Food and Chemical Toxicology 161 (2022) 112842

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

High throughput screening of bisphenols and their mixtures under conditions of low-intensity adipogenesis of human mesenchymal stem cells (hMSCs)

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ARTICLE INFO

Handling Editor: Dr. Jose Luis Domingo

Keywords:
Adipogenesis
Bisphenol
Mesenchymal stem cell
Endocrine disrupting chemicals (EDCs)
High content screening
Obesity

ABSTRACT

In vitro models of adipogenesis are phenotypic assays that most closely mimic the increase of adipose tissue in obesity. Current models, however, often lack throughput and sensitivity and even report conflicting data regarding adipogenic potencies of many chemicals. Here, we describe a ten-day long adipogenesis model using high content analysis readouts for adipocyte number, size, and lipid content on primary human mesenchymal stem cells (MSC) sensitive enough to compare bisphenol A derivatives quantitatively in a robust and high throughput manner. The number of adipocytes was the most sensitive endpoint capable of detecting changes of 20% and was used to develop a benchmark concentration model (BMC) to quantitatively compare eight bisphenols (tested at 0.1–100 μM). The model was applied to evaluate mixtures of bisphenols obtaining the first experimental evidence of their additive effect on human MSC adipogenesis. Using the relative potency factors (RPFs), we show how a mixture of bisphenols at their sub-active concentrations induces a significant adipogenic effect due to its additive nature. The final active concentrations of bisphenols in tested mixtures reached below 1 μM, which is within the concentration range observed in humans. These results point to the need to consider the toxicity of chemical mixtures.

1. Introduction

Obesity poses an increasing health problem that has reached the level of pandemics. It is associated with various other co-morbidities like type 2 diabetes, cardiovascular diseases, and musculoskeletal disorders like osteoarthritis and some types of cancers (WHO, 2020). While main factors contributing to obesity are defined as the lack of physical activity, high-calorie diet and genetic factors, it is increasingly being recognized that some chemicals in our surroundings, that we are constantly exposed to in our modern life, can contribute to the development of obesity (Franks and McCarthy, 2016; Legler et al., 2020). This is mainly claimed to happen through the interaction of chemicals with nuclear hormone receptors, introducing a disbalance in the hormonal system, leading to the classification of such compounds as endocrine disrupting chemicals (EDCs). So far, many chemicals have been reported to affect various systems in the body regulated by hormones, such as reproductive, neurodevelopmental, thyroid system, and energy metabolism. Metabolism disrupting chemicals (MDCs) contributing to the excess development and growth of adipocytes and adipose tissue in the whole organism were also named obesogens (Heindel and Blumberg, 2019). Chemicals showing a stimulating effect on adipogenesis (e.g., *in vitro*) are known as adipogens.

In contrast to signalling pathways from estrogen and androgen receptors, as well as thyroid- and steroidogenesis-related activities (known together as EATS mechanisms), affecting mainly the reproductive system and organism development, current regulatory risk assessment has no mandatory assays to test new chemicals on obesogenicity or adipogenicity and several other important endocrine endpoints (OECD, 2018, ECHA/EFSA/JRC et al., 2018). Current European Horizon 2020 research project GOLIATH aims to deliver new possibilities for regulatory toxicity testing of new chemicals that would involve the assessment of their metabolic disruption effects, including adipogenicity (Legler et al., 2020).

Bisphenol A (BPA) is a chemical with many applications, from the production of plastic items (bottles, food packaging) to metal coatings

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https://doi.org/10.1016/j.fct.2022.112842
Received 2 August 2021; Received in revised form 13 January 2022; Accepted 25 January 2022
Available online 31 January 2022

