate was split into 25 g aliquots (3). From a 25 g homogenate, a 3 g aliquot was taken for the chemical extraction (4). B) Chemical extraction and chromatographic separation protocol. The α -fraction corresponds to the first 11 minutes of semi-preparative HPLC chromatographic elution, which has been previously shown to contain the most persistent and lipophilic compounds, while avoiding endogenous hormones.

Briefly, 3 g of placenta homogenate was split in two 5ml Falcon tubes with 1.5 g each. Then, 1.5 mL of distilled water was added into the tubes, which were vortexed for 1 min (Figure 1.B). Successively, the mixture was extracted by adding 3 mL of ethyl acetate and vortex-shaked again for 10 min. Afterwards, the mixture was centrifuged for 10 min at $4050 \times g$. The two supernatants were pooled in a clean glass vial, and the extract was evaporated to dryness at room temperature under a nitrogen stream. The dried extract was dissolved with hexane (700 μL) and dried once more under nitrogen stream. Then it was dissolved in 400 μL of hexane and injected twice (200 μL) into a column to undergo preparative HPLC. The placental extract was eluted by a specific gradient of two mobile phases: n-hexane (phase A) and n-hexane:methanol:2-isopropanol (40:45:15)(v/v) (phase B) at a flow rate of 1.0 mL/min. Specifically, the α -fraction represents the first 11 min of elution of the chromatographic run.

The whole process was repeated 5 times and the obtained α -fractions were sent on dry ice to each participant laboratory (Figure 1.B). The available knowledge to date has shown the presence of common persistent and lipophilic pollutants in the α -fraction, and the absence of steroid endogenous hormones [27,32,33]. Examples of chemical compounds present in different minutes of elution of the α -fraction can be consulted in Table S1 of the Supplementary Material.

2.4 Bioassays

Each laboratory reconstituted the dried α -fractions and made specific dilutions to be tested following the requirements of each specific bioassay (e.g. sensitivity, range and cytotoxicity threshold). To facilitate the comparison among bioassays, the concentrations of placental α -fractions tested were expressed as mg of placenta extracted per well volume in mL ($\text{mg}_{\text{placenta}}/\text{mL}$). The corresponding calculations for each bioassay are explained in Table S2 of the Supplementary Material.

E-Screen

MCF-7 cells were used to assess the proliferative effect induced by placental α -fractions as described previously [27]. Cells were seeded at a density of 4×10^3 cells/well in 96-well plates (obtained by Falcon®, VWR International Eurolab, Barcelona, Spain) in culturing medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) with phenol red supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) and left at 37°C with 5% CO₂. After 24 h, culturing medium was replaced with experimental medium, consisting of phenol red-free DMEM supplemented with 10% dextran-coated charcoal-stripped FBS (DCCS-FBS) (Gibco, Invitrogen, Spain). Dried α -fractions were reconstituted in 1 mL of experimental medium, vigorously shaken, left to rest for 30 min, filtered through a 0.22 μm filter, and diluted

1, 5 and 10 times, and tested in triplicates. Each well plate included 150 μL of experimental medium together with 50 μL of placental α -fractions. Dilutions x1, x5 and x10 corresponded to 750 $\text{mg}_{\text{placenta}}/\text{mL}$, 150 $\text{mg}_{\text{placenta}}/\text{mL}$ and 75 $\text{mg}_{\text{placenta}}/\text{mL}$, respectively (Table S2). After 6 days of exposure, cells were fixed, stained with sulforhodamine B (Sigma-Aldrich, MO, USA) and the solubilized bound dye was read at 492 nm in a Titertek Multiscan plate reader (Flow, Irvine, CA, USA). 17 β -estradiol was tested as a positive control at concentrations of 0.1 pM-1000 pM. Experimental medium was used as negative control.

Estrogen receptor (ER) reporter gene assay

The activation of ERs by placental α -fractions was evaluated with the stably transfected human breast adenocarcinoma MVLN cells carrying the estrogen response element luciferase reporter vector (provided by M. Pons, France). The procedure followed was previously described by Bjerregaard-Olesen and colleagues [34]. Briefly, cells were seeded at a density of 8.5×10^4 cells/well in 96-well plates with culture medium that included phenol red-free DMEM (LONZA, Belgium) supplemented with 1% DCCS-FBS (HyClone, Belgium), 6 $\mu\text{g}/\text{L}$ insulin (Sigma, USA), 64 mg/L hexamycin (Sandoz, Denmark), 4 mM glutamine (Sigma, USA), and 20 mM HEPES (Gibco, UK), and were left to incubate at 37°C with 5% CO_2 overnight. The experimental medium consisted on phenol red-free DMEM (LONZA, Belgium) containing 0.5 % DCCS-FBS. Dried α -fractions were reconstituted in 44 μL EtOH:H₂O:DMSO (50:40:10, V/V/V) and from this reconstituted 44 μL , we used 20 μL that were subsequently diluted in experimental medium 55, 275, and 550 times. Successively, 100 μL of these diluted extracts were added into the well and analyzed in triplicate. The dilutions 55, 275 and 550 correspond to 1240 $\text{mg}_{\text{placenta}}/\text{mL}$, 248 $\text{mg}_{\text{placenta}}/\text{mL}$ and 124 $\text{mg}_{\text{placenta}}/\text{mL}$, respectively (Table S2). After 24 h of exposure, the luciferase activity was measured with automatic injection of luciferase substrate (D-luciferin, free acid, Molecular Probes, L2911, Invitrogen) using a LUMIstar luminometer (BMG Labtech, RAMCON). Cell protein levels were quantified to correct for differences in cell numbers in the well. Protein was quantified by fluorometric measurements using a WALLAC Victor2 (Perkin Elmer). Cell viability was assessed visually at the

microscope, and for cells with visual cytotoxicity, low protein levels were also observed. As a quality control, a dose-dependent 17 β -estradiol curve at 1.5 pM-300 pM was tested in parallel.

