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Report on results of the selected biomarkers of combined effect [Proof of concept]

Deliverable Report

D14.4

WP14 - Biomarkers of Effect

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1 Abstract/Summary

Background: Humans are continuously and simultaneously exposed to mixtures of chemicals from different sources that enter the organism by different routes. Hence, there is a need to identify and develop biomarkers that assess the combined effect of chemical mixtures present in exposed individuals.

Objective: The aim of Deliverable D14.4 was to conduct different ex vivo bioassays of potential utility as biomarkers of combined effects using the same biological matrix, human placentas, in order to prove that the combined effect of chemical mixtures isolated from human samples can be assessed by quantifying the signal elicited in different bioassays.

Methods: To fulfil this purpose, human placental extracts were tested in six bioassays that assessed biological activities of complex mixtures: i) E-screen for estrogenic activity, ii) Luciferase-reporter gene assay for antiandrogenic activity (UGR); iii) evaluation of estrogenic and iv) antiandrogenic activity exerted by the mixtures (AU); v) Xenopus Laevis assay to assess combined thyroid activity; and vi) the aryl hydrocarbon receptor (AhR) reporter gene assay to determine the capacity of extracts to induce AhR activity. Additionally, two biomarkers related to oxidative stress were investigated in placental samples: i) telomere length, as an effect biomarker of biological ageing (UH); and ii) 8-OHdG, as an effect biomarker of global DNA damage.

Results: Placenta extracts showed varying xeno-estrogenic activities in both the E-Screen and Xenobiotic transactivity bioassays. Placental extracts also revealed anti-androgenic activities in the Xenobiotic transactivity assay. Moreover, placenta extracts also evidenced an antagonistic effect on thyroid hormone signalling in the XETA assay, and 10 out of 24 placenta extracts induced or inhibited AhR activity in the reporter gene assay. All placental tissue studied had measurable and variable 8OHdG concentrations and telomere lengths.

Conclusions: Different ex vivo cell-based bioassays can be used to assess the combined effect of chemical mixtures isolated from placenta samples providing relevant information on specific signalling pathways of estrogenic, anti-androgenic, anti-thyroid, and AhR activity systems. For some novel bioassays tested, additional optimisation will be required. Given the need to evaluate the possible harmful effects of chemical mixtures on human health, the use of biomarkers of combined effect could add value to HBM programs.

Future steps: The methodology developed here will be applied to minimally invasive human samples and matrices employed in routine HBM, such as serum and urine. In future works in collaboration with Work Package 16 potential chemical-biological activity relationships will be investigated (Additional Deliverable AD14.4).

2 Introduction

Exposure to mixtures of environmental chemicals

The European population is exposed to an increasing volume of chemicals substances. Pollution patterns have changed since several decades ago to nowadays, from higher levels of a low number of chemical families, locally produced and mostly in occupational settings, to lower levels of a wide range of chemical substances, produced and used worldwide. The composition of this mixture of old and new chemicals, each one present at low levels, includes many of the so-called endocrine disruptors (Kortenkamp, 2014), which have been associated with reproductive and neurodevelopmental disorders (Balabanic et al., 2011; Mustieles et al., 2015), cardiovascular diseases (Khalil et al., 2014; Vafeiadi et al., 2016), endocrine alterations (Fini et al., 2017; Mustieles et al., 2018) and higher cancer risk (Montes-Grajales et al., 2016, (Claus Henn et al., 2014). The list of endocrine and suspect endocrine disrupting chemicals is long and some of them are used as biocides, cosmetic ingredients, in tools for daily life activities or in the food industry, as food packaging or additives (Vandenberg et al., 2007; Wittassek et al., 2011). Therefore, these chemical species such as polychlorinated dioxins, persistent organic pollutants, phthalates, bisphenols, multiple pesticide residues, heavy metals, polybrominated and poly/per-fluorinated compounds and polycyclic aromatic hydrocarbons and/or their metabolites, can be easily measured in several human matrices.

Biomarkers of exposure, effect and combined effect within a human biomonitoring context

Biomarkers of exposure, defined in 1987 by the National Research Council Committee on Biological Markers (NRC) as '' the chemical or its metabolite or the product of an interaction between a chemical and some target molecule or cell that is measured in a compartment in an organism'' (Silbergeld and Davis, 1994) are a critical tool to estimate population exposure to environmental chemicals (DeCaprio, 1997). However, estimation of exposure alone is not enough to ensure harmful effects on human health. For this reason, apart from exposure biomarkers, there is a need to assess effect biomarkers, defined by the NRC as "a measurable biochemical, physiologic, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease''. In other words, effect biomarkers are biological markers that can detect an altered physiological structure or function before the disease occurs (DeCaprio, 1997; Silbergeld and Davis, 1994). Therefore, the use of both exposure and effect biomarkers is needed in human biomonitoring (HBM) studies to assess population exposure to the most important environmental chemicals and their possible effects on human health.

Biomarkers of effect can include both traditional effect biomarkers such as hormones, metabolic parameters and inflammatory cytokines among many others, as well as more novel effect biomarkers including epigenetic marks and other OMIC biomarkers. Interestingly, there exists another category of effect biomarkers that has been less explored. These types of biomarkers are commonly referred to as "biomarkers of combined effect", "biomarkers of combined biological activity", or "biomarkers of combined internal dose". The common fact shared by these descriptions is the "combined" aspect, which refers to how mixtures of different chemicals, normally extracted from human biological samples (serum, placenta, adipose tissue…), can act in a combined way to exert a particular biological activity. This biological activity is normally assessed in cell-based bioassays (Estrogenic-screen, Antiandrogenic-screen) but can also be measured using *in vivo* models (X. Laevis assay). In this deliverable we will use the term "biomarkers of combined effect" to designate the biological effect or activity of mixtures of chemicals extracted from human samples, in this case, placental samples.

Human placenta as an exposure and effect biological matrix

Human placenta has many advantages. It is the only human organ that can be obtained in a non-invasive manner. Each placenta weights around 500 grams, its storage at biobanks is perfectly feasible and there is no issue with its availability, since most university hospitals have far more than 1,000 deliveries per year. The placenta contains a large variety of cell types, which allow its main functions: nutrition, respiration and elimination of metabolic wastage of the fetus. However, many toxic agents including environmental chemicals can cross the placenta and accumulate in it (Grabic et al., 2006; Kantola et al., 2000; Reichrtová et al., 1998). Therefore, the placenta constitutes a very valuable biological matrix for the study of chemical mixtures and its possible adverse health effects. On the other hand, this biological complexity also means that there are several issues to account for when working with placental samples, such as the presence of endogenous hormones that may mask or interfere with the effects of xenobiotics.

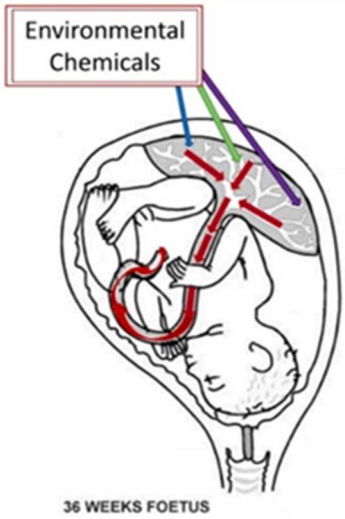

Figure 1. Foetus in its 36th week of gestation being exposed to mixtures of environmental chemicals pollutants from the mother through the placenta

Assessing the combined effect of chemical mixtures

Combined exposures to multiple chemicals are understudied, probably due to the practical and theoretical challenges that the assessment of mixture effects exerted by the additive interactions of a large number of chemicals families can suppose (Kortenkamp, 2014). However, this trend is changing. Thus, Hass et al., found adverse reproductive effects in male offspring Wistar rats treated with chemical mixtures, and Fini et al., found neurodevelopmental adverse effects in Xenopus embryos, after exposing them to chemical mixtures simulating real-life mixtures previously reported in human amniotic fluid (Fini et al., 2017; Hass et al., 2007). Regarding epidemiological studies, Vilahur et al. extracted chemical mixtures from placental samples and tested their combined estrogenic activity using a cell-based assay (E-screen), finding that higher combined xeno-estrogenic activities were associated with altered birth weight and neurodevelopment among boys from the INMA Spanish birth cohort study (Vilahur et al., 2014; 2013). Additionally, a case-control study by Arrebola and colleagues compared the anti-androgenic activity exerted by extracts of chemical mixtures obtained from placentas of children born with urogenital malformations (cases) *vs.* normal children (controls). A higher anti-androgenic activity of the placenta extracts was associated with a higher risk of urogenital malformations (Arrebola et al., 2015).

Aim of the study

There is a need to identify and develop biomarkers of combined effect to mixtures. Current advances in the characterization and isolation of chemicals from human biological samples, together with cell-based models, allow the development of biomarkers of combined effect that could be implemented in HBM programs. Therefore, our main objective was to conduct different bioassays and/or possible biomarkers of combined effect using the same biological matrix, human placenta, in order to provide the most complete picture regarding different biological activities in response to combined exposures.

3 Planning and Distribution of Tasks

Twenty-five placentas from healthy women participating in the INMA birth cohort were recruited at the *San Cecilio University Hospital*, Granada (Spain). From each placenta, UGR partner obtained placental homogenates, alpha-extracts, placental pieces and placental biopsies. Additionally, aliquots of urine samples taken during each trimester of pregnancy from the same women that donated the placentas were obtained for future tasks (Figure 2).

A preparative high-performance liquid chromatography method (HPLC) was used to separate environmental chemicals present in the placenta homogenates from endogenous hormones without destroying them. Placenta extracts were eluted by a specific gradient with two mobile phases, collecting two eluates: the so-called alpha (min-1 to min-11) and beta (min-13 to min-32) fractions. A normal-phase column separated chemicals according to their polarity, with the most lipophilic compounds eluting in the shortest time; this alpha fraction was used for the investigations together with placenta homogenates, placenta biopsies and urine from selected patients when required.

Before the shipment, UGR set the tasks for each partner based on its previous knowledge and expertise. As a result, each partner received: i) CNRS: 24 alpha-extracts, ii) CEA: 24 alpha-extracts, iii) AU: 24-alpha extracts ad 25 placental homogenates, iv) DTU: 24 alpha extracts and 25 placental homogenates, v) MU: 25 placental pieces and 85 urine samples, vi) UH: 25 placental biopsies, vii) INSERM: 25 placental homogenates and viii) INRA: 25 placental homogenates (Figure 2).

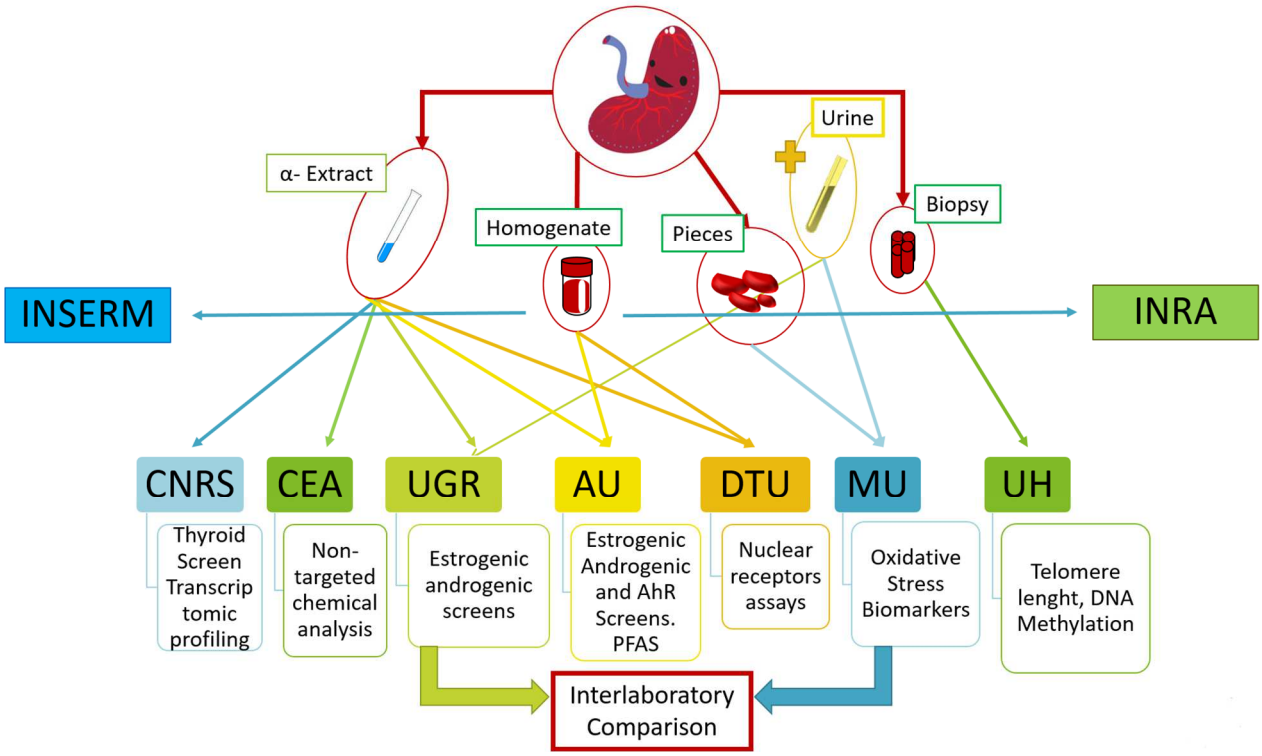

Figure 2. Planning and distribution of tasks among partners.