Abbreviations

3T3-L1	murine preadipocyte cell line
ADIPOQ	adiponectin
AIC	Akaike Information Criterion
BMC	benchmark concentration
BMCL	lowest limit of benchmark concentration confidence interval
BMCU	upper limit of benchmark concentration confidence interval
BM-MSC	bone marrow derived mesenchymal stem cells
BMP	bone morphogenetic protein
BPA	bisphenol A
BPAF	bisphenol AF
BPB	bisphenol B
BPC	bisphenol C
BPF	bisphenol F
BPS	bisphenol S
CAR	constitutive androstane receptor
CEBPα	CCAAT enhancer binding protein alpha
CED	critical effect dose (concentration)
CES	critical effect size
CI	confidence interval
CSS	charcoal stripped serum
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxid
DPBS	Dulbecco's phosphate balanced saline
EDC	endocrine disrupting chemical
EF1A	elongation factor 1-alpha
EFSA	European Food Safety Authority
ER	estrogen receptor
ERR	estrogen related receptor
FABP4	fatty acid binding protein 4
FBS	fetal bovine serum
GR	glucocorticoid receptor
HCS	high content screening
hMSC	human mesenchymal stem cells
IBMX	isobutylmethylxanthine
LPL	lipoprotein lipase
LXR	liver X receptor (oxysterols receptor)
mBM-MSC	murine bone marrow mesenchymal stem cells
MDC	metabolism disrupting chemical
MSC	mesenchymal stem cell
NOEC	no observed effect concentration
PPARγ	peroxisome proliferator activated receptor gamma
PR	progesterone receptor
PXR	pregnane X receptor
ROSI	rosiglitazone
RPF	relative potency factor
RT-PCR	real time polymerase chain reaction
SD	standard deviation
TBBPA	tetrabromobisphenol A
TBT	tributyltin chloride
TCBPA	tetrachlorobisphenol A
TR	thyroid hormone receptor

and thermal receipts. It is one of the most widely studied obesogens, with reported adipogenic and obesogenic effects from *in vitro, in vivo* and epidemiological studies (Wu et al., 2020, Rubin, Schaeberle and Soto, 2019). Its endocrine disrupting effects led to significant restrictions of its use to reduce the constant human exposure. This, in turn, led to increased effort from the chemical industry to replace bisphenol A in their products. While shops are filling up with BPA-free claimed products, without a mandatory regulatory toxicity test for adipogenicity or obesogenicity and several other endocrine adverse outcomes, the replacement chemicals are unfortunately not sufficiently tested before reaching the market even to deserve the title of being any better than the chemical they are aiming to replace. Indeed, some BPA replacement derivatives on the market were already found to exert adipogenic and obesogenic effects similar to BPA (Ji et al., 2021; Andújar et al., 2019). In perinatal and chronic exposure, bisphenol S (BPS) induced overweight, hyperinsulinemia and hyperleptinemia in male mice offspring (Ivry Del Moral et al., 2016). The systematic analysis of available regulatory and academic human epidemiological and *in vivo* studies on BPS indicated many similarities and from some aspects even worse toxicological profile compared to BPA (Beausoleil et al., 2021). Bisphenol B (BPB), bisphenol C (BPC), bisphenol AF (BPAF) and a few other BPA substitutes were, like BPA, highly potent in activating several nuclear receptors (constitutive androstane receptor (CAR), glucocorticoid receptor (GR), estrogen receptor α (ERα), estrogen receptor β (ERβ) and estrogen-related receptor γ ERRγ) (Liu et al., 2019), some of which also play a role in adipogenesis (Lee, 2017). Although with variable potencies, adipogenic effects in *in vitro* models have been reported for BPA, bisphenol S (BPS), bisphenol F (BPF), BPB and tetrabromobisphenol A (TBBPA) (Andújar et al., 2019; Ramskov Tetzlaff et al., 2020; Riu et al., 2011). The consequences of the lack of full regulation in this area show the uttermost need for a reliable screening method to quantitatively compare new chemicals aimed to replace the old ones, shown to induce adverse effects.

The process of adipogenesis consists of two main phases: the commitment of cells to adipocyte lineage turning stem cells into preadipocytes; and the differentiation of preadipocytes into adipocytes (Lee, 2017). Current *in vitro* research methods capable of detecting potential obesogens mainly involve murine 3T3-L1 preadipocyte cell line, which can be differentiated into adipocytes *in vitro*. Apart from being of non-human origin, which may result in species-related differences in the assessment of adipogens, the main disadvantage of 3T3-L1 cells is that they differ between batches from different vendors, making them difficult to define as a universal test system (Kassotis et al., 2017). Other cells used as *in vitro* systems for adipogenesis include human and rodent primary cells: mesenchymal stem cells (MSCs) from bone marrow or adipose tissue and preadipocytes (Nunes et al., 2018). The so far reported detection methods almost exclusively rely on fluorimetry readouts of total fluorescence of fluorescently labelled lipid droplets as a measure of adipogenesis. Since these assays are most often performed in 24-well format, they mainly have medium to low throughput.