Androgen receptor (AR) reporter gene assay

The AR reporter gene assay was performed as previously described [35] with few modifications, to evaluate the anti-androgenic activity of placental α -fractions. The AR-EcoScreen cell line (JCRB Cell Bank, cat. No. JCRB1328), cultured in DMEM-F12 without phenol red (Life Technologies, CA, USA) and supplemented with 5% DCCS-FBS (Life Technologies, CA, USA), 50 units/mL penicillin, 50 μ g/mL streptomycin (Life Technologies, CA, USA), 200 μ g/mL zeocin (Invivogen, CA, USA), and 100 μ g/mL hygromycin (Invitrogen, CA, USA), was seeded at a density of 0.9×10^4 cells/well in 96-well plates (Costar, Corning, USA). The experimental medium consisted of DMEM-F12 without phenol red supplemented with penicillin/streptomycin (but without zeocin or hygromycin), and 5% DCCS-FBS (Sigma Aldrich, MO, USA). Before exposure was initiated, plates were left in the incubator for 24 h at 37°C with 5% CO₂. Dried α -fractions were reconstituted in 400 μ L of hexane, which was split in four glass vials and left to evaporate until dryness. Evaporated α -fractions were reconstituted with 250 μ L of experimental medium, left to rest for 30 min, filtered with a 0.22 μ m filter and diluted 60-, 180-, and 600 times. Successively, 50 μ L of diluted fractions together with 150 μ L of experimental medium were added into wells. Dilutions 60, 180 and 600 corresponded to 12.5 mg_{placenta}/mL, 4.2 mg_{placenta}/mL and 1.25 mg_{placenta}/mL (Table S2). All treatments, including controls were co-treated with 0.1 nM metribolone (R1881) (PerkinElmer, MA, USA), a known inductor of the AR activity. After ~20 h of exposure, 100 μ L Dual-Glo® Firefly Luciferase Reagent, prepared according to manufacturer's protocol (Dual-Glo® Luciferase Assay System, Promega, USA) was added to wells, and plates were left on a shaker table for 10 min. Then, the luminescence was measured using a BioOrbit, Galaxy luminometer to assess AR activity. Successively, 50 μ L of Dual-Glo® Stop & Glo® reagent was added to wells, plates were left on shaker table for 15 min, and luminescence was measured (BioOrbit, Galaxy) to assess cell

viability, measured by the stably transfected construct of Renilla luciferase. This assay provides a direct measure of cytotoxicity in the cells. The assay was performed in three independent experiments with technical triplicates for each treatment within the experiment.

Hydroxyflutamide (OHF), a known antagonist of AR activity, was used as quality control and tested in concentrations ranging from 1×10^3 - 5000×10^3 pM in all experiments.

Aryl hydrocarbon receptor (AhR-CALUX) reporter gene assay

The induction of AhR is known for leading to the transcription of metabolizing enzymes [36]. To evaluate the activation of the AhR receptor, a stably transfected rat hepatoma (H4IIE-CALUX) cell line was used [37]. Briefly, 2.2×10^4 cells/well were seeded in 96-well plates and incubated for ~22 h at 37°C with 5% CO₂ in culture medium, which consisted of minimum essential medium α (MEM α) supplemented with 5% FBS, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL Gibco Amphotericin B (Life Technologies). The FBS content was reduced to 1% during experiments (experimental medium). Dried α -fractions were reconstituted in 400 μ L of hexane, split into four glass vials, and left to evaporate until dryness. Evaporated α -fractions were reconstituted in 250 μ L of experimental medium, left to rest for 30 min, filtered through a 0.22 μ m filter, and diluted 100-, 300-, and 1000 times. Successively, 50 μ L of diluted fractions together with 150 μ L of experimental medium were added into wells and tested in two independent experiments. Dilutions corresponded to 7.5 mg_{placenta}/mL, 2.5 mg_{placenta}/mL and 0.75 mg_{placenta}/mL, respectively (Table S2). After ~22 h of exposure, cells were lysed and the luminescence was measured by the addition of 40 μ L luciferin solution containing 0.5 mM luciferin and 0.5 mM ATP in lysis buffer. Cell viability was tested following the protocol above, but seeding only 1.1×10^4 cells/well. At assay termination, medium was removed and 50 μ L of fresh medium was added. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added leading to a final concentration of 0.45 mg/mL and incubated for 1.5 h. Afterwards, medium was removed and 50 μ L/well isopropanol was added. Plates were left on a shaker table for 5 min after which absorbance was measured. 2,3,7,8-

tetrachlorodibenzo-p-dioxin (TCDD) was used as positive control and tested in concentrations ranging from 0.5 pM-3000 pM.

Xenopus eleutheroembryonic thyroid assay (XETA)

The XETA is a miniaturized and relatively high throughput *in vivo* assay to detect disruption of thyroid hormone (TH) signaling through variations in the fluorescence emitted by transgenic tadpoles [38]. To assess whether placental α -fractions inhibit or activate TH signaling during embryonic stages, Nieuwkood and Faber (NF) stage 45 *Xenopus laevis* tadpoles (1 week old) from the *Tg(thbz:eGFP)* line were used, as previously described [39]. The construct contains the promoter of the TH- sensitive TH/bZIP promoter coupled to a Green Fluorescent Protein (GFP) reporter gene to capture disruption of TH signaling that may involve multiple points of regulation of the thyroid axis. Briefly, dried α -fractions were reconstituted in 16 μ L of DMSO and 400 μ L of Evian water. Reconstituted α -fractions were then split into two parts of 208 μ L each which were added to wells and tested in duplicates within four independent experiments. Each well was completed with experimental medium up to 8 mL, reaching a concentration equivalent to 187.5 mg_{placenta}/mL. Fifteen tadpoles per well were placed in 6-well-plates (TPP Switzerland) containing 8 mL of either control solvent (DMSO) in Evian water, thyroid hormone triiodothyronine T3 (5 nM), or α -fractions spiked with T3 (5 nM). DMSO (0.01%) was present in all treatments and plates were placed at 23 °C. After 72 h of exposure, tadpoles were anesthetized and color images of each tadpole manually positioned in a well of a 96-well plate were acquired using an Olympus AX-70 binocular equipped with long pass GFP filters and a Q-Imaging Exi Aqa camera (25x objective, and 3 sec exposure). QC Capture pro (QImaging) software was used for image acquisitions and quantifications were carried out using ImageJ. Quantifications were carried out in a region of interest (ROI) containing the whole head area while excluding the non-specific auto-fluorescence emitted from the gut area (Figure S1, Supplementary Material).

2.5 Data handling and statistical analysis

All bioassay data were normalized to the mean of solvent plate control(s). If more than one independent experiment was conducted, the means from independent experiments were pooled. Normal distribution analysis was conducted (D'Agostino & Pearsons omnibus test). XETA results were not normally distributed, and the difference between experimental samples and controls was performed using the Kruskal-Wallis test (Dunn's post hoc test). For *in vitro* data, and based on the number of replicates for each sample, the distribution could not be evaluated. Therefore, classification of samples as positive in the bioassays was based on cut-off values calculated from the limit of detection (LOD). For the E-Screen, LOD value has previously been standardized to >2.0 [40]; for the remaining agonist assays, LOD was defined as 1 plus 3 times the standard deviation (SD) of the vehicle control, and for the antagonist assays, the LOD was calculated as 1 minus 3 times the SD of the vehicle control. Positive controls were fitted to a four-parameter non-linear regression curve with the vehicle controls constrained to 1 and in the case of inhibitors, the lower limit constrained to >0 . The maximum efficacy (E_{\max}) obtained for the positive controls or α -fractions was defined as the observed maximum change compared to the vehicle control, and calculated as the percentage difference between the highest fold change reached minus that of the vehicle control (set to 1). All data processing was performed in GraphPad Prism 8.1.