Table 1 shows the bioassays and effect biomarkers that were planned for the present deliverable. Additional results and the continuation of this work will be reported in Additional Deliverable AD14.4 ‘‘First report on the state of development of novel biomarkers of effect’’.

Table 1: Summary of biomarkers of combined effect and effect biomarkers developed in the present deliverable

Summary of Bioassays			
PARTNER	**Responsible**	**Biological shipped material**	**Bioassays/Biomarkers**
UGR	Nicolás Olea	24 α-Fraction	**E-screen**. Total Effective Xenoestrogen Burden (TEXB) to estimate the total xenoestrogenic activity of the placenta samples, expressed as estradiol equivalents. **Luciferase reported gene assay:** Total Androgenic Xenobiotic Assay (TAXB) through androgen receptor within PALM cell line, to estimate the total androgenic activity of the placentas.
AU	Eva Cecilie Bonefeld Jorgensen	24 α-Fraction 25 Placenta Homogenate (≈20g)	**Estrogenic and anti-androgenic** effects of alpha-fractions derived from the placentas: Estrogenic, Androgenic screens.
CNRS	Jean-Baptiste Fini	24 α-Fraction	**Thyroid hormone disruption** exerted by mixtures of compounds present in alpha-fractions obtained from the placentas using the Thyroid Embryo Xenopus Assay (TEXA).
DTU	Anne Marie Vinggaard	25 α-Fraction 25 Placental Homogenate (≈20g)	The Aryl Hydrocarbon receptor expression (AhR) assay was used by DTU to test the effect of alpha-fractions derived from placenta extracts
MU	Ludek Blaha	25 Placenta pieces Paired pregnancy urines	**8-OHdG is an effect biomarker of oxidative DNA damage.** MU has fine-tuned the methodology for the measurement of 8-OHdG in placental pieces.
UH	Tim Nawrot	25 Placenta Biopsies	UH presented preliminary results of **telomere length** measured in placental biopsies.

4 Methods

Six experiments were conducted by several Work Package 14 partners with previous expertise in the characterization of environmental chemicals exposure and its toxicological effect [University of Granada (UGR), Spain; Aarhus University (AU), Denmark; The National Center of Scientific Research (CNRS), France; Technical University of Denmark (DTU), Denmark; University of Hasselt (UH), Belgium; and Masaryk University (MU), Czech Republic]. UGR obtained 25 placental homogenates, 25 UGR, AU, CNRS and DTU evaluated the combined biological activity of 24 alpha-fractions extracted from 24 human placentas. UH received 25 placental biopsies from UGR, and measured telomere length, an effect biomarker of biological aging. Finally, MU received 25 placenta pieces from UGR, and MU fine-tuned a methodology for the measurement of 8OHdG, a marker of oxidative DNA damage, in these placenta pieces.

4.1 Estrogenic and (anti)-Androgenic Screens bioassays (UGR)

4.1.1 Placental Extraction

4.1.1.1 Placenta Protocol:

Placentas, without deciduas basalis and chorionic plate, were collected at the time of delivery and immediately frozen at −20°C and then stored. Months later, placentas were moved from the biobank and stored at -80°C. Before analysis, the placenta was almost defrosted and half of the placenta was mechanically homogenized and stored again in small aliquots (20-25 g) at -80ºC (Lopez-Espinosa et al., 2009; Vilahur et al., 2013).

4.1.1.2 Extraction protocol:

1.5 g of placenta homogenized with 1.5 mL of water by shaking on a vortex for 1 min. The homogenate was extracted by adding 3 mL of ethyl acetate and shaking again for 10 min and the mixture was then centrifuged for 10 min at 5000 rpm (4050 × g). The underlying organic layer was transferred to a clean glass vial and evaporated to dryness at room temperature under a nitrogen stream. This process was repeated twice (3 g of placental in total), and all the dried extracts were dissolved with hexane (0.7 mL) and collected together. Then, the mix was dried under nitrogen stream again. The residue was dissolved in 400 microliters of hexane and then injected twice (200 microliters) into the preparative HPLC.

The preparative liquid chromatography method was applied to allow the separation of the more lipophilic xenoestrogens from natural estrogens without destroying them. Extracts were eluted by a gradient with 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. The gradient programme was based on a previously described method from (Jimenez-Diaz et al. 2011) with some modifications. Working conditions were: t-0 min, 100% phase A; t-17 min, 60% phase A; t-25 min, 100% phase B; t-32 min, 100% phase A. Three pooled fractions, named "alpha", "x" and "beta", were separated by High Performance Liquid Chromatography (HPNC). The alpha fraction was collected in the first 11 minutes.

4.1.2 Chemicals, materials, and instrumentation

For *in vitro* cell assays, reference standards methyltrienolone (R1881), puromycin, geneticin (G418) and luciferin (sodium salt), were obtained from Sigma-Aldrich Inc. (St Louis, MO). Stock solutions R1881 and procymidone were prepared in ethanol, and successive dilutions were performed in culture medium. Stock solutions were kept at -20 °C, and dilution series were freshly prepared before each experiment. Culture medium and foetal bovine serum (FBS) came from

Gibco (Invitrogen, Barcelona, Spain), and all cell culture plastics were supplied by Falcon (VWR International Eurolab, Barcelona, Spain).

Finally, for PALM assays, an infinite M200 luminometer (Tecan, Barcelona, Spain) was used to measure luciferase activity in intact cells.

4.1.3 MCF-7 cell line and proliferation cell assay

For *in vitro* cell assays, dry alpha fractions were re-suspended in 1 ml of experimental medium [phenol red-free DMEM supplemented with 10% dextran-coated charcoal-FBS (10% DCC-FBS)], vigorously shaken and left at rest for 30 min, then filtered through a 0.22 µm filter and tested (200µl were added per well) on MCF-7 cells at 1:1, 1:5 and 1:10 dilutions. Each sample was tested in triplicate. A dose-response curve (0.1 pM-1000 pM) for E2 and negative control (cell treated only with hormone-free medium) and solvent controls (blank and solvent) were included in each experiment (Lopez-Espinosa et al., 2009).

4.1.4 PALM cell line and culture conditions

Cells used in this study were cultured as previously reported (Molina-Molina et al. 2013; 2014). In brief, human prostate cancer PALM cells were cultured in Ham's F12 supplemented with 10% FBS, 1 mg/ml G418, and 1 µg/ml puromycin. Experiments were performed in a test culture medium of Ham's F12 supplemented with 6% dextran-coated charcoal-FBS (6% DCC-FBS) and 1% antibiotic, in a 5% CO2 humidified atmosphere at 37 °C.

4.1.5 PALM cell luciferase assay

PALM cells, according to previously described protocols (Molina-Molina et al. 2013, 2014), were seeded at a density of 5 x 104 cells per well in 96-well white opaque tissue culture plates in 200 µl test culture medium.

Dry alpha fractions were re-suspended in 1 ml of test culture medium, vigorously shaken and left at rest for 30 min, then filtered through a 0.22 µm filter) and 200 µl were added per well at 8 h after seeding at 1:1, 1:5 and 1:10 dilutions. On each plate, alongside the test samples, serial dilutions of the agonist R1881 (1 pM-10,000 pM) were included as positive and negative controls (test culture medium alone or with solvent). PALM cells were incubated for 40 h at 37 °C, and the medium was then removed and replaced by test culture medium containing 0.3 mM luciferin. Next, the 96-well plate was introduced into a luminometer for 2 s to measure luminescence from intact living cells.

hAR-agonistic activities were tested at 1:1 to 1:10 dilutions of alpha fractions, performing tests in quadruplicate for each dilution. Maximal luciferase activity (100%) was obtained in the presence of 10 nM R1881. The antagonistic activity of extracts was determined by co-incubation with R1881 agonist (0.3 nM). Results were expressed as percentage of maximal luciferase activity. Finally, the luciferase activity in each sample extract was calculated as a percentage of the maximal luciferase activity obtained with R1881 or procymidone and transformed into R1881 or procymidone equivalent units (R1881eq or Proceq, respectively) by reading from dose-response curves of R1881 or procymidone (standard serial dilutions) included on each plate. R1881eq or Proceq were calculated from the dilution obtaining the greatest induction or inhibition of luciferase activity, respectively. Derived R1881eq or Proceq were corrected for the dilution factor and reported as R1881eq/g or Proceq/g of the original placenta samples.

4.2 Xenoestrogenic transactivity of placenta extracts (AU)

4.2.1 ER Transactivation Assay.

In this study, the established ER transactivation assay was applied using the human cancer cell line MVLN which is derived from the human breast carcinoma MCF-7 cell line, and is stably transfected with a reporter gene construct, pVit-tk-Luc. The gene construct contains a luciferase gene under control of the estrogen response element (ERE).

The ER-transactivation of placenta samples was determined using the stable transfected MVLN cell line. Briefly, 8.5x104 cells were seeded in each well in a 96-well plate and left at 37º C in the incubator overnight. The next day, the diluted samples with and without 17 β-estradiol (E2) co-exposure were added to the 96-well plate in triplicate, using 100 µL/well. After 24 hours incubation at 37º C, the cells were harvested, the luciferase activity was determined in a LUMIstar luminometer (BMG, RAMCON, Denmark), and the protein content was determined by fluorometric measurements using a WALLAC Victor2 (PerkinElmer, USA). The ER-transactivation data were expressed as relative light units per count of protein (RLU/prot).

4.2.1.1 Dissolution of samples

The obtained placenta extract samples were yellow solid in glass vials. The samples were stored in freezer before analysis. The dry placenta extracts were reconstituted in 44 µL ethanol (EtOH) and gently shacked overnight. The next day, 20 µL was used for the xenoestrogenic transactivity assay.

4.2.1.2 Dilution of placenta samples

For the dissolution of placenta extract, we added 44 µl EtOH to each dry placenta sample.

Then the ethanol solution of placenta sample was diluted 50 times with Dulbecco's modified Eagle's media without phenol red (DMEM, Lonza) containing 0.5% charcoal-dextran stripped fetal bovine serum (CD-FBS). Thus, the samples contained 2% ethanol (EtOH). These placenta sample solution (1/50) were further diluted 5 times and 10 times to get 1/250 and 1/500 dilutions, which contained 0.36% and 0.18% EtOH, respectively (Table 4.2.1.2.1). Solvent control was prepared following the same procedure for the samples by using culture medium plus ethanol instead of placenta sample, and no toxicity was observed for the solvent control.

Table 4.2.1.2.1: Dilution of samples for xenoestrogenic transactivity of biological samples

Procedure used to dilute the placenta extracts: **The dry placenta extracts were reconstituted in 44 µL EtOH (20 µL for ER assay and 20 uL for AR assay)**				
	Dilution factor	**Extract**	**Medium (µL)**	**EtOH concentration in solution on cells**
	1/10	20 µL ↑	200	9 %
	1/50	100 µL ↑	400	1.82 %
Dilution 1	1/250	100 µL ↑	400	0.36 %
Dilution 2	1/500	150 µL ↑	150	0.18 %

4.2.1.3 Xenoestrogenic transactivity measurement

The MVLN cells were exposed to samples and controls for 24 h. The induced luciferase activity was determined using a luminometer (BMG LUMISTAR) with automatic injection of luciferase substrate and expressed as relative light units (RLU). The luciferase data were corrected for cell density as the protein content in each well was determined by addition of 50 µl/well of fluorescamine diluted in acetonitrile (500 mg/l), followed by fluorometric measurements in a Wallac VICTOR2 (Perkin Elmer) at 355/460 nm wavelength, according to a standard curve of bovine serum albumin (BSA, Promega).

The placenta extracts were tested alone to assess the effect on the ER function (termed as XER). To mimic the effect in vivo assessing the ability of the placenta extract to compete with the endogenous estrogen, we determined the effect of placenta extracts in the presence of the ER agonist 17β-estradiol (E2) and termed as XERcomp.

The ER agonist 17β-estradiol (12.5, 25 pM and 100 pM) were used as parallel positive controls on each 96-well microtiter plate.

4.2.2 Xenoandrogenic transactivity of placenta extracts

A luciferase reporter gene assay was employed to assess the xenoandrogenic transactivity of TEXB-α placenta extracts.

The androgenic and anti-androgenic transactivities were assessed using the Chinese hamster ovary cell line CHO-K1 (ATCC no. CCL-61), transiently co-transfected with the MMTV-LUC reporter vector (kindly provided by Dr. R. M. Evans, Howard Hughes Medical Institute, USA), and with the human AR expression plasmid pSVAR0 (kindly provided by Dr. A. O. Brinkmann, Erasmus University, Rotterdam, NL). Cells were exposed to serial dilutions of the placenta extracts for 20 hours with and without dihydrotestosterone (DHT) before harvest and measurement of xenoandrogenic transactivity.