The high content analysis offers immense possibilities to markedly increase the sensitivity of these methods since image analysis can be directed to measure the fluorescence of particular structures within the cell (e.g. lipid droplet, nucleus) (Kamstra et al., 2014), which can greatly reduce the background noise and the impact of non-specific staining found in classical total fluorescence measurements. In addition, image analysis makes it possible to distinguish between an increase in adipose mass due to an increase in adipocyte recruitment (adipocyte number) or in adipocyte cell size. These two different fates of storing excess lipids have been shown to have a profound effect on the whole organism, where adipocyte hypertrophy (increase in adipocyte size) is linked to insulin resistance and inflammation further contributing to metabolic syndrome, whereas adipocyte hyperplasia (increase in adipocyte number) characterizes a healthier way of managing excess lipids in the organism (Stenkula and Erlanson-Albertsson, 2018; Longo et al., 2019). Still, it was shown that, on average, obese people have more adipocytes than lean people, and their number cannot be reduced (Spalding et al., 2008). Therefore, even increasing the number of adipocytes in

conditions where no new adipocytes are needed (e.g. in exposure to obesogenic chemicals) creates a cell depot in the body, ready to easily accumulate lipids, which can contribute to quicker weight gain.

Mesenchymal stem cells are pluripotent cells that in the body differentiate into adipocytes, osteoblasts, chondrocytes, and myocytes (Pittenger et al., 1999). They can be isolated from bone marrow or various target tissues for their differentiation. In addition, they offer a very reliable source of cells as they can be cultured and propagated for a much longer time than most other primary cells, which enables the use of the same batch over a few years. The process of MSC differentiation into adipocytes *in vitro* includes both commitment and differentiation phases of adipogenesis and usually requires 2–3 weeks of differentiation.

Adipogenic effects of BPA have been studied in human MSCs and several related cell types. Its reported effects are usually of lower intensity and are sometimes not even detectable (Andrews et al., 2020). When detected, active concentrations of BPA are most often observed at medium micromolar concentrations (Nunes et al., 2018) but in some reports even reach low nanomolar concentrations (Dong et al., 2018). Many conflicting data make the comparisons of adipogenic potencies across the studies difficult, and comparative studies on bisphenols within one model in the same study are rare. A systematic assessment of these *in vitro* models is needed to define robust experimental conditions that could provide strong grounds for successful *in vitro/in vivo* correlation studies and also deliver practical answers for the reproducible assessment of a multitude of chemicals on the market.

The aim of this study was to develop a quantitative *in vitro* method using high content analysis on primary human cells that can be used for screening of adipogenic effects of chemicals and their mixtures and apply this method for a quantitative comparison of bisphenol A to its most prominent replacement chemicals and structural analogues. In the first part, we describe the development of the method, where we used three known obesogens: rosiglitazone, tributyltin chloride and bisphenol A to direct the method development to increase its sensitivity using shortened 10-day differentiation protocol. In the second part, we show how this method was applied in the quantitative assessment of adipogenic effects of bisphenol A derivatives and their mixtures.

2. Methods

2.1. Test compounds

Rosiglitazone (ROSI), tributyltin chloride (TBT), bisphenol A (BPA), bisphenol AF (BPAF), bisphenol B (BPB), bisphenol C (BPC), bisphenol F (BPF), bisphenol S (BPS), tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) were purchased from Sigma (CAS and product numbers and purity of test compounds are listed in the supplementary information, Table S1), and stock solutions were prepared in DMSO (Sigma) at 1000x higher concentrations than test concentrations and kept at −20 °C. Dexamethasone (Sigma) was dissolved in DMSO at 1 mM and IBMX (Sigma) at 50 mM in 0.1 M NaOH. Insulin was purchased from Sigma as 10 mg/mL solution.

2.2. Cell culture and differentiation

Human bone marrow-derived mesenchymal stem cells (hMSCs) were purchased from PromoCell in passage 2 and cultured in MSC growth medium 2 (PromoCell) with 10% Supplement Mix (PromoCell) according to manufacturer's instructions. Briefly, upon reaching 70–90% confluency, cells were rinsed with room temperature HEPES Buffered Saline (PromoCell) and allowed to detach with room temperature Accutase solution (PromoCell) 1 mL per 75 cm^2 for 2–5 min. Then, one millilitre of cell culture medium was added, and cells were counted using a hemocytometer, plated at the density of 2000–4000 cells/cm^2 and passaged again after 3–4 days with medium change after 2–3 days. Cells were used in experiments in cell passages 5–8. Cells from 4 donors (2 males and 2 females, all >60 years old) have been used in experiments.