To assess correlation between bioassay responses, all samples were normalized to vehicle controls constrained to 1, and then fold-change values were expressed as percentages, in order to represent values for both agonist and antagonist effects. Firstly, Pearson correlation coefficients were calculated between the effect magnitude (percentage of biological activity) of the different bioassays. Secondly, and to assess the shape of correlations within relevant findings, we performed linear regression models categorizing the independent variable into tertiles. Thus, placentas eliciting the lowest signals in the bioassay of interest were categorized in the first tertile (T1), while placentas with the highest signal were located in the third tertile (T3). The coefficient of determination (r^2) was calculated for each regression to estimate the proportion of the variance in the dependent variable that is predicted by the independent

variable. All correlations between biomarkers were conducted using the SPSS v24.0 (IBM, Chicago, IL), significance level was set at $p \leq 0.05$ and borderline significance was set at $p \leq 0.1$.

3. Results

3.1 Study population

In this study, 44% of the newborns were boys and 56% were girls; mean (SD) gestational age was 39 (1.2) weeks. Mean (SD) head circumference was 34.4 (1.7) cm, birth weight and birth length were 3.35 (0.4) kg and 50.94 (2.1) cm, respectively. No infant was born preterm (<37 week) or with low birth weight (<2.5 kg). Mean (SD) age, pre-pregnancy weight and height of mothers was 30 (4.9) years, 68.1 (15.9) kg and 1.65 (0.1) m, respectively; and with a mean (SD) pre-pregnancy BMI of 24.9 (4.5) kg/m². Half of mothers (48%) received higher education (university studies/professional formation), 84% did not consume tobacco and 52% were primiparous.

3.2 Positive controls and cut-off values

Results obtained for positive controls known to induce or inhibit the tested endpoints are shown in Figure 2. The half maximal effect concentration (EC₅₀) and the half maximal inhibitory concentration (IC₅₀)-values were determined based on a 4-parameter curve fit. Briefly, EC₅₀ values were 0.02 nM (17 β -estradiol), 0.03 nM (17 β -estradiol), 0.05 nM (metribolone) and 0.04 nM (2,3,7,8-tetrachlorodibenzo-p-dioxin) for the E-Screen, and the ER, AR, and AhR reporter gene assays, respectively. The IC₅₀ value was 0.79 nM (hydroxyflutamide) in the AR reporter gene assay. Maximum efficacies for positive controls tested in each *in vitro* assay were as follows, E-Screen: 580%, ER: 213%, AR: 694% metribolone and 66% hydroxyflutamide and AhR: 904% (Figure 2). Cut-off values to classify a placenta extract as positive were based on LODs (illustrated with dotted line in Figure 3). For E-screen, ER and AhR agonism, cut-off values were set to >2.0 [40], >1.5 and >1.8-fold change, respectively. For AR antagonism, the cut-off value was set to <0.8-fold change.

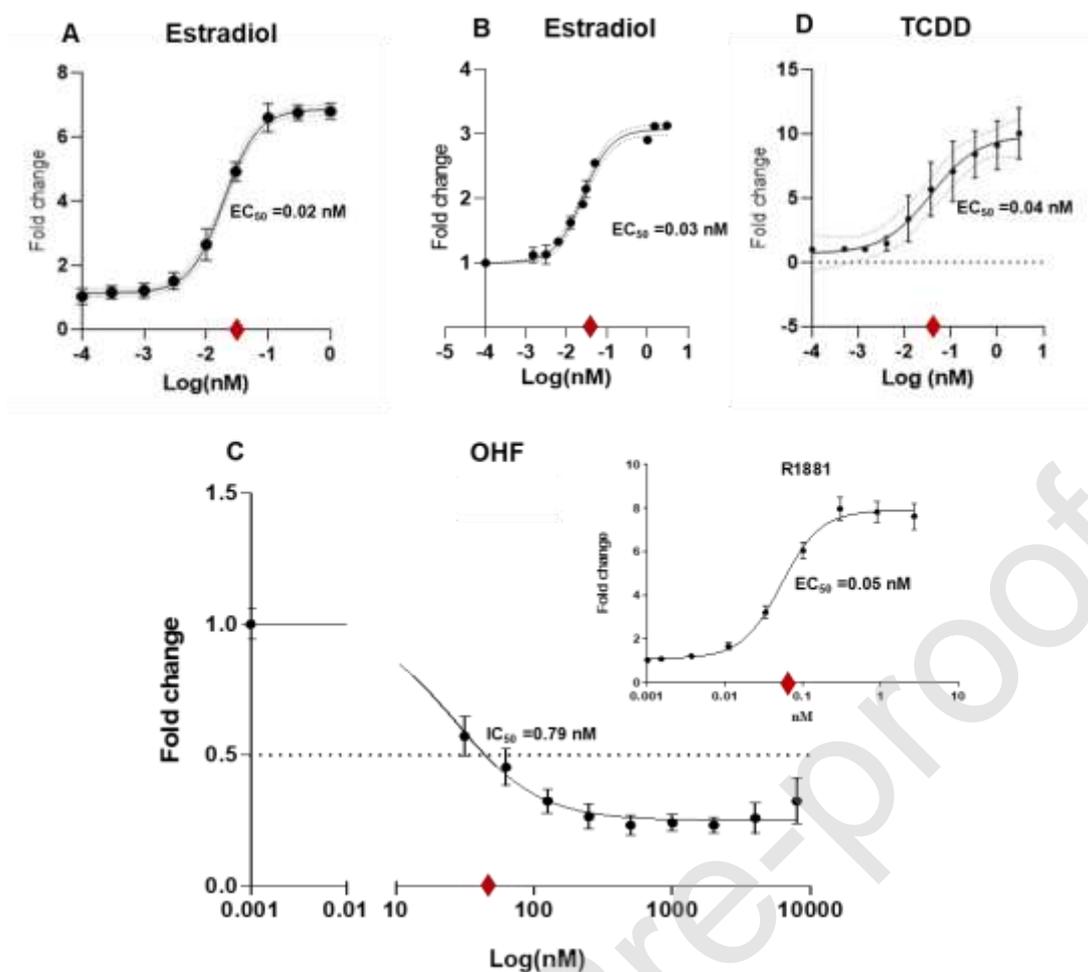


Figure 2. Concentration-response curves and standard deviations (SD) for positive controls tested in *in vitro* bioassays. (A) 17β -estradiol (E_2) for the E-Screen assay. (B) E_2 for the ER transactivation assay. (C) Hydroxyflutamide (OHF) and metribolone (R1881) for the AR reporter gene assay. (D) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for the AhR reporter gene assay. Data points represent means from 1 (Fig. A and, B), 2 (Fig. D) or 3 (Fig. C) independent experiments. Diamonds point out the EC_{50}/IC_{50} values.