The AR agonist, dihydrotestosterone (DHT), was obtained from Sigma-Aldrich. The AR antagonist hydroxyflutamide (HF) was from MikroMol GmbH (Luckenwalde). DHT and HF were used as dose–response controls in the AR transactivation assay. HF was dissolved in 96% extra pure ethanol into 20 mM stock solution and DHT was dissolved in dimethyl sulfoxide (DMSO, Thermo Scientific) to produce a 10 mM stock solution.

CHO-K1 cells were seeded 24 h before transfection, in white 96-well microtiter plates (Perkin Elmer) with a density of 8000 cells per well in DMEM/F-12 with supplements and 10% CD-FCS (HyClone). The transfection was carried out for 5 h in DMEM/F-12 without supplements and serum, using 0.3 µl per well of the transfection reagent FuGene (Roche) and 150 ng cDNA per well of the AR expression plasmid pSVAR0 and the MMTV-LUC reporter vector in a ratio of 1:100.

After 5 h of transfection, the test solutions (see description of dilution of samples below) and controls were added, and the cells were exposed for 20 h. The induced luciferase activity was determined using a luminometer (BMG LUMISTAR) with automatic injection of luciferase substrate and expressed as relative light units (RLU). The luciferase data were corrected for cell density as the protein content in each well was determined by addition of 50 µl/well of fluorescamine diluted in acetonitrile (500 mg/l), followed by fluorometric measurements in a Wallac VICTOR2 (Perkin Elmer) at 355/460 nm wavelength, according to a standard curve of bovine serum albumin (BSA, Promega).

A DHT concentration-response control (2–200 pM) was performed in parallel in the assay, and additionally, a DHT-EC50 (12 pM) served as a control at each 96-well microtiter plate. A HF

concentration-response control (0.5–5000 nM) was included in the assay as well to serve as inhibitor concentration-response control.

4.2.2.1 Dissolution and dilution of samples

Dry extracts were diluted in extra pure EtOH. The dry placenta extracts were reconstituted in 44 µL EtOH and left to shake overnight. The next day, 20 µL was used for the xenoestrogenic transactivity assay and the remains were stored in dark at 4ºC for 6 days before the xenoandrogenic transactivity assay (Arrebola et al. 2015).

The samples were tested in three dilutions prepared as seen in table 4.2.2.1.1 The received placenta extracts were tested alone and upon co-treatment with 12 pM DHT. Each sample was tested in three dilutions in duplicates with 100 uL of the dilution added to each well, which already contained 100 µL transfection medium.

Table 4.2.2.1.1: Dilution Table. Final concentration in the well is half of the concentration in the tube, as the samples are added to the well with 100 µL transfection medium

	Dilution factor of solution	Final dilution factor in well on cells	Extract	Medium (µL)	EtOH Concentration in solution	Final EtOH in wells on cells
*Our normal dilution of **serum extracts** (obtained from 3 mL serum)*						
		The dry extracts were reconstituted in 20 µL DMSO:H2O:EtOH (10:40:50)				
	1/10	1/20	20 µL ↑	200	4.55 %	
Test solution	1/50	1/100	100 µL ↑	400	0.91 %	0.46%
*Procedure used to dilution the **placenta extracts***						
		The dry extracts were reconstituted in 44 µL EtOH (20 µL for ER assay and 20 µL for AR assay)				
	1/10	1/20	20 µL ↑	200	9 %	
	1/50	1/100	100 µL ↑	400	1.82 %	
Dilution 1	1/100	**1/200**	200 µL ↑	200	0.91 %	0.46 %
Dilution 2	1/250	**1/500**	160 µL ↑	240	0.36 %	0.18 %
Dilution 3	1/500	**1/1000**	150 µL ↑	150	0.18 %	0.09 %

4.3 Aryl Hydrocarbon receptor expression (AhR) assay (DTU)

The AhR reporter gene assay was already established in the laboratory at the National Food Institute. However, the protocol for cell viability assessment was changed to the MTT assay. Stably transfected rat hepatoma (H4IIE-CALUX) cells provided by Dr Michael Denison (University of California, USA) were used for both the AhR reporter gene assay and the cell viability test (Long et al., 2003; Ronsenmai et al., 2017).

4.3.1 Cell culture

Cell were cultured in minimum essential medium (MEMα) supplemented with 5% foetal bovine serum (FBS) and 1% penicillin/streptomycin/fungizone (PSF). The same medium was used for the experimental set-up. Cells were kept at 37ºC in 5% CO2 in a humidified atmosphere and sub-cultured approximately twice a week.

4.3.2 Cell treatment

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was used as a positive control and was tested in nine dilutions ranging from 0.5-3000 pM. The vehicle was DMSO and was constant at 0.1% across all exposure groups and the vehicle control group. Both vehicle control and TCDD were tested in triplicates.

Twenty-four placentas from normal Spanish women were received from Granada University partner (UGR). The 400 µL placenta extracts, each prepared from 3 g placenta, were aliquoted into four 100 µL vials. One vial of 100 µL was evaporated for the AhR reporter gene assay and cell viability testing in the H4IIE cell-line. The evaporated extract was solubilized in 250 µL cell culture medium by vigorously shaking. Successively, the extracts were left to rest for 30 min after which they were filtered through a 0.22 µm filter.

The placenta extracts were tested in triplicates in concert with a medium control, which was tested in six replicates per 96-well plate. From the 250 µL solubilized placenta extract, a 1000-, 300-, and 100-fold dilution was prepared and tested. This means that from the initial 3 g placenta, the final concentration was 0.3 mg placenta/well, 1 mg placenta/well, and 3 mg placenta/well. The placenta extract exposure dilutions were selected based on initial screening, where compromised viability was observed visually at higher concentrations. Both the AhR reporter gene assay and the cell viability test in the H4IIE cell-line were tested in two independent experiments.

4.3.3 AhR activity

Cells were seeded in a concentration of 22*103 cells/well in white clear bottomed 96-well plates (100 µL per well) and left to incubate for ~22 h after which cell culture medium was removed and placenta extract and TCDD was added in varying concentrations. After approximately 24 h exposure, cell culture medium was removed, cells were washed with PBS, and lysed with 25 µL lysis buffer containing 25 mM triphosphate, 15% glycerol, 1% triton X, 1 mM dithiotreitol, and 8 mM MgCl2 for approximately 20 min on a shaker table. Finally, luminescence was measured after addition of 40 µL luciferin solution containing 0.5 mM luciferin and 0.5 mM ATP in lysis buffer.

4.3.4 Cell viability

Cell viability was examined by the MTT assay. Cells were seeded at a concentration of 11 × 103 cells/well and after approximately 22 h exposed to placenta extracts and TCDD as above. After 24 h exposure, cell culture medium was removed from wells and 50 µL fresh medium was added. MTT was added leading to a final concentration of 0.45 mg/mL and incubation was performed at 37ºC for 1.5 h. Successively, medium containing MTT was removed and 50 µL isopropanol was added to each well after which cell culture plates was left on shaker table for 5 min after which absorbance was measured.

4.4 Xenopus Embryonic Thyroid-signaling Assay (XETA) (CNRS)

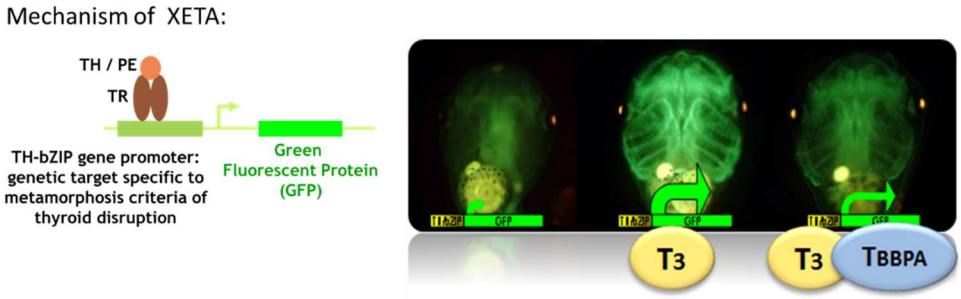

Figure 4.4.1: The XETA can detect the activity of Thyroid Hormone (TH) agonists that activate TH receptors, as well as antagonists of thyroid axis (e.g. Tetrabromobisphenol A (TBBPA)

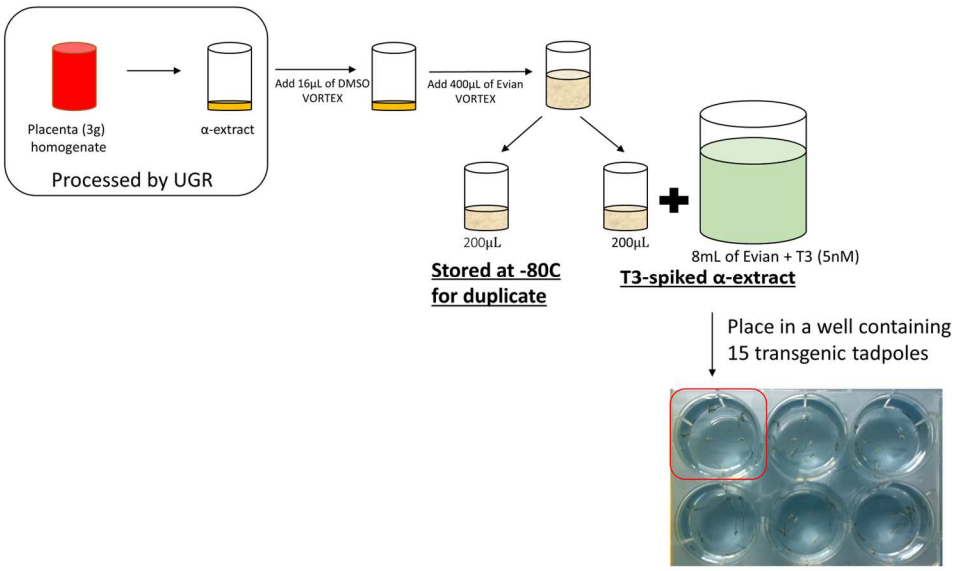

Figure 4.4.2: Methodology followed for the XETA

Alpha-extracts from 3 g of homogenized placenta samples of varied estrogenic properties, as evaluated by UGR's E-screen bioassay in terms of *Estradiol* equivalent quotient (Eeq) (Pm)/g placenta were selected for each experiment. Each α-extracts was resuspended in 16 µL of DMSO and 400 µL of Evian water, of which only half was used to allow for duplicates. Screening for thyroid disrupting properties of the α-extracts was carried out using XETA as previously described using stage NF45 tadpoles (1 week old) from Tg(thibz:eGFP) transgenic X. laevis (Fini JB et al.,2007 and 2012). Fifteen tadpoles were placed in each well of 6 well plates (TPP Switzerland), containing 8 mL of either control solvent (DMSO) in Evian, the thyroid hormone triiodothyronine T_3 (5nM) or a T_3 (5nM) -spiked α-extract. Plates were placed at 23 °C. DMSO concentration was 0.01% in all treatments. Solution were not renewed due to scarcity of α-extracts.

At regular 24 h intervals for a total of 72 hours, GFP readings were acquired with a 25x objective and 3 s exposure using an Olympus AX-70 binocular equipped with long pass GFP filters and a Q-Imaging Exi Aqa video camera. QC Capture pro (QImaging) software was used for image acquisitions and quantifications were carried out using ImageJ. All pictures of a group were

stacked, the 3 layers of RGB pictures were split and the red and blue channels subtracted from the green channel to exclude non-specific signals. Quantifications were carried out in a region of interest (ROI) to exclude the auto-fluorescence of the intestines which is variable during the first 24h, and data expressed in relative units of fluorescence (RFU).

Results are presented as a bar chart normalized to the Control group (ctrl) (100%) for each experiment at the 72 hour time point (figure 5.4.1) and as a bar chart displaying the reduction of T3-induced fluorescence divided by the specific T3 induction (T3 normalized to control) of each experiment (figure 5.4.2). GraphPad Prism 7 software was used for graphs and statistical analysis. Experiments were validated with a column to column comparison between the control and the T3 group at the same time point using the Welch T test, and between α-extracts and the T3 group using either Dunn's (experiment A) or Dunnett's (experiment B, C & D) multiple comparison test. Differences with the control group were considered significant at $p < 0.05$(#), $p < 0.01$ (##), $p < 0.001$(###) and $p < 0.0001$(####), and differences with the T3 group at $p < 0.05$(*), $p < 0.01$ (**), $p < 0.001$(***) and $p < 0.0001$(****).

4.5 Oxidative stress biomarkers of effect (MU)

4.5.1 Chemicals

Standard 8-hydroxy-2'-deoxyguanosine (8-OHdG, 98 %) and 2-deoxyguanosine monohydrate (2dG, 99-100 %) were obtained from Sigma-Aldrich (Merck); 15N5-8-hydroxy-2′-deoxyguanosine (>95 %) were obtained from Cambridge Isotope Laboratories; MS grade acetonitrile, isopropanol and formic acid (FA, 99%) were purchased from BIOSOLVE BV (Netherlands).