For differentiation into adipocytes, hMSCs were plated in the inner wells of black-walled 96-well plates with microclear bottom (CellStar, Greiner-BIO One) at the density of 4200 cells/well in 100 μL of plating medium consisting of DMEM with high glucose, Glutamax, phenol red, no pyruvate (Gibco, Prod. No. 61965) supplemented with 10% heat-inactivated FBS (Gibco). Sterile DPBS (200 μL) was added to the outer wells. After reaching confluency in 72 h, the medium was gently replaced with 180 μL/well of differentiation medium consisting of DMEM supplemented with 10% CSS (Charcoal Stripped Serum, Gibco, not heat inactivated), 1 μg/mL insulin, 0.25 μM dexamethasone and 0.5 mM IBMX. Medium was replaced after 3 and 7 days of differentiation and cells were stained and fixed on day 10.

In some experiments during method development, FBS was used instead of CSS in the differentiation medium, medium volume per well was 100 μL instead of 180 μL and the experiment was completed on day 7 or day 17 instead of day 10. Such exceptions are clearly mentioned in the results.

2.3. Cell exposure to test compounds

hMSCs in plates were exposed to test compounds during the whole course of differentiation into adipocytes. Compounds were added from 1000x concentrated stocks in DMSO to the differentiation medium prior to adding to the cells. Solubility of test compounds was monitored visually and no precipitation was observed in the tested concentration range of applied compounds. DMSO was added to negative control samples. All wells in all experiments contained 0.125% of DMSO (0.1% coming from the test compound stock solution or DMSO (for negative controls) and 0.025% coming from the dexamethasone stock solution for the differentiation medium). Rosiglitazone at the concentration of 1 μM was used as a maximum positive control for the assay, and 1 nM tributyltin chloride was used as MDC positive control for assay sensitivity. Fresh solutions of compounds in the differentiation medium were prepared at the start of differentiation and on day 7. Each concentration of test compounds and positive controls was tested in triplicate wells, whereas negative control had six wells evenly distributed on the left and right sides of the plate.

2.4. Staining of adipocytes

After 10 days of differentiation, adipocytes were stained according to a slightly modified published procedure (Fink and Zachar, 2011) with 1 μg/mL BODIPY493/503 and 2 μg/mL Hoechst 33342 in plating medium (DMEM supplemented with 10% FBS) for 1 h at 5% CO_2 and 37 °C. Hoechst 33342 stock solution was sonicated in an ultrasonic bath for 20 min prior use. After staining, cells were carefully washed three times with 150 μL per well of room temperature DPBS with Ca^{2+} and Mg^{2+} (Gibco) and fixed with 100 μL per well of 4% paraformaldehyde in PBS at +4 °C for 15 min. Plates were then washed once with DPBS, and finally, 100 μL of DPBS was added to cells.

2.5. Imaging and image analysis

Stained and fixed cells in plates were imaged using ImageXpress Micro high content analysis system (Molecular Devices) using 10× objective. BODIPY493/503 was imaged in the FITC channel (Ex 482/35, Em 536/40) and Hoechst 33342 in the DAPI channel (Ex 377/50, Em 447/60). Twelve sites were imaged per well. Sites were distributed evenly across the well to represent both the middle and the edge of the wells (Supplementary Information Fig. S7).

Images were analysed in the MetaXpress software (Molecular Devices) using the Transfluor HT module for the analysis of granular objects to obtain integrated granule intensity of all lipid droplets and the Cell Score module for the quantification of BODIPY493/503 positive cells to obtain the percentage of adipocytes and total cell count. Image

acquisition and analysis setups and examples are provided in the supplementary information (Table S3, Figs. S3–S6). Obtained integrated granule intensity values were divided by cell count to calculate relative lipids per cell in culture. The number of adipocytes was calculated by multiplying the percentage of adipocytes with the total cell count divided by 100. Finally, the relative measure of lipids per adipocyte, reflecting adipocyte size, was calculated by dividing the values of integrated granule intensities of all lipid droplets by the number of adipocytes.

To evaluate the quantitative response and separation of positive and negative controls on each plate, Z' factor was calculated as follows (Zhang, Chung and Oldenburg, 1999):

$$Z' = 1 - \frac{(3*SD_{C+} + 3*SD_{C-})}{|\mu_{C+} - \mu_{C-}|}$$

where SD_{C+} is the SD of the positive control (ROSI 1 μM); SD_{C-} is the SD of the negative control (DMSO); μ_{C+} is the mean value of positive controls, and μ_{C-} is the mean value of negative controls, all on the same plate.