3.3 Combined biological activities exerted by placental α -fractions

Estrogenic activity

All twenty-four placental α -fractions elicited a concentration dependent proliferative effect (PE) at varying degrees in the E-Screen assay (Figure 3.A), and all α -fractions were above the cut-off value (dotted line, Figure 3.A) at the highest concentration tested. Samples led to maximal effects at $750 \text{ mg}_{\text{placenta}}/\text{mL}$, with 14 out of 24 samples reaching a proliferation efficacy above 450%.

In the ER transactivation assay, all α -fractions at both concentrations of 124 and 248 $\text{mg}_{\text{placenta}}/\text{mL}$, were above the cut-off value, with the exception of placentas # 2, 21 and 25 (dotted line Figure 3.B). Notwithstanding, at least one of the concentrations tested for placentas # 2 and 25 were above the cut-off value and most placentas (23 out of 24) were thus deemed positive for ER activity (Table 2). Most placental α -fractions tested at the highest concentration (1240 $\text{mg}_{\text{placenta}}/\text{mL}$) compromised cell viability, except α -fraction # 2. Fractions tested at 124 and 248 $\text{mg}_{\text{placenta}}/\text{mL}$ induced increased ER transactivity compared to the negative control, which appeared concentration dependent for placental α -fractions # 5, 7 and 14. More than half of the α -fractions led to an efficacy above 200%, with the exception of fractions # 2, 7, 12, 15, 16, 20, 22, 23, 24, and 25.

Anti-androgenic activity

Most α -fractions showed an inhibitory effect on the AR activity at the highest concentration tested (12.5 $\text{mg}_{\text{placenta}}/\text{mL}$). However, six α -fractions compromised cell viability at this concentration (# 1, 7, 8, 9, 12 and 16), and three (# 2, 21 and 25) showed error bars slightly above the cut-off value. Consequently, 14 placentas were considered as positive for AR antagonism activity (Figure 3.C) uniquely based on the cut-off criteria. Among the positives, about half of the placental fractions led to E_{max} values above 70% of AR activity inhibition, with α -fractions # 10, 11, 14, and 15 being among the most efficacious. If the criteria were based on the presence of a concentration-dependent response together with the presence of an average response below the cut-off value, 22 out of the 24 placental fractions would be classified as antiandrogenic. No compromised cell viability was observed at the lowest (1.25 $\text{mg}_{\text{placenta}}/\text{mL}$) and medium (4.2 $\text{mg}_{\text{placenta}}/\text{mL}$) concentrations tested.

Aryl Hydrocarbon Receptor (AhR) Activity

Although increases in AhR-transactivity were observed for placental α -fractions # 1, 8, 14, and 20, only placenta 14 was above the cut-off value (Figure 3.D). Cell viability was compromised in α -fractions # 5, 8 and 11 at the highest concentration tested (7.5 $\text{mg}_{\text{placenta}}/\text{mL}$).