4.5.2 Extraction of placenta samples

DNA for analyses of 8-OHdG (and also 2-deoxy-guanosin - 2dG as a reference) was extracted from placenta samples by using DNA isolation kit (QIAGEN) according to the manufacturer instructions. Approximately 30 mg of tissue was homogenized in cold ATL buffer 3-times 20 seconds using glass beads in a homogenizer (Homogenizer MP Bio FastPrep®-24) and the homogenate was incubated with proteinase K and RNase A for 1h at 56°C. The purification of DNA from homogenate was done using DNeasy Mini spin columns. The yield of DNA isolated from the sample (in 0.175 mL of Tris-HCl buffer; pH> 8) was calculated based on the absorbance at 260/280 nm measured in NanoDrop ND 1000. Samples of extracted DNA were stored at - 80 °C.

Enzymatic digestion of isolated DNA was performed by 8-OHdG Assay Preparation Reagent Set (WAKO). Briefly, extracted DNA was denaturated (98°C, 2min) and then hydrolysed in a sodium acetate buffer for 60 minutes with nuclease P1 at 37°C. Further, the digested DNA was incubated in Tris-HCl buffer with alkaline phosphatase for 30 minutes at 37°C. Finally, to remove enzymes and other macromolecules, hydrolysates was ultrafiltrated through centrifugal filter units (AmiconUltra-0.5mL; 3,000 MWCO) at 15 000 x g for 20 min at 4°C. Supernatant was transferred to glass vial with inserts for analyses of 8-OHdG. For analyses of 2dG, samples were further diluted in glass vials 150-times by using 0.1% formic acid (FA). All samples were analysed immediately.

4.5.3 Analyses of 8-OHdG and 2dG in placentas by LC-MS/MS

Analyses of 8-OHdG and 2dG were performed with LC-MS system as described above. The determination of 8-OHdG and 2dG by LC MS/MS were done in two separate analyses.

The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile acidified by 0.1% formic acid (B). The binary pump gradient was linear (5% B at 0-1 min, then increase from 5% B at 1 min to 80% B at 5 min; and 80% B was kept for 2 min) followed by 4 min column equilibration to

the initial conditions (5% B). The flow rate was 0.2 mL/min. Injection used 8 µL of individual sample with added 2 µL of internal standard (8 ng/mL; 15N5 -8-OHdG) from the thermostatted autosampler (10°C). The ionization parameters were as follows: capillary voltage, 2.5kV; the source temperature and the desolvation temperature, 150 and 750 °C, respectively; the cone gas flow, 150 (L/h); the cone voltages, 30 V; the desolvation gas flow, 750 (L/h); and the collision gas flow, 0.15 mL/min. The collision energy was optimized for each analyte (Table 4.5.3.a). Retention time was 3.1 min for 8-OHdG and limit of quantification (S/N>10) was 0.05 ng/mL.

To avoid the potential oxidation in ion source and contamination of detector, the LC flow was diverted from 0 to 2.5 min (retention time of 2dG was 2.3 min) and from 5 to 10 min to waste if 8-OHdG was analysed. Concentration of 8-OHdG and 2dG were corrected for content of internal standard (15N5 -8-OHdG) and further expressed as 8-OHdG per 105 2dG (molar ratio) and also as ng 8-OHdG per g tissue or ng 8-OHdG per mg DNA.

Table 4.5.3.a: LOD and MS parameters for 8-OHdG and internal standard for analyses in placenta samples

Compound	MRM transitions	Collision energy (V)
8-OHdG	284.1 → 168.1	14
	284.1 → 140.1	28
^{15}N5 -8-OHdG	289.1→ 173.1	14
	289.1→ 145.1	28
2dG	268.0→ 152.0	18
	268.0→ 135.0	38

4.6 Telomere Length (UH)

Telomeres are nucleoprotein structures that cap the end of chromosomes. They can consist of several thousands of tandem-repeated TTAGGG sequences. With each cellular division, telomeres shorten. Telomere length (TL) has been associated with age-related diseases and mortality and is considered a marker of biological aging. Telomere length is also associated with environmental and lifestyle factors that influence the oxidative stress and inflammatory status in humans.

DNA was extracted from placental biopsies. For placental telomeres, the triplicates of the telomere runs showed a CV of 0.55%, those of the single-copy gene runs a CV of 0.36%, and those of T/S ratios a CV of 7.6% (Martens et al., 2016).

5 Results

5.1 Estrogenic and (Anti)-Androgenic Screens bioassays (UGR)

5.1.1 E-screen Assay

The total estrogenic xenobiotic burden (TEXB) in alpha fraction ranged from <1 pM Eeq to 68.95 pM Eeq/g of placenta. TEXB-α values were positive (≥LOD) in 100% of placenta samples. TEXB-α values are reported in the table and graphic below.

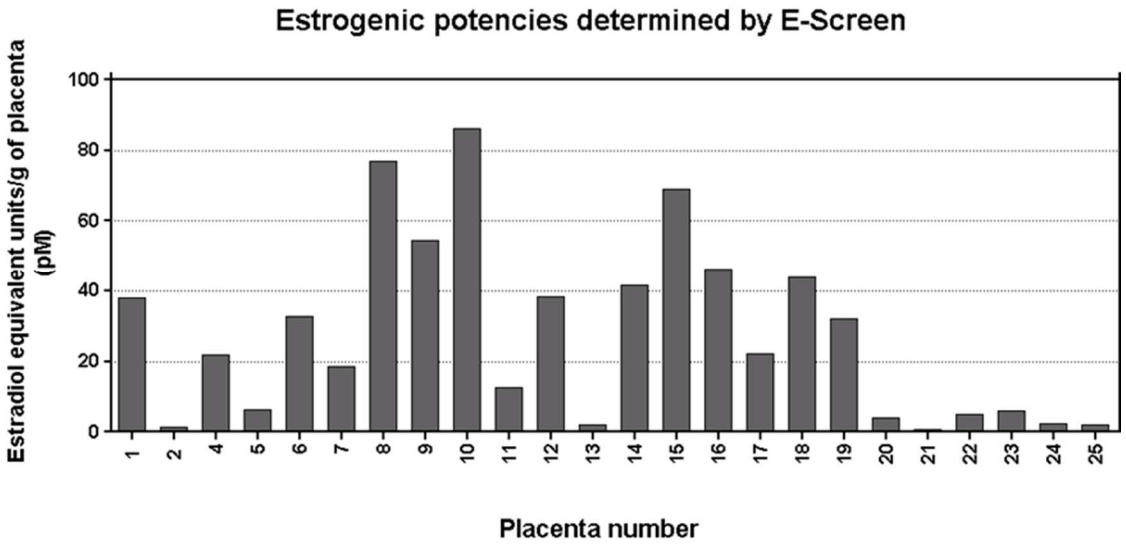

Fig. 5.1.1.1: Results from E-Screen in the 24 alpha fractions from placenta samples

Table 5.1.1.1.: Estrogenicity and anti-estrogenicity of α-fractions in the E-Screen bioassay. (-) Placenta samples without statistically significant hormonal activity. Placenta number 3 was unable to process.

Placenta	E-Screen bioassay	E-Screen bioassay
	Eeq(pM)/g placenta	ICIEq/g (pM)
1	38.10	-
2	1.41	-
4	21,66	-
5	6.36	-
6	32.83	-
7	18.50	-
8	77.00	-
9	54.44	-
10	86.16	-
11	12.51	-
12	38.27	-
13	1.92	-
14	41.61	-
15	68.95	-
16	46.11	-
17	22.05	-
18	43.80	-
19	31.91	-
20	4.08	-
21	0.58	-
22	5.02	-
23	5.92	-
24	2.47	-
25	1.83	-

5.1.2 Luciferase Reported gene Assay

All placenta extracts were unable to activate luciferase expression in PALM cells after 40 h of exposure. Total antiandrigenic xenobiotic burden (TAXB)-α values were negative (<LOD) in 100% of placenta samples.

5.2 Xenoestrogenic transactivity of placenta extracts (AU)

5.2.1 ER Transactivation Assay

5.2.1.1 Assay 1

In this pilot assay, the xenoestrogenic transactivity of dilution of 1/50 was measured.

After adding 44 µl EtOH to each dry placenta sample extract and shaking overnight, most samples were not totally dissolved. We transferred 20 µl of the solution to a brown test tube and further

diluted the placenta ethanol solution 50 times with culture medium to measure the estrogenic transactivity.

The growth of MVLN cells treated with 1/50 dilution was insufficient. Many cells were dead and there were many particles under the microscope. The cells exposed to solvent control (containing up to 2% ethanol) grew adequately, suggesting the cell death was not caused by 2% ethanol. It seems others factors such as substances or particles of the placenta samples are cytotoxic to MVLN cells.

As shown in Figure. 5.2.1.1.2, most samples showed significantly lower ER transactivity compared to solvent control. Sample 2# showed significantly agonistic ER transactivity. The low responses of placenta samples may be related to the cytotoxicity of other substances in the placenta tissue.

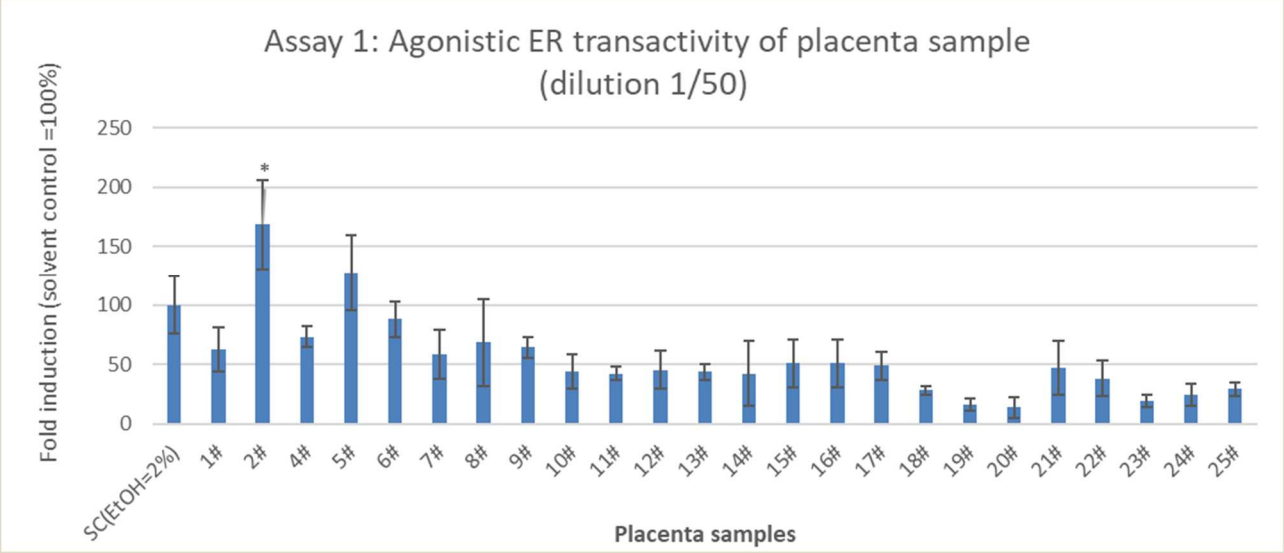

Figure 5.2.1.1.1: The xenoestrogenic transactivity of 1/50 dilution placenta sample alone (XER)

When the MVLN cells treated with the placenta samples in the presence of 25 pM estradiol (E2), almost all samples showed significantly lower ER transactivity compared to 25 pM E2 + solvent control, which may relate to the cytotoxicity (Figure 5.2.1.1.2).

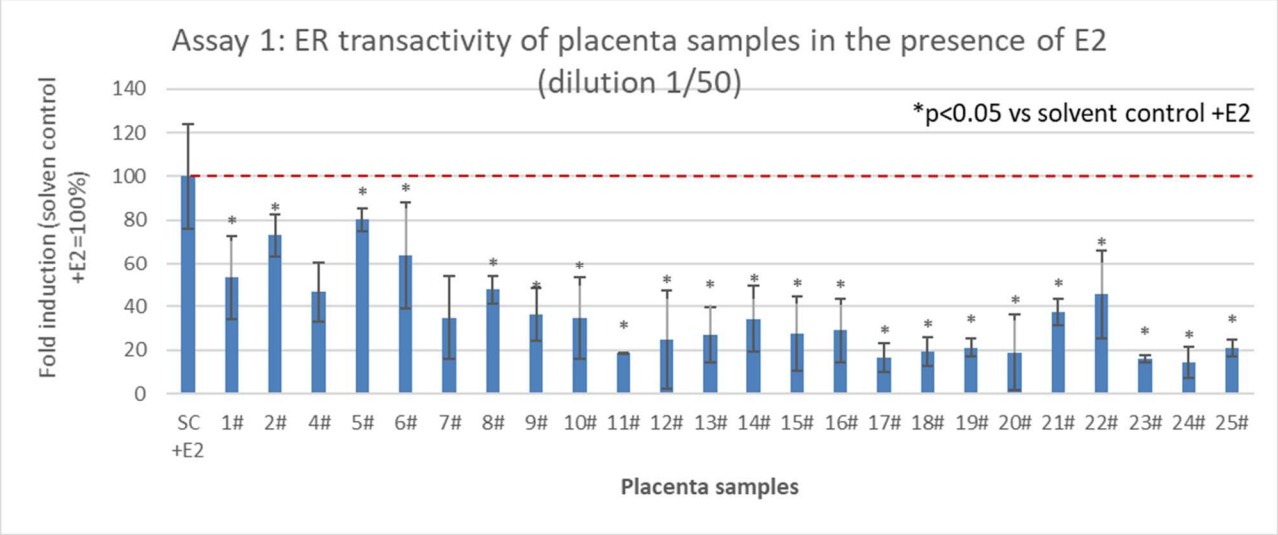

Figure 5.2.1.1.2: The xenoestrogenic transactivity of 1/50 dilution placenta sample in the presence of 25 pM E2 (XERcomp)

From the experience of Assay 1, the ER transactivity can't be measured in the 1/50 dilution of placenta samples, which might because some factors or substances can be toxic to the cells. Therefore, we further ran Assay 2 by measuring the ER transactivity of 1/250 and 1/500 dilution of placenta samples.