2.6. Benchmark concentration modelling

The benchmark concentration (BMC) approach was used for dose-response assessment, according to the European Food Safety Authority (EFSA) guidelines (EFSA et al., 2017). The benchmark modelling was performed using the R-package PROAST (v70.0, The Netherlands' National Institute for Public Health and the Environment, RIVM) in R-version 3.6.3 (R Development Core Team). The nominal concentrations in the wells were employed as the independent variable (predictor). The dependent variable was the % adipocyte fold change, averaged over the average of the different replicates. The critical effect size (CES) was 20%, corresponding to a 1.2-fold change (fold induction). The standard 5% change was deemed not an adequate threshold for adversity, and the new CES was corroborated by a within-group SD-adjusted CES suggestion of 16.7% (Slob, 2017; EFSA et al., 2017).

Four different families of models were fit to the data (exponential, Hill, inverse exponential and log-normal). The Akaike Information Criterion (AIC) was set to 2, and the employed covariates were the different bisphenols. Model averaging was employed, weighing the models by estimated AIC in a total of 1000 iterations (Wheeler and Bailer, 2007, EFSA et al., 2017). The resulting confidence intervals for the individual models and after model averaging were expressed in terms of BMCL and BMCU, the lower and upper bound of the 90% confidence interval, respectively.

For calculating the relative potency factors, the median BMC resulting from the 1000 iterations of model averaging was employed. The index chemical was BPA, and the following equation was employed:

$$RPF = \frac{CED_{20}\ \text{index chemical (BPA)}}{CED_{20}\ \text{study compound}}$$

where CED_{20} corresponds to median BMC.

The relative potency approach was employed to scale and compare the potency of the various bisphenols in relation to BPA for this particular endpoint. It converts the concentration of each bisphenol into the BPA-equivalent concentrations, estimating a factor (Bosgra et al., 2009). BPA was used as an index chemical (RPF = 1), as it is the most well-studied bisphenol. Thus, an RPF <1 indicates lower potency than that of BPA.

2.7. Quantitative RT-PCR

To analyse adipocyte gene expression, hMSCs were seeded in 12-well plates at a density of 44200 cells per well and exposed to bisphenols in 2 mL medium with CSS per well, as described for lipid accumulation (sections 2.2 and 2.3). Cells were lysed for RNA isolation on day 7 of differentiation. An additional sample of non-differentiated MSCs was taken on day 0 of differentiation. RNA was isolated using Aurum Total RNA Mini Kit (Bio-Rad), transcribed into cDNA with iScript™ cDNA Synthesis Kit (Bio-Rad), and quantitative RT-PCR was run on QuantStudio 3 (Applied Biosystems) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). The selected genes and their primer sequences, designed in PrimerExpress software (Applied Biosystems), are shown in the Supplementary Information Table S5. Gene expression was calculated using EF1A and GAPDH as housekeeping genes, and DMSO treated sample as a comparator in the ΔΔCt method.

2.8. Cell viability measurements

Differentiation medium was collected after 3, 7 and 10 days of differentiation and the impact of test compounds on the cell membrane permeability was assessed by measuring the release of cytosolic enzyme adenylate kinase into the cell medium using Toxilight kit (Lonza). Briefly, 10 μL of culture medium was mixed with 50 μL of Toxilight reagent in a white 384-well plate, and luminescence was read after 10 min of incubation at room temperature. An increase in luminescence higher than 1.5-fold over negative control (DMSO-treated cells) was considered cytotoxic.

2.9. Data analysis and statistics

In experiments where only baseline adipogenesis (only with DMSO in differentiation medium) was monitored, results are expressed as a percentage of cells that become adipocytes (% adipocytes). MDC effects are expressed relative to the negative control (vehicle DMSO) as fold over negative control (or fold induction). Therefore, DMSO control had the fold value of 1. Mean fold induction values for % adipocytes in each experiment were calculated from triplicate (test compounds) or hexaplicate (negative controls) samples, respectively, from the same plate. If not specified differently, experiments were repeated three times and the mean values of all repeats were calculated.

For statistical analysis, the values of folds induction, as well as percentage of adipocytes in some experiments, were $\log_{10}$-transformed and analysed in paired two-tailed *t*-test or one-way ANOVA with Sidak's multiple comparisons test or Dunnett's post-test using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. Results

3.1. Impact of cell differentiation conditions on the recruitment of adipocytes

In order to obtain a sensitive method for detecting adipocyte changes, we have first analysed the impact of several common cell culture conditions on the extent of adipocyte recruitment from hMSCs. Factors such as charcoal stripping of serum, the volume of medium per well, HEPES in medium, final cell density per well, hMSC passage number, and the length of differentiation were investigated in parallel, searching for conditions that lower the baseline intensity of adipogenesis.