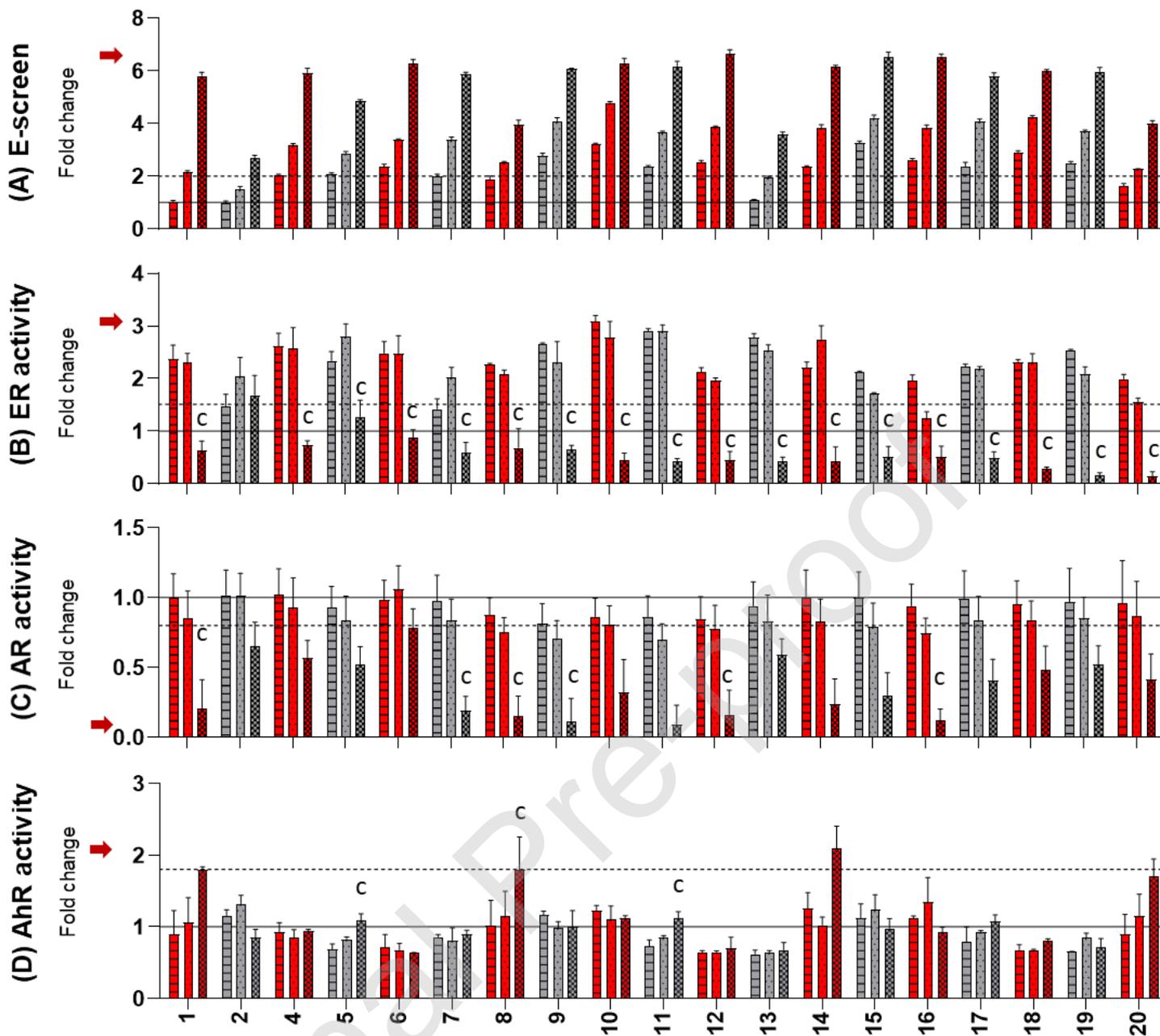


Figure 3. In vitro activity exerted by placental α -fractions determined by the estrogenic-proliferative effect (E-Screen), estrogen receptor (ER) transactivity, androgen receptor (AR) antagonism, and aryl hydrocarbon receptor (AhR) activity gene assays. (A) E-Screen data for placental α -fractions tested at 75 mg_{placenta}/mL, 150 mg_{placenta}/mL and 750 mg_{placenta}/mL in duplicates in a single independent experiment. (B) ER transactivation assay data for placental α -fractions tested at 124 mg_{placenta}/mL, 248 mg_{placenta}/mL, and 1240 mg_{placenta}/mL in duplicates in a single independent experiment. (C) AR antagonism assay for placental α -fractions tested at 1.25 mg_{placenta}/mL, 4.2 mg_{placenta}/mL, and 12.5 mg_{placenta}/mL in triplicates in three independent experiments. (D) AhR reporter gene assay data for placental α -fractions tested at 0.75 mg_{placenta}/mL, 2.5 mg_{placenta}/mL, and 7.5 mg_{placenta}/mL in triplicates in two independent experiments. All graphs are presented as data normalized to vehicle controls (mean \pm SD), set to 1 (continuous line). Graph C and D data were normalized in independent experiments and then mean values were pooled. C=compromised cell viability/cell toxicity. Arrows highlight the maximum efficacy exerted by α -fractions.

Thyroid Activity

Placental α -fractions # 5, 24 and 25 were not tested in this *in vivo* assay, due to lack of sample availability. The positive control group was exposed to triiodothyronine (T_3) hormone at 5 nM. All data was normalized to vehicle control, which was normalized to 1, shown in continuous line (Figure 4).

The inhibition of the thyroid activity was consistent across all twenty-one α -fractions tested after 72 hours of exposure (Figure 4). All α -fractions, except # 8, 9, 15 and 23 induced a statistically significant decrease ($p < 0.05$) of the thyroid activity when compared with the positive control group. The highest antagonistic thyroid activity was shown for α -fractions # 1, 2, 12, 13, 14 and 20.

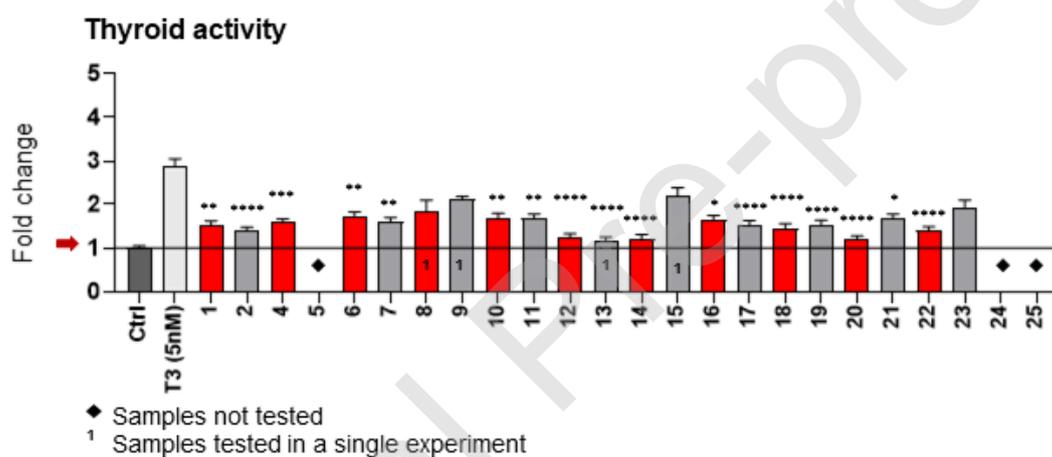


Figure 4. Antagonistic thyroid activity exerted by 21 placental α -fractions tested in the Xenopus Embryo Thyroid-signaling Assay (XETA), after 72 hours of exposure. Samples were tested at a concentration equivalent to 187.5 mg_{placenta}/mL in two independent experiments. Data was normalized to the negative control group (mean \pm SE), set to 1 (continuous line). SE= standard error. Each α -fraction was tested in two experiments except for α -fractions 8, 9, 13 and 15. Samples 5, 24 and 25 were not tested due to lack of sample availability. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The arrow highlights the maximum efficacy exerted by α -fractions.

3.4 General overview of bioassay activities

A summary of the results obtained from the 5 bioassays is shown in Table 2.

Table 2. Effect of 24 placental α -fractions tested in the E-screen assay, the estrogen receptor (ER), androgen receptor (AR) and aryl hydrocarbon receptor (AhR) reporter gene assays, and

the thyroid function assay (XETA). E-screen, ER and AhR assays were performed in agonist mode, whereas the AR reporter gene assays and XETA were performed in the antagonist mode.

α -fractions	E-Screen	ER	AR	AhR	XETA
1	POS	POS	NEG	NEG	POS
2	POS	POS	NEG	NEG	POS
4	POS	POS	POS	NEG	POS
5	POS	POS	POS	NEG	--
6	POS	POS	NEG	NEG	POS
7	POS	POS	NEG	NEG	POS
8	POS	POS	NEG	NEG	NEG
9	POS	POS	NEG	NEG	NEG
10	POS	POS	POS	NEG	POS
11	POS	POS	POS	NEG	POS
12	POS	POS	NEG	NEG	POS
13	POS	POS	POS	NEG	POS
14	POS	POS	POS	POS	POS
15	POS	POS	POS	NEG	NEG
16	POS	POS	NEG	NEG	POS
17	POS	POS	POS	NEG	POS
18	POS	POS	POS	NEG	POS
19	POS	POS	POS	NEG	POS
20	POS	POS	POS	NEG	POS
21	POS	NEG	NEG	NEG	POS
22	POS	POS	POS	NEG	POS
23	POS	POS	POS	NEG	NEG
24	POS	POS	POS	NEG	--
25	POS	POS	NEG	NEG	--

(Red, POS): the placental α -fraction affects activity above the cut-off value and is deemed positive, in the case of XETA, it was statistically significant when compared to control. (Blue, NEG): the placental α -fraction affects activity below the cut-off value and is deemed negative, in the case of XETA, it was not statistically significant when compared to control. (White, --): the placental α -fraction was not tested.