5.2.1.2 Assay 2

MVLN cells growth status after exposed to placenta extract and controls

The cells exposed to E2 and solvent control exhibit normal growth (Figure 5.2.1.2.a, 5.2.1.2.b). When placenta samples further diluted to 1:500, cell growth was better than dilution of 1/50, but still elicited toxic to the cells to some extent (Figure 5.2.1.2.c, 5.2.1.2.d).

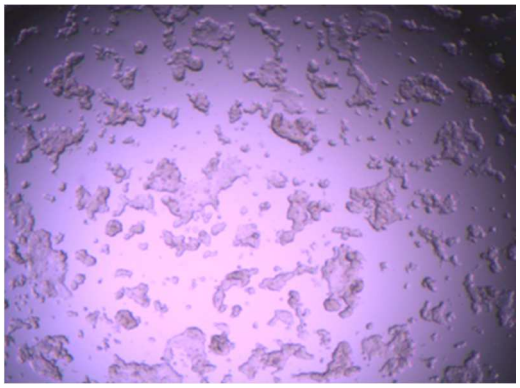

Fig.5.2.1.2.a: Positive control (25 pM E2)

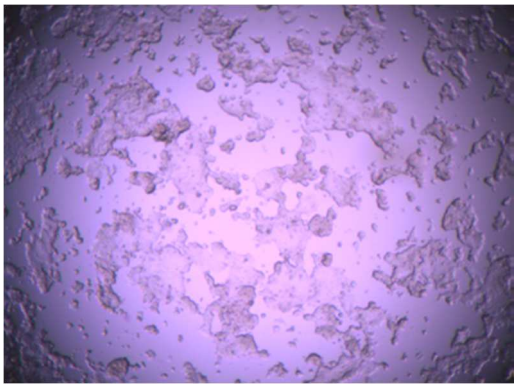

Fig. 5.2.1.2.b: Solvent control (0.18%))EtOH)

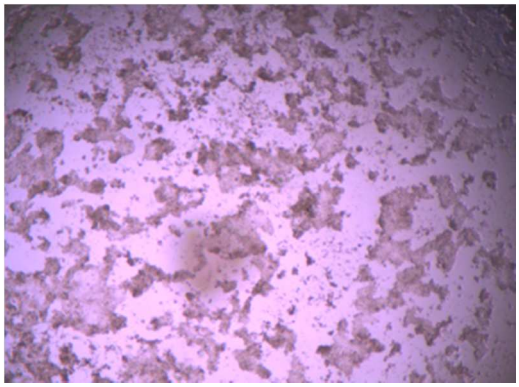

Fig. 5.2.1.2.c: Placenta sample 24#

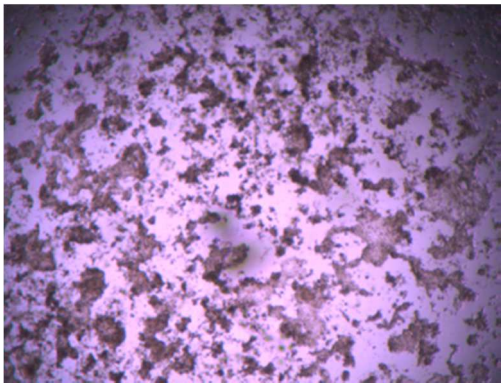

Fig. 5.2.1.2.d: Placenta sample 24# + 25 pME2 (dilution 1/500)

The agonistic ER transactivity (XER)

Xenoestrogenic activity (XER) was determined by exposure of MVLN cells to placenta extracts alone. The solvent control of XER activity were sample solvent only. As shown in Figure 5.2.1.3, almost all placenta samples elicited significantly estrogenic transactivity both in the dilution of 1/250 and 1/500. The fold induction of dilution 1/500 was similar with that of 1/250 dilution for half of samples. Sample 16 #, 20#, 23# and 25 # in the dilution of 1/250 elicited non significant activity. When they were diluted to 1/500, significant estrogenic transactivity were shown (Figure 5.2.1.3).

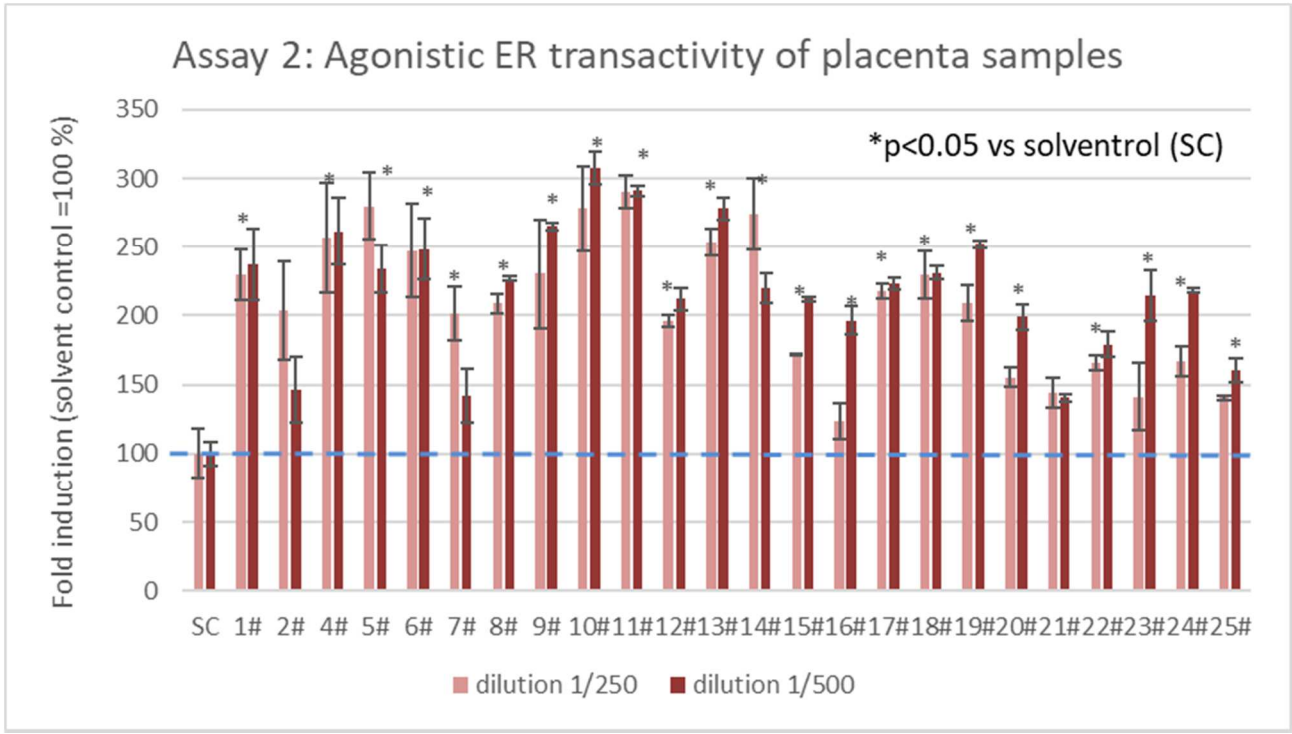

Figure 5.2.1.3: ASSAY-2: The xenoestrogenic transactivity of 1/250 and 1/500 dilution placenta sample alone (XER)

Competitive estrogenic activity (XERcomp) of placenta samples

XERcomp activity was measured upon co-exposure of MVLN cells to placenta extract of 1/500 dilution in the presence of 25 pM E2. As shown in Figure 5.2.1.4, most of samples elicited lower transactivity compared to that of 25 pM E2. Sample 16# significantly inhibited effect of E2 transactivity suggesting anti-estrogenicity.

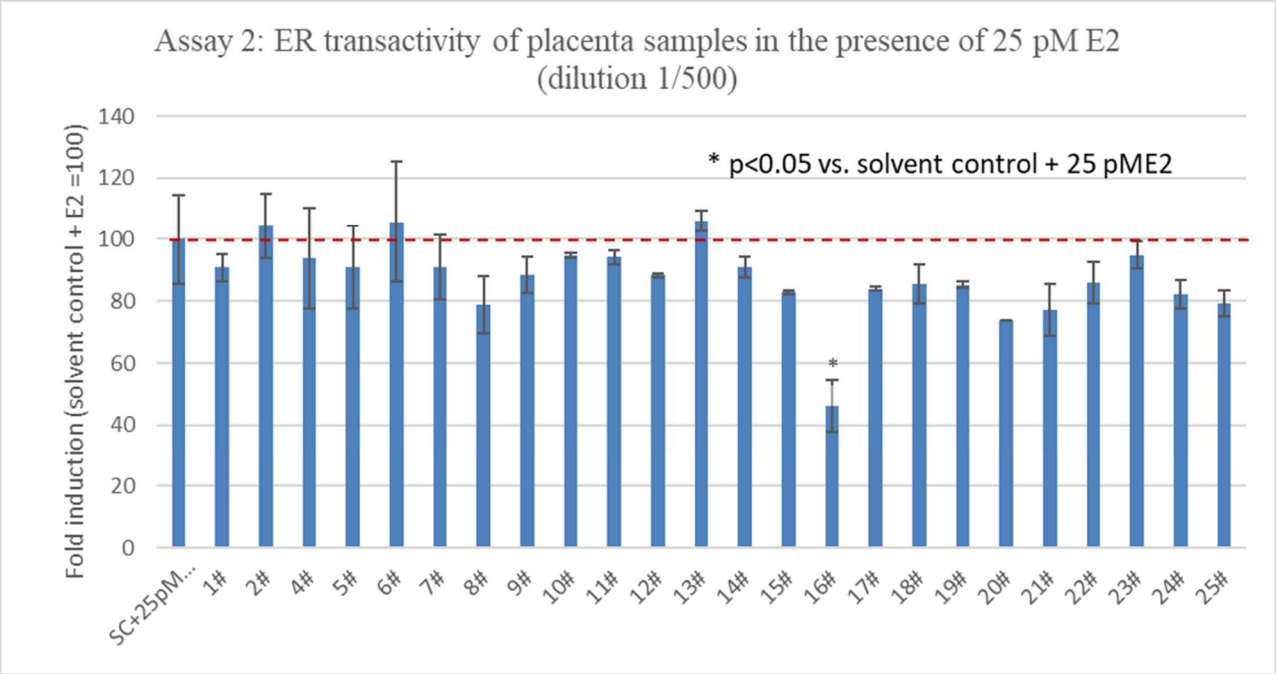

Figure 5.2.1.4: The xenoestrogenic transactivity of 1/500 dilution placenta sample in the presence of 25 pM E2 (XERcomp)

5.2.1.3 Comparison of Assay 1 and Assay 2

It is shown that the dilution factor influence the response of ER transactivity. As shown in Figure 5.2.1.3 A, the lowest response was found in the dilution 1/50, which might relate to its possible cytotoxicity. There is no big difference for the response of dilution 1/250 and 1/500, suggesting the response of these dilutions might reach the plateau.

Upon co-treatment with ER ligand, 17β-estradiol, the placenta sample at 1/50 dilution showed lower response compared to the solution of 1/500 (Figure 5.2.1.3 B). This further suggests some substances in the placenta extract might be toxic to MVLN cells. Therefore, it is important to find out the optimal solvent and optimal dilution factor.