During the adipogenic differentiation of hMSCs in the applied conditions, only a fraction of all cells become adipocytes, and they are quite unevenly distributed in the well. In the spectrum of conditions tested here, this fraction spanned from 2 to 58% and was affected by culturing conditions during differentiation. As shown in Fig. 1a, replacing non-stripped serum with charcoal-stripped serum significantly reduces basal adipocyte recruitment from hMSCs. Increasing cell medium volume per well (Fig. 1b) had the same statistically significant effect; just the intensity of the effect seems lower. We have also found that cell monolayers with a lower cell density of 100–200 cells per image (corresponding to a final density of 16600-33200 cells/cm^2, measured at the

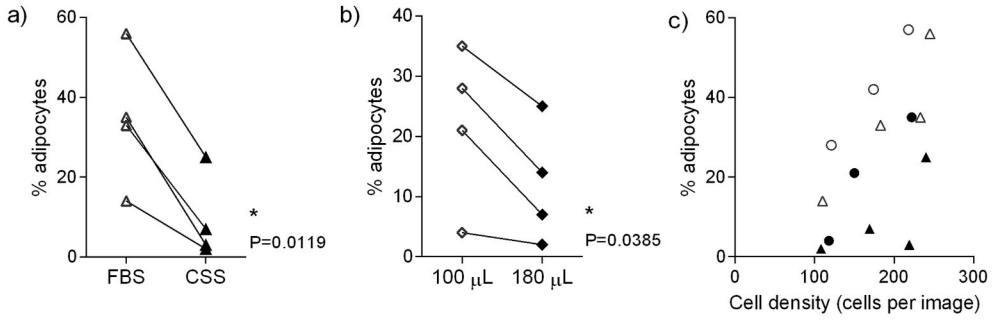

Fig. 1. Impact of a) serum stripping, b) volume of cell medium per well, and c) cell density on the baseline recruitment of adipocytes from hMSCs during the differentiation for 10 days. Pairs of data points connected with lines are from the same experiment differing only in a) type of serum (open triangles – non-stripped serum (FBS); closed triangles – charcoal-stripped serum (CSS)), or b) volume of medium per well (open diamonds – 100 µL; closed diamonds – 180 µL). In c) total cell density at the end of differentiation, shown as cell count per image, is related to the observed percentage of adipocytes (open circles – at 100 µL per well with FBS; open triangles – at 180 µL per well with FBS; closed circles – at 100 µL per well with CSS; closed triangles – at 180 µL per well with CSS). The percentage of cells that became adipocytes (% adipocytes) was measured by high content analysis. Shown data are mean values of triplicate samples from four independent experiments conducted on cells from one donor in passages 6 and 7. Statistical analysis in a) and b) was performed by the paired two-tailed *t*-test on $\log_{10}$-transformed values. P values are given.

end of the experiment), also tended to have lower percentages of adipocytes (Fig. 1c). At higher cell densities (200–300 cells per image corresponding to 33200-49800 cells/cm^2), especially in medium with CSS, the impact of cell density on adipocyte recruitment seemed less pronounced. In addition, cell passages 5 to 8 have been tested in these experiments, but no consistent effect has been observed. It is possible that in the comparisons between experiments, some other factors, for example, cell density, may have masked the effect of the cell passage number on the baseline differentiation in some experimental conditions. Similarly, no consistent effect on baseline differentiation was observed when HEPES was added to the differentiation medium (data not shown). However, after passage 8 we have in preliminary experiments observed a very low basal differentiation (% adipocytes <0.5), and these experiments were excluded from further analysis (data not shown), and cells were no longer used beyond passage 8.

3.2. Impact of cell differentiation conditions on assay sensitivity to adipogenic chemicals

Next, we investigated whether the cell culture conditions that result in lower baseline adipocyte recruitment, as described in the previous section, are more sensitive to detect the adipogenic effects of obesogenic metabolism disrupting chemicals. We hypothesised that the culture conditions that produce sub-maximal baseline differentiation of adipocytes could be further induced with other stimuli (such as obesogenic chemicals) to greater extent than in conditions giving higher baseline response.