3.5 Correlations among bioassays

Pearson correlation coefficients among bioassay activities are presented in Table 3.

Additionally, the bioassay data taken as independent variable was categorized into tertiles to assess the shape of the correlation among biological activities (Figure 5). Overall, α -fraction concentrations showing no cytotoxicity and with the highest magnitude responses were selected to perform the correlation analyses. Thus, chosen concentrations were: 750 mg_{placenta}/mL for the E-Screen, 124 mg_{placenta}/mL for the ER reporter gene assay, 4.2 mg_{placenta}/mL for the AR reporter gene assay and 2.5 mg_{placenta}/mL for the AhR reporter gene assay. For XETA, only one concentration was tested and, therefore used for the analysis. Notwithstanding, a correlation

matrix among all bioassay dilutions that showed no cytotoxicity can be consulted in Table S3 of the Supplementary Material.

The E-Screen assay activity showed a positive and significant correlation with the ER reporter gene assay activity, and a positive borderline-significant correlation with the AR reporter gene assay activity (Table 3). When the E-Screen was categorized into tertiles from lower to higher ER-mediated proliferative effect, a linear positive dose-response correlation was observed between responses in both estrogenic bioassays (Figures 5.A and 5.C).

The ER reporter gene assay activity showed a positive and significant correlation with the E-screen assay activity and a positive borderline-significant correlation with the AR reporter gene assay activity (Table 3). After categorizing the ER reporter gene assay into tertiles from the lowest to highest ER transactivity, only the correlation with the E-Screen assay showed a linear dose-response shape (Figures 5.C and 5.D).

The AR reporter gene assay activity showed a positive borderline-significant correlation with both, the E-Screen and the ER reporter assay activities (Table 3). When the AR reporter gene assay was categorized into tertiles from lower to higher AR antagonistic effect, results showed linear dose-response correlations for both the E-screen and the ER reporter gene assay activities (Figures 5.E, and 5.F). In other words, a higher xenoestrogenicity tended to be correlated with a higher anti-androgenicity.

The AhR reporter gene assay activity did not show any significant correlation with other bioassays (Table 3). After categorizing the AhR reporter gene assay into tertiles from lower to highest AhR activity, a linear dose-response correlation between AhR and ER activity was observed (Figure 5.G).

The XETA results were not correlated with any of the remaining bioassays (Table 3). Figure 5.H shows a non-linear correlation between the ER reporter gene assay and XETA values categorized into tertiles (Figure 5.H).

In general, coefficients of determination (r^2) in the regression models were modest (Figure 5).

E-Screen (%)	-	0.492*	0.347†	-0.103	-0.058
ER (%)	0.492*	-	0.333†	-0.300	-0.230
AR antagonism (%)	0.347†	0.333†	-	0.086	-0.081
AhR (%)	-0.103	-0.300	0.086	-	0.009
XETA antagonism (%)	-0.058	-0.230	-0.081	0.009	-

Table 3. Pearson correlation coefficients assessing the relationships among bioassays when treated as continuous variables.

Concentrations selected for the main analyses were: 750 mg_{placenta}/mL for the E-Screen; 124 mg_{placenta}/mL for the ER reporter gene assay; 4.2 mg_{placenta}/mL for the AR reporter gene assay; 2.5 mg_{placenta}/mL for the AhR reporter gene assay; and 187,5 mg_{placenta}/mL for XETA.

* $p \leq 0.05$ † ≤ 0.10

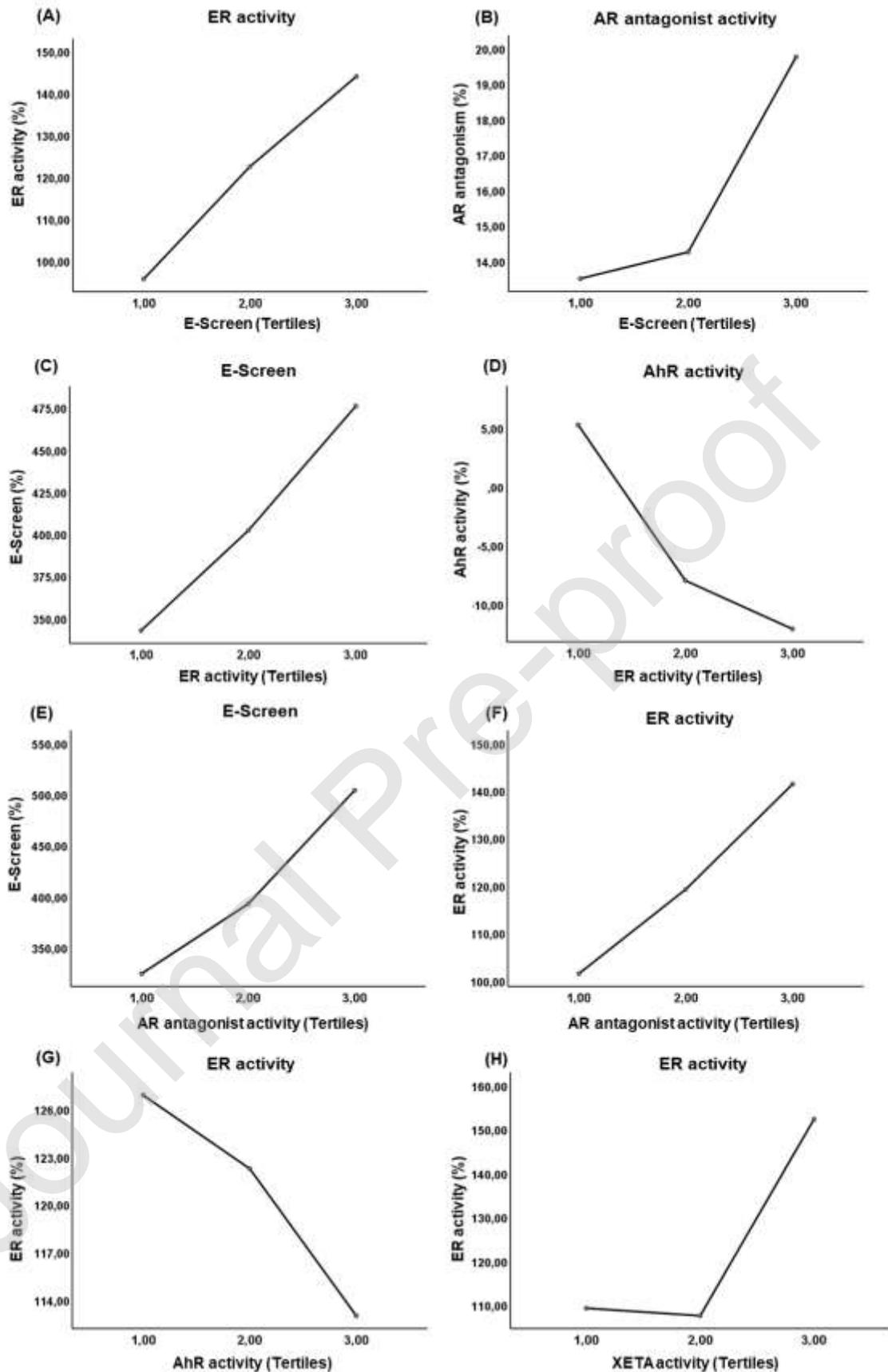


Figure 5. Linear regression models categorizing the independent variable into tertiles from lower to higher signals obtained in each biomarker of combined activity. *Data shown makes*