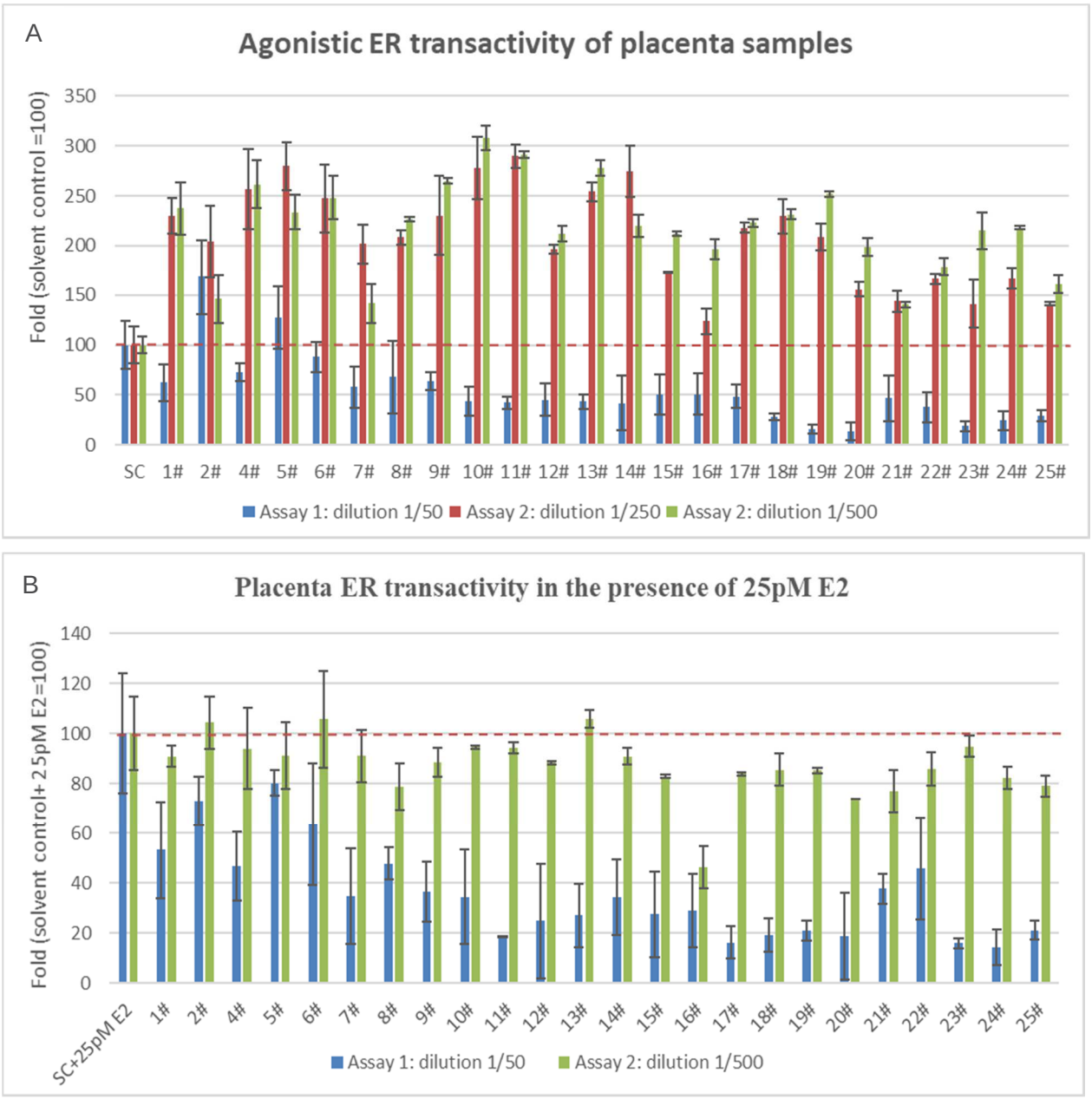

Figure 5.2.1.3: Response of different dilutions of placenta samples alone (A) and in the presence of E2 (B)

5.2.2 AR Transactivation Assay.

Viabilty of the cells was assessed using a microscope. Cells exposed to the placenta extracts showed some cytotoxicity (figure 5.2.2.1). The cells exposed to the highest concentration of placenta extracts (Dilution 1) were clearly damaged, and some of the samples were toxic in dilution 2 (1/500) as well. In Dilution 3 (1/1000), the cells looked more healthy, but still with some apoptotic and dying cells.

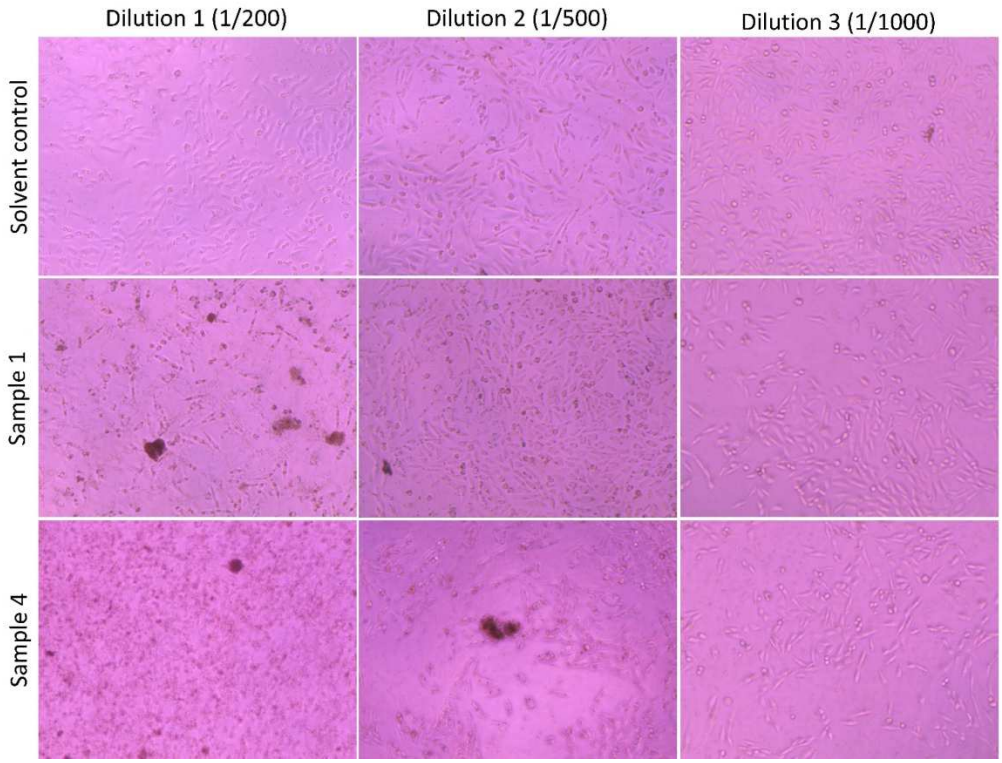

Figure 5.2.2.1: Pictures of the cells exposed to solvent control, sample #1 and sample #4 in the three different dilutions without DHT. The pictures are taken just before harvest.

The results from the AR transactivity assay with samples tested alone are shown in figure 5.2.2.2, No luciferase activity was observed in the cells exposed to the highest concentration of the placenta extracts (Dilution 1 (1/200)). For Dilution 2, the androgenic transactivities were also low for most of the samples (2.6–57.3 % of the solvent), but three samples (#2, #5, and # 6) were non-significantly higher than the solvent control (103.6–132.6% of the solvent control). At the lowest concentration of the placenta extracts tested (Dilution 3 (1/1000), 14 samples were significantly below the solvent control (19.9–66.8% of solvent control), 7 samples were non-significantly different from the solvent control (80.9–119.1% of the solvent), and 3 samples were significantly higher than the solvent control (136.2–157.9% of the solvent control) (figure 5.2.2.2).

For all samples, a dose-response trend was found, being the lowest androgenic transactivity at the highest concentrations (Dilution 1 (1/200)) (figure 5.2.2.2). This could indicate that the dose-response is due to the toxicity of the samples, and not due to androgenic transactivity.

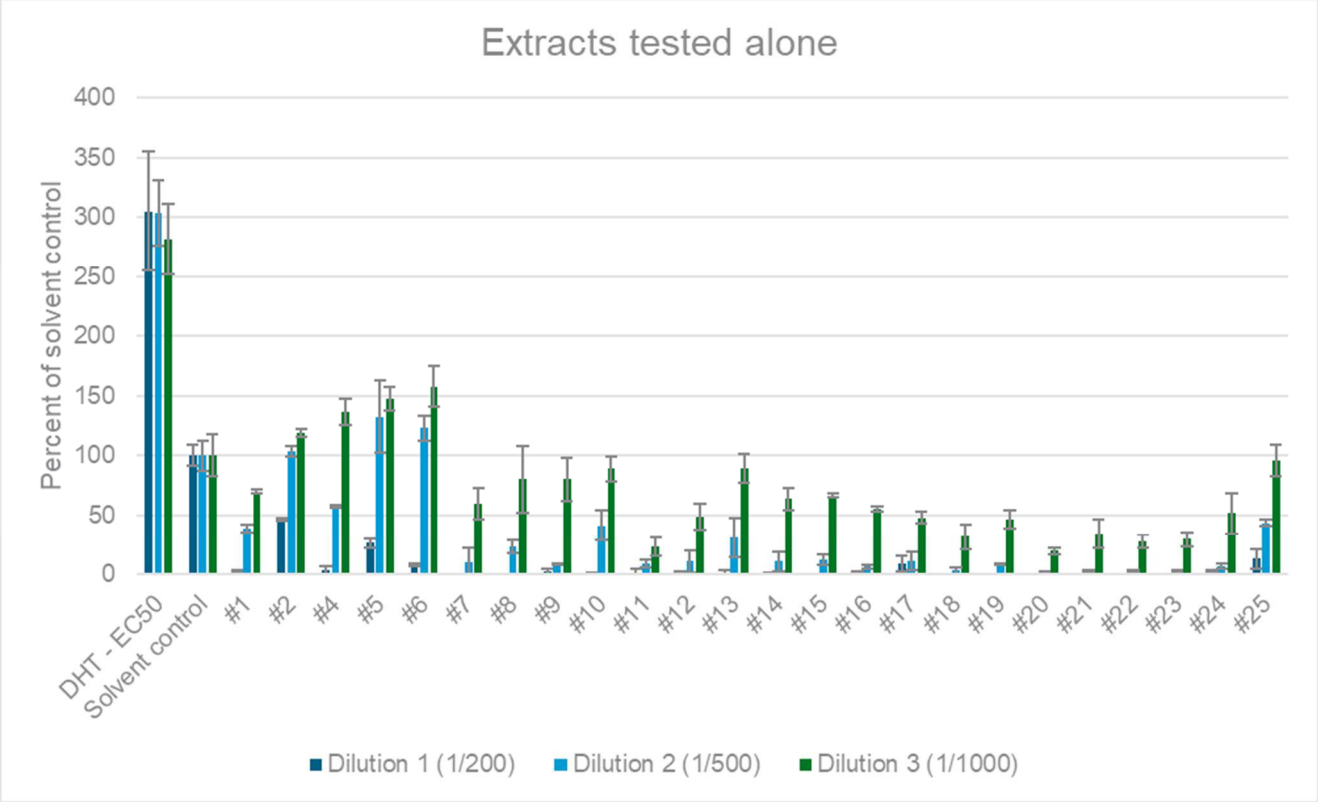

Figure 5.2.2.2: Results from the testes with extracts alone in three dilutions (Dilution 1 (1/200), Dilution 2 (1/500), and Dilution 3 (1/1000)). The solvent control for each dilution were set to 100 %.

Additionally, the anti-androgenic transactivity of the samples was tested in the presence of DHT (12 pM) (figure 5.2.2.2). As in the tests without DHT, the highest concentrations of placenta extracts (Dilution 1) were toxic to the cells, and we did not observe any androgenic transactivity. For Dilution 2 (1/500), all the samples were below the solvent control + DHT (1.99–37.9 % of solvent control + DHT). In Dilution 3 (1/1000), the lowest tested concentration of the placenta extracts, the androgenic activity ranged from 9.1–65.7% of the solvent control (figure 5.2.2.2). The results may indicate a strong antagonizing effect of the placenta extracts on the AR, but with the present results, it is not able to eliminate the possibility that the low androgenic responses were due to cytotoxicity of the cells or inhibition of the transfection.

5.3 Aryl Hydrocarbon receptor expression (AhR) assay (DTU)

5.3.1 AhR activity

TCDD gave comparable results between the two experimental runs in the AhR reporter gene assay with maximum efficacy of 11.0 and 8.7 and EC_{50} of 24.1 and 64.5 pM for experiment 1 and 2, respectively (Figure 5.3.1). The variations are within the normal range of this assay for TCDD.

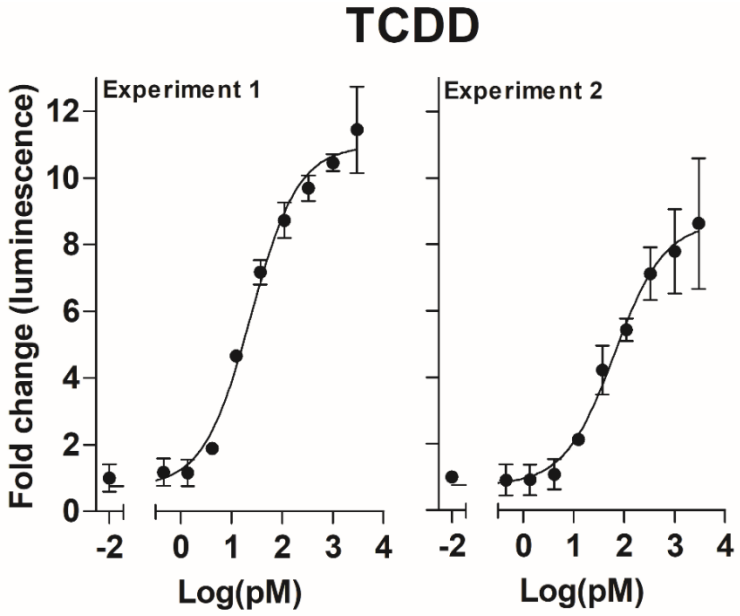

Figure 5.3.1: Concentration-response curves for the positive control, TCDD, from the two independent experiments. Data presented as mean ± SD. The four parameter curve was fitted in Graph Pad Prism 5 and maximum efficacy and EC_{50} values are derived from this curve.