As shown in Fig. 2, rosiglitazone induced an increase in lipids per cell (Fig. 2a), % adipocytes (Fig. 2c) and lipids per adipocyte (Fig. 2d) compared to the negative control (baseline adipogenesis with vehicle DMSO). In addition, the effect of rosiglitazone was found significantly more pronounced when the assay was conducted with 180 µL rather than 100 µL of medium per well in the same experiment. At the same time, total cell count remained not significantly affected by neither rosiglitazone nor medium volume (Fig. 2b).

The impact of serum stripping was investigated on rosiglitazone, as well as two obesogenic metabolism disrupting chemicals (MDCs): tributyltin-chloride (TBT) and bisphenol A (BPA). Replacing the non-stripped serum (FBS) with stripped serum (CSS) resulted in a significant increase in the extent of compound effects on lipids per cell and percentage (number) of adipocytes (Fig. 3a and Fig. 3c). In addition, effects of 1 nM TBT and 30 µM BPA on these two endpoints became detectable and quantifiable in medium with CSS. Whereas all three tested compounds induced an increase in the percentage of cells that became adipocytes indicating induction of adipocyte recruitment, only rosiglitazone also increased lipids per adipocyte, reflecting an increase in adipocyte size (Fig. 3d).

The length of differentiation *in vitro* was found to negatively affect the extent of MDC effects, with the strongest effects of MDCs on lipids per adipocyte (supplementary information, Fig. S1a) and percentage of adipocytes (Fig. S1c) recorded on day 7 of differentiation. In addition, even though cell count remained stable for all treatments (Fig. S1b) indicating no cytotoxicity, shrinkage of adipocyte size has been noted with longer differentiation in treatments with TBT and rosiglitazone as evidenced by the reduction of lipids per adipocyte (Fig. S1d).

The impact of cell density on the measured potency of rosiglitazone did not show a clear trend, likely because comparisons were made across experiments introducing variability due to other factors (data not

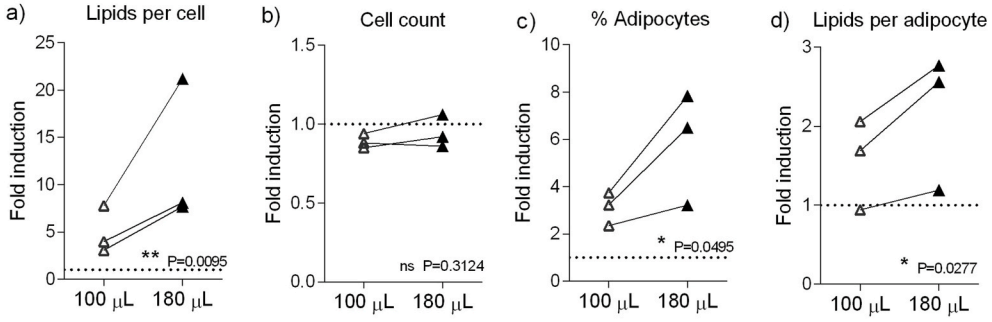

Fig. 2. *Impact of the volume of cell culture medium* per *well on the intensity of adipogenic effects of rosiglitazone. hMSCs were differentiated into adipocytes in the presence of* 1 µM *rosiglitazone with either* 100 µL *(open triangles) or* 180 µL *(closed triangles)* per *well in medium with CSS for 10 days. Adipogenesis endpoints were measured by high content screening as a) Lipids* per *total cells - indicating total accumulation of lipids in samples, b) Total cell count - indicating cell viability, c) Percentage of adipocytes – indicating adipocyte recruitment (number), and d) Lipids* per *adipocyte – indicating adipocyte size. Results are expressed as fold induction over negative control (vehicle DMSO – dotted line) for each of the three independent experiments. Pairs of data points connected with lines are from the same experiment. Experiments were conducted on cells from 1 donor in passages 6 and 7. Statistical analysis was conducted by paired two-tailed t-test on $\log_{10}$-transformed values of fold induction. P values are given.*