reference to significant or borderline-significant correlations found in Table 3, to test the functional shape of the correlations. (A) E-screen correlation with ER reporter gene assay; (B) E-Screen correlation with AR reporter gene assay; (C) ER gene reporter assay correlation with the E-Screen; (D) ER reporter gene assay correlation with AhR reporter gene assay; (E) AR reporter gene assay correlation with the E-Screen; (F) AR reporter gene assay correlation with the ER reporter gene assay; (G) AhR reporter gene assay correlation with the ER reporter gene assay; (H) XETA correlation with the ER reporter gene assay. Coefficients of determination (r^2): 5.A: $r^2=0.202$; 5.B: $r^2=0.095$; 5.C: $r^2=0.166$; 5.D: $r^2=0.228$; 5.E: $r^2=0.121$; 5.F: $r^2=0.105$; 5.G: $r^2=0.030$; 5.H: $r^2=0.151$.

4. Discussion

4.1 Chemical mixtures interact with diverse signaling pathways

In this screening study, HPLC α -fractions containing “real-world” mixtures of lipophilic and persistent chemicals were isolated from 24 human placentas and tested in 5 different bioassays, in an effort to comprehensively address the biological effect of chemical mixtures on different biological endpoints. Overall, most placental fractions elicited estrogenic activities in both the E-Screen and the ER reporter gene assay. While 14 out of 24 placental α -fractions were clearly positive for anti-androgenic activity based on the cut-off value, most fractions (22 out of 24) were considered positive for AR inhibition when both a concentration-dependent response as well as average responses below the cut-off were accounted for. Additionally, most placental α -fractions showed a clear inhibition of thyroid activity in the *in vivo* XETA, while subtle induction effects were noticed in the AhR gene assay.

Differences in sensitivity across bioassays were noticed, since effects were observed at placental concentrations $< 12.5 \text{ mg}_{\text{placenta}}/\text{mL}$ in the AR reporter gene assay, whereas in the estrogen assays higher placental concentrations were needed to induce effects. However, the range of effects observed was higher in the E-Screen assay, which allowed to detect a wide variability among the α -fractions tested. Interestingly, when correlation analyses were conducted, the highest correlation was found between the two estrogenic bioassays, while a positive trend was observed between xeno-estrogenic and anti-androgenic activities. This is most likely due to the similar information reported by the E-Screen and the ER-reporter gene assay, since both of them evaluate the same signaling pathway. Additionally, the positive correlation observed between

both estrogenic assays and the anti-androgenic assay is not unexpected since compounds eliciting estrogenic activity often tend to behave as antiandrogens as previously shown, including a potential cross-talk between ER and AR signaling [41–44]. In addition, placental fractions with the highest estrogenicity also tended to show the lowest AhR activity, which could be due to the inhibitory cross-talk reported between AhR and ER [45]. The XETA results hardly correlated with any other selected bioassays, suggesting that this assay is responsive to chemical families whose biological effects may not be captured by the remaining assays. Despite the observed correlations among bioassays, r^2 coefficients were modest (Figure 5), suggesting that the variance in biological activity was only partially predicted by the remaining assays, and that consequently, various bioassays are needed to characterize the effects of complex chemical mixtures. To the best of our knowledge, this study represents the first attempt to comprehensively map the biological effects of chemical mixtures present in the same human placental samples.

4.2 Bioassays as biomarkers of combined effect in epidemiologic studies

One of the potential applications of these bioassays is their use as biomarkers for characterizing the combined effect of chemical mixtures in human samples. Previous epidemiologic studies have tested the cumulative estrogenic and anti-androgenic activities elicited by α -fractions extracted from placenta samples. For example, a nested case-control study within the Spanish INMA Granada mother-child cohort [27], reported for the first time that children with a higher placental xeno-estrogenicity, assessed by the E-Screen, had higher risk to develop urogenital malformations: cryptorchidism and/or hypospadias [27]. Similarly, Arrebola et al. (2015), found that children with higher placental anti-androgenic activity had higher risk to develop the same urogenital malformations [20]. Interestingly, when comparing our results with the abovementioned studies that studied placenta samples collected years ago, the present screening study found similar estrogenic and anti-androgenic combined effects exerted by persistent chemical mixtures extracted from human placentas at similar magnitudes (Figures 2.A and 2.C). Taken together, the observed biological activities suggest that unborn children are prenatally

exposed to complex mixtures of chemicals that may exert potential harmful effects during this critical window of development.

In a series of research studies from the Spanish INMA prospective birth cohort assessing placental α -fractions from different Spanish areas (Asturias, Gipuzkoa, Sabadell, Granada and Valencia), higher levels of xeno-estrogenicity were associated with increased birth weight [25], more behavioral problems in boys [24], as well as differences in genome-wide DNA methylation and repetitive elements [46,47]. Taken together, the combined estrogenic activity exerted by placental mixtures of lipophilic chemicals have been associated with several adverse health effects in children, supporting the utility of this approach for generating hypotheses between chemical mixtures and human health.

Our current findings complement the abovementioned information, suggesting that bioassays related to different signalling pathways and specific receptor activities (e.g. XETA and AhR) may be needed to comprehensively characterize the whole biological fingerprint of exposure to complex mixtures. For instance, our results showed that placental α -fractions induced a weak AhR activity, which could be due to the observed decreasing trends in exposure to dioxin and dioxin-like compounds in the Spanish population [48,49]. Moreover, dioxins present in placental α -fractions could exert an anti-estrogenic activity, thus potentially explaining the not significant but inverse dose-response correlation observed between the AhR assay and the ER reporter gene assay, as previously suggested in other studies [50]. Although not in placenta samples, previous studies have used the AhR reporter gene assay in similar contexts (for a recent overview see [29]). In a case-control study using serum samples, higher POPs-induced AhR activity was found in breast cancer cases compared to controls [14]. Another case-control study showed that the activity of the AhR elicited by amniotic fluid extracts was not associated with the risk of autism spectrum disorder (ASD) in children [51].