An increased activity was observed for placenta extract 1, 8, 14, and 20 in both independent experiments (Figure 5.3.2). The increase was concentration dependent for extract 1 and 8 in experiment 2. For several extracts a decreased activity was observed across both independent experiments. This included placenta extracts 6, 12, 13, 18, 24, and 25.

5.3.2 Cell viability

The cell viability with exposure to the placenta extracts varied between the two independent experiments (Figure 3). In experiment 1 none of the extracts led to a decreased response of more than 0.9 of controls and no extracts affected cell viability. In experiment 2 three placenta extracts led to decreased cell viability by more than 25%, indicated with the 0.75 dotted line in Figure 5.3.3. These were placenta extract 5, 8, and 11. Overall, no extracts caused cytotoxicity at the two lowest test dilutions in none of the experiments.

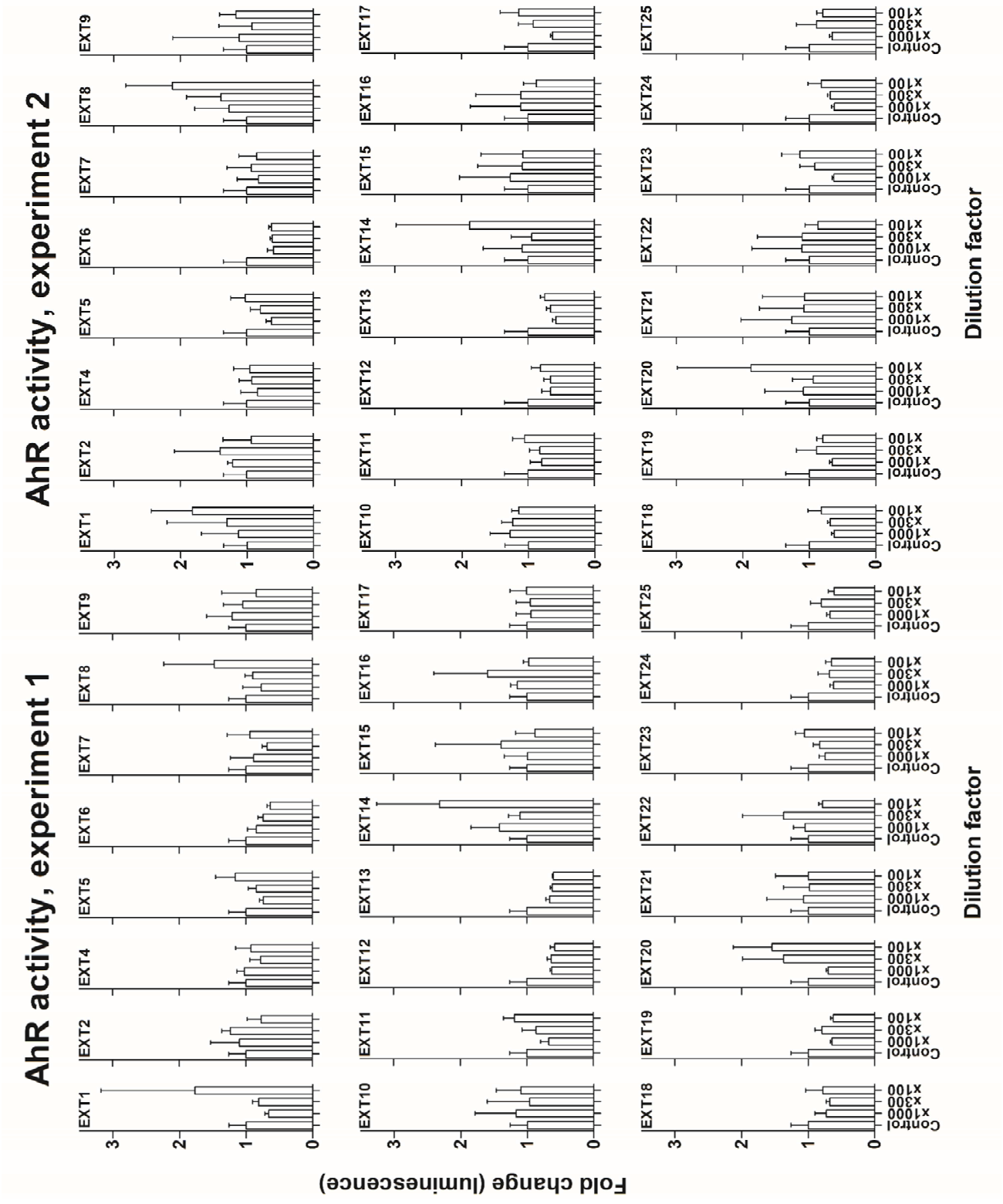

Figure 5.3.2: AhR activity of placenta extract dilutions of 1000, 300, and 100 times equivalent to 0.3 $mg_{placenta}$/well, 1 $mg_{placenta}$/well, and 3 $mg_{placenta}$/well, respectively. Data are from two independent experiments and presented as mean ± SD. All placenta dilution were tested in triplicates and normalized to medium controls pooled from all plates (n=24).EXT=extract.

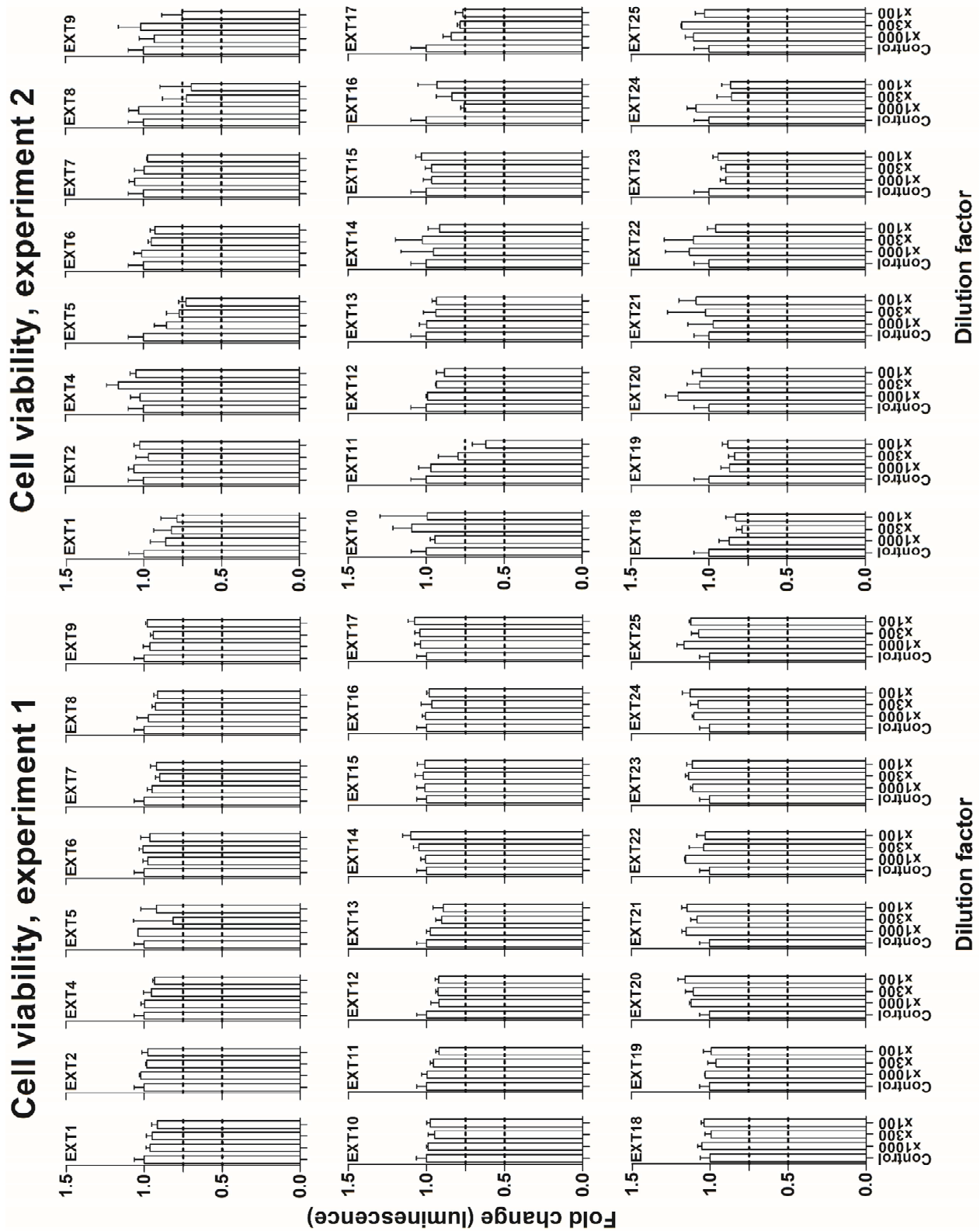

Figure 5.3.3: Cell viability of placenta extract dilutions of 1000, 300, and 100 times equivalent to 0.3 $mg_{placenta}$/well, 1 $mg_{placenta}$/well, and 3 $mg_{placenta}$/well, respectively. Data are from two independent experiments and presented as mean ± SD. All placenta dilution were tested in triplicates and normalized to medium controls pooled from all plates (n=24).EXT=extract.

5.4 Xenopus Embryonic Thyroid-signalling Assay (XETA) (CNRS)

T_3 induced a significant increase of fluorescence compared to the control after 72h of exposure, ranging from 214% and 224% for experiment B and D up to 319% and 335% for experiments A and C, respectively (figure 5.4.1).

A statistically significant difference between the T3 group and several T3-spiked α-extracts was determined in every experiment, however stronger antagonistic effects on T3 signaling were found in experiments A and C which feature a higher T3 induction of fluorescence (figure 5.4.1). To take into account these variations across experiments, the reduction of fluorescence by each T3-spiked α-extract was divided by the level of fluorescence induced by T3 in each experiment (figure 5.4.2).

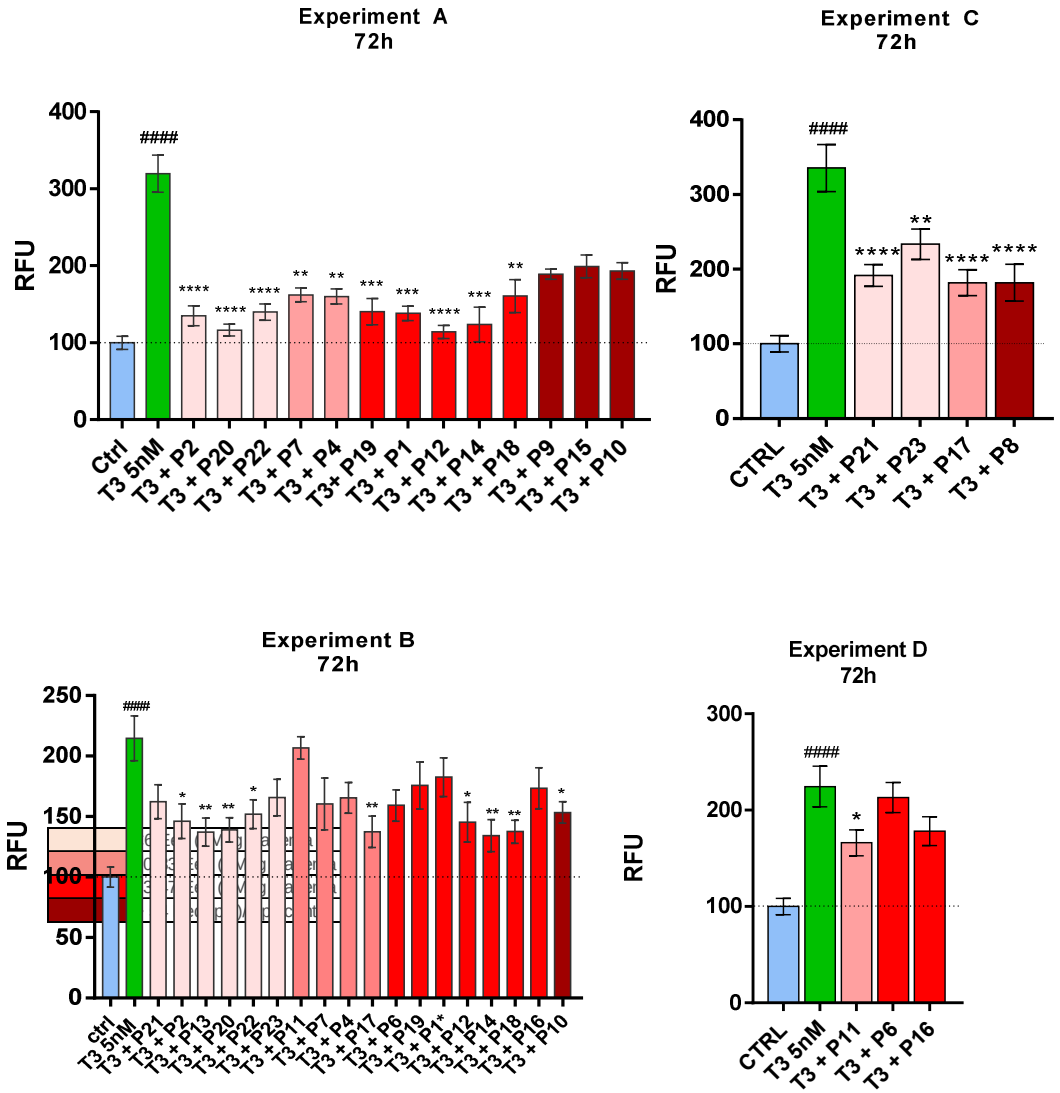

Figure 5.4.1: Thyroid disrupting activity of α-extracts from placenta samples assessed with four XETA experiments at 72 hours of exposure.

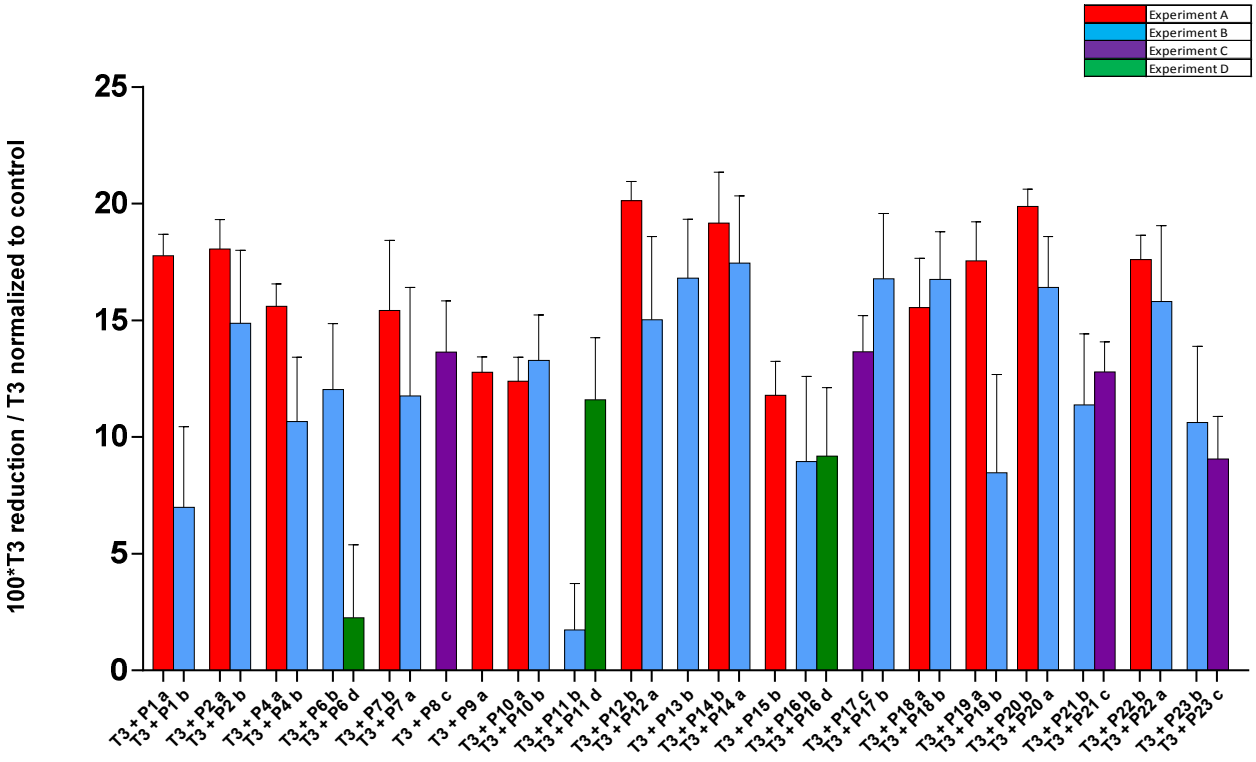

Figure 5.4.2: Reduction of T3-induced fluorescence by α-extracts from placenta samples assessed with four XETA experiments at 72 hours of exposure weighed by experiment-specific levels of T3 induction.

5.5 Oxidative stress biomarkers of effect (MU)

Concentrations of measured OH-dG in placenta samples are presented in Table 5.5.1

Table 5.5.1: 8-OHdG level present in placenta samples

Sample	Placenta samples n=26		
CODES	8-OHdG /10^5 2dG (molar ratio)	8-OHdG ng /g tissue	8-OHdG ng / mg DNA
1	0.90	1.37	2.84
2	1.69	1.90	4.40
3	1.50	1.29	4.09
4	1.14	1.06	3.10
5	1.94	1.62	4.01
6	1.19	0.63	2.31
7	2.14	2.11	6.27
8	1.70	1.30	4.01
9	1.52	1.30	3.61
10	1.42	1.17	3.15
11	1.50	2.10	3.90
12	1.10	1.25	3.15
13	0.98	0.74	1.43
14	1.48	1.49	3.34
15	1.71	1.73	4.72
16	1.81	1.28	4.69
17	0.47	0.77	1.75
18	1.27	1.07	4.75
19	2.05	1.94	3.17
20	1.21	0.84	3.18
	1.88	1.08	4.25
21	1.93	1.79	7.34
22	2.05	2.58	4.86
23	1.14	1.19	3.82
24	2.52	3.62	4.80
25	1.32	1.22	3.88

5.6 Telomere length (UH)

In the proof of concept including 25 placental samples we successfully measured telomere length. The average relative telomere length was 1.02 and ranged from 0.51 to 1.75 in placental tissue. See the figure below:

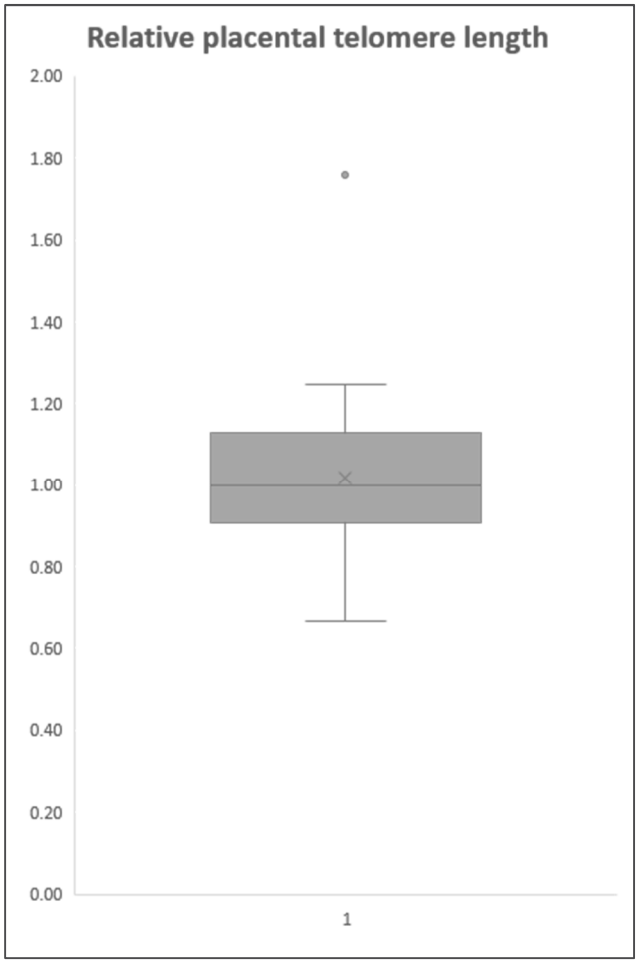

Figure 5.6: Determination of the relative placental telomere Length from placental biopsies

6 Conclusions

Different ex vivo cell-based bioassays can be used to assess the combined effect of chemical mixtures isolated from placenta samples providing relevant information on specific signalling pathways of estrogenic, anti-androgenic, anti-thyroid, and AhR activity systems. For some novel bioassays tested, additional optimisation will be required. Given the need to evaluate the possible harmful effects of chemical mixtures on human health, the use of biomarkers of combined effect could add value to HBM programs.

In the present Deliverable D14.4, human placenta was the biological matrix of choice, mainly due to the non-invasive availability of large samples (≈ 500 g), allowing the UGR to send aliquots of placenta homogenates, placenta pieces and/or placenta extracts to several HBM4EU partners for performing different biological analyses in the same samples. Results for the bioassays and biomarkers of combined effect, using the same biological matrix, showed that:

1. The use of E-Screen to measure the estrogenicity of placenta samples appears to be a reasonable strategy to assess maternal exposure to xenobiotics and to estimate exposure of the fetus, and it may be more valuable as a biomarker of effect than the study of individual residues.
2. With regard to the androgen activity measured in the placenta, it is difficult to draw conclusions on the transactivity levels of the extracts. We could not determine whether the low androgenic transactivity response observed in the samples was due to actual anti-androgenic effects of the extracts, to cytotoxicity of the cells, or to inhibition of the transfection. In future studies, extracts should be tested at even lower concentrations, and the cytotoxicity of the cells should also be tested. AU recommends sending a recommended extract dissolution protocol together with the extracts for future experiments.
3. Four out of twenty-four placenta extracts (nº 1, 8, 14 and 20) induced AhR activity. However, one of these (no. 8) showed compromised cell viability of more than 25% at AhR receptor-activating concentrations. In addition, six out of twenty-four placenta extracts inhibited AhR activity.
4. The T3-induced increase in fluorescence in the XETA was significantly reduced by exposure to α-extracts obtained from human placentas, pointing to an antagonistic effect of thyroid hormone signalling, even at our high dilution factor of the α-extracts. While no correlations between XETA and E-screen results were found, chemical analysis of the α-extract could reveal other correlations. These results support the future use of a wider range of α-extract concentrations relevant to human exposure. Gene expression analysis could clarify the effect on the HPT axis. Given the known adverse effects of disruption of this axis on brain development, mRNA expression of biomarkers of relevance for human neurobehaviour & development could be investigated as complementary to behavioural studies on mobility.
5. The proposed integrative oxidative stress biomarkers have enormous potential to complement both (i) information on exposures and (ii) responses of other – perhaps more specific – biomarkers.
6. Telomere length is a long-term effect biomarker that integrates potential effects over time. Its relevance for DOHaD and public health is evident, given that telomere length at birth has been shown to predict adult telomere length (Bijnens et al 2017). Precaution must be taken, because the DNA must be freshly extracted.

Given the need to evaluate the possible harmful effects of chemical mixtures on human health, the validation of biomarkers of combined effect could add value to HBM programs. The methodology developed in this proof of concept will be applied to non-invasive human samples and matrices employed in HBM, such as serum and urine.

7 Future Steps

Deliverable D14.4 constitutes the first attempt within Work Package 14 to explore the implementation of effect biomarkers of combined effect to chemical mixtures, with the same biological samples being studied by different HBM4EU partners. The continuation of this work will be reported in Additional Deliverable AD14.4 ‘‘First report on the state of development of novel biomarkers of effect’’.

This last deliverable devoted to placenta extracts will include:

- Assessment of the hormonal profile from placental samples (INRA)
- Assessment of persistent lipophilic chemicals present in the alpha-fraction (UGR).
- A non-targeted chemical analysis of organohalogenated substances in placenta extracts in order to identify preliminary exposure-activity relationships (CEA).
- Fine-tuning of an LC-MS methodology to assess 8OHdG concentrations in urine samples by MU, and assessment of 8OHdG concentrations in the same urine samples by UGR for inter-laboratory comparisons. Urine samples were gathered during different trimesters of pregnancy and paired with the placental samples used in the current D14.4 (by MU and UGR).
- Testing of gene expression, epigenetic and omics biomarkers in the 25 placenta samples, done by INSERM. Some of the experimental techniques to be developed include:
    i. Untargeted metabolomics using high-resolution mass spectrometry,
    ii. Gene expression analyses,
    iii. OMICS approach,
    iv. Gene sequencing,
    v. Western blot to assess proteins expressed in the placenta,
    vi. DNA damage,
    vii. Micro-RNA expressions in placentas,
    viii. Analyses of xenoestrogenic potential of placental extracts using transgenic zebrafish with aromatase enzymatic activity in placenta;
    ix. Analyses of certain metals (As, Cd, Cu, Mn, Pb, Ms, Se, Zn), to assess preliminary trends,
    x. Preliminary test specifically for metabolomics testing of bioassay sensitivity,
    xi. Recording of placenta metabolomics and identification of xenobiotics that will lead to the identification of prenatal exposure (INSERM).

A natural follow-up of this work on combined effect biomarkers applied to chemical mixtures would be:

a) To apply these biomarkers of proven utility to other biological matrices more frequently used in HBM studies, such as serum and urine

b) To explore the isolation of specific chemical families from human samples, i.e., the chemical isolation of PFAS compounds from placenta and serum samples, in order to test their combined biological activities, which will be of a major interest for both HBM and risk assessment.

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