4.3 XETA as a potential novel biomarker of combined effect

Few studies have tested the xeno-activity of mixtures on the thyroid axis [52,53]; furthermore, the XETA has not been used before to assess the effect of chemical mixtures isolated from human samples. Moreover, there are no validated *in vitro* assays to assess thyroid disruption, and in contrast to the previous endocrine modalities tested, the XETA may detect endocrine disruption arising from multiple levels of regulation [54,55]. In the present screening study, placental α -fractions showed a strong inhibition of thyroid signaling, in line with previous anti-thyroid effects exerted by chemicals mixtures made *ad hoc* based on human amniotic fluid exposure levels [56]. While *ad hoc* mixtures can only test previously known chemicals, extraction protocols in human samples coupled to bioassays are able to assess biological activities of “real-world” mixtures with both known and unknown environmental chemicals. In the current study, the XETA was not correlated with any other implemented bioassays, suggesting that the observed anti-thyroid activity was not related to the remaining biological activities. On the one hand, this is in line with thyroid receptors not being structurally related to steroid hormone receptors. On the other hand, the XETA *in vivo* assay presents a higher level of biological complexity compared to *in vitro* models, and observed effects may be driven by diverse action mechanisms not limited to the receptor level of the hypothalamus-pituitary-thyroid axis. XETA results highlight that different biomarkers of combined activity, representing different signaling pathways, should be used to fully characterize the cumulative effects elicited by complex chemical mixtures in human populations. The XETA has been recently validated at the OECD level (TG248) as a Tier 3 assay [38], and our findings highlight it as a promising biomarker of combined anti-thyroid effect which should be further investigated in population-based studies, particularly in relation to neurological endpoints.

4.4 Identification of the chemicals responsible for biological effects

Sample preparation and extraction is critically important, especially when investigating environmental chemicals in human samples. As extraction methods are rarely optimized for bioassays but rather adopted from chemical analysis, this may result in a misrepresentation of

the actual biological activity [15]. HPLC α -fractions obtained from human samples allows the assessment of the combined effects elicited by the most lipophilic and persistent compounds while avoiding the interference of endogenous hormones [26,57,58]. As previously reported, if the endogenous hormones are present in the mixture, they can saturate the endpoint of the *in vitro* or *in vivo* assay. Thus, the biological activity of chemical mixtures would be importantly masked, leading to a reduction in the variability of the signal elicited as well as an underestimation of the combined effect [59,60]. This is not the case with the extraction protocol used. Several studies have demonstrated the absence of endogenous steroid hormones and identified some chemical families present in the α -fraction: mainly lipophilic and persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), including lindane, hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE), among others [14,27,32,58]. The chemical compounds detected by previous studies in the α -fraction are listed in Supplemental Table 1.

Although some chemical groups have been identified in α -fractions, its full chemical composition has not been elucidated yet, and the number of chemical families and metabolites contained is anticipated to be much higher. A step forward for both environmental toxicology and epidemiology would be the identification of those chemical compounds responsible of such biological effects, including the characterization of both unknown and emerging contaminants. In this regard, it has been proposed that non-targeted chemical analyses can help to decipher the chemical composition of complex lipophilic mixtures isolated from human samples [61]. Indeed, suspect and non-target methodologies are being developed inside the HBM4EU project [62]. Interestingly, effect directed analysis (EDA) coupled with non-target chemical analysis can be used to overcome some limitations, allowing an efficient identification of biologically active chemicals through sub-fractionation processes, thus facilitating non-target chemical analysis [63]. Further research is ongoing using EDA coupled to non-targeted chemical analysis

on these placental α -fractions in order to characterize the bioactive chemicals responsible for the observed combined biological activities.

4.5 Strengths and limitations

A major strength of this study is the application of five different biological assays on the same set of human placentas, mapping different signaling pathways and exploring their relationships. Additionally, this is the first study which has assessed human samples with the *in vivo* XETA, showing a clear anti-thyroid activity, which adds valuable information regarding the effect of lipophilic environmental contaminants on this important pathway. Finally, this is the first attempt to correlate various biological activities exerted by the same mixture of lipophilic compounds extracted from human samples in order to explore potential relationships among them. This study also presents some limitations. The relatively small number of placenta samples analyzed limited our ability to observe correlations among bioassays. Although the α -fraction provides a good representation of the most lipophilic and persistent compounds, the full chemical exposome was not addressed. In addition to the known chemical families previously detected in the α -fraction, non-targeted chemical methodologies will be needed to unravel its complex chemical composition. Another limitation is that the tested biological assays do not measure the biological effects elicited by mixtures of lipophilic compounds at the same levels of biological complexity. For instance, the *in vivo* XETA evaluated the highest level of biological complexity; the E-screen assessed cell proliferation after 6 days of exposure; and the reporter gene assays tested the ability of the mixture to agonize or antagonize a particular nuclear receptor. These disparities and other methodological differences (e.g. different dilutions, medium for reconstitution of dried extracts, etc.) should be considered when interpreting the results of this screening study. Finally, the centralized isolation of α -fractions in the same laboratory under controlled conditions (e.g. glassware material, work inside flow-chambers) limited potential background contamination. These measures, together with the strong and variable biological activities observed for most -but not all- of the placentas in the selected bioassays (estrogenic, anti-androgenic, anti-thyroid and AhR activities), and at different

dilutions, makes unlikely that results could be explained in terms of sporadic background contamination.

This work represents a multidisciplinary effort to evaluate human exposure to chemical mixtures by synergizing the toxicological and epidemiological fields. Isolation and assessment of chemical mixtures from human samples may help to identify bioactive chemicals responsible for a given biological activity, as well as identify novel bioactive chemicals through complementary methodologies. In parallel, a battery of *in vitro* and *in vivo* bioassays should play a crucial role in the assessment of human exposure to complex mixtures, since they can determine the joint activity elicited by complex mixtures through a specific mode of action [53]. The use of this combined approach together with the information provided by additional biomarkers of effect (e.g. hormone levels, biochemical parameters, etc.) would help to strengthen the weight of evidence in observational studies linking chemical exposures to health outcomes.

5. Conclusions

A panel of different bioassays is needed to provide a comprehensive assessment of “real-world” chemical mixtures. The biomarkers of combined activity investigated in this work enabled us to better characterize the signaling pathways through which mixtures could elicit adverse health outcomes in humans. Epidemiological studies should also include these bioassays as a complementary tool to improve the causal inference of exposure-effects relationships in the context of complex mixtures. Further research is needed to characterize the full composition of complex chemical mixtures present in human samples.

6. Declaration of Competing Interest

The authors declare no actual or potential conflicts of interest. The funders had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

Declaration of interests

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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