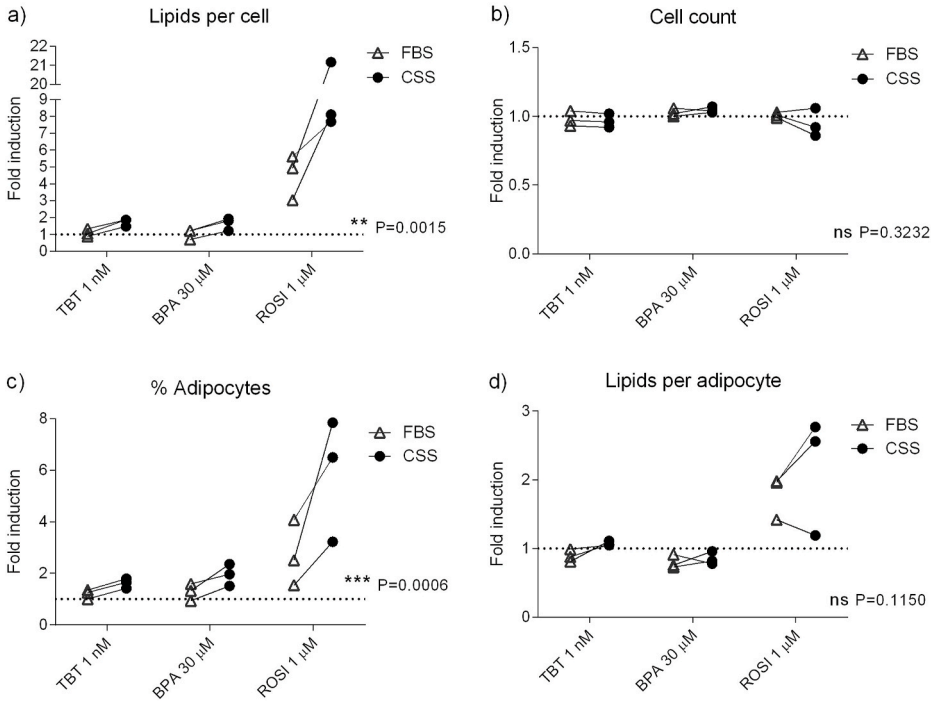

Fig. 3. *Impact of serum stripping on the sensitivity of the adipogenesis assay to obesogenic MDCs. hMSCs were differentiated into adipocytes in the presence of* 1 nM TBT, 30 μM *BPA or* 1 μM *rosiglitazone in medium with either non-stripped serum (FBS) – open triangles, or charcoal-stripped serum (CSS) – closed circles, in* 180 μL *medium* per *well for 10 days. Adipogenesis endpoints were measured by high content screening as a) Lipids* per *cell – indicating total accumulation of lipids in samples, b) Total cell count - indicating cell viability, c) Percentage of adipocytes - indicating adipocyte recruitment (number) and d) Lipids* per *adipocyte - indicating adipocyte size. Results are expressed as fold induction over negative control (vehicle DMSO – dotted line) for each of the three independent experiments. Pairs of data points connected with lines are from the same experiment. Experiments were conducted on cells from 2 donors in passages 6 and 7. Impact of treatments and sera was analysed by the two-way ANOVA. Shown collective P values are for the comparisons between the samples with CSS and FBS.*

shown).

3.3. Applicability of the HCS adipogenesis method for the screening of bisphenols and their mixtures

To evaluate the applicability of the developed *in vitro* model for the assessment of adipogenicity of chemicals and their mixtures, we have selected 8 bisphenols: BPA, BPAF, BPB, BPC, BPF and BPS - all known to have similar applications as BPA, and TBBPA and TCBPA - brominated flame retardants, structurally related to BPA. According to the results obtained in the method development part, the following conditions were selected for the screening of test chemicals: CSS, 180 μL medium per well, plating 4200 cells per well, differentiation for 10 days and hMSC cell passages 5–8 (Fig. 4). Rosiglitazone of 1 μM concentration was used as positive control on each 96-well plate and DMSO as the negative control. These experimental conditions lead to low-intensity adipogenesis with a baseline of 2–11% cells developing into adipocytes in the negative control (supplementary information, Fig. S2a). Rosiglitazone had EC_{50} of 13.8 nM for % adipocytes, 7.5 nM for lipids per adipocyte and 11.6 nM for lipids per cell (Fig. 5). Lowest observed effect concentrations (LOEC) obtained for rosiglitazone was 1 nM for % adipocytes and lipids per cell and 10 nM for lipids per adipocyte. The total cell count was not affected in the tested concentration range (Fig. 5).

3.3.1. Cell viability

The impact of bisphenol derivatives on cell viability was assessed using two parameters: total cell count obtained from image analysis of stained nuclei on day 10 of differentiation, and cell membrane permeability measured as the release of a cytosolic enzyme adenylate kinase on days 3, 7 and 10.

In the tested concentration range of 0.1–100 μM, only 100 μM BPC and 100 μM BPAF were identified as cytotoxic by the membrane permeability assay at day 7 and day 10 of differentiation (data in the supplementary information, Table S2). The same two treatments were also confirmed by image analysis to have a significantly lower (<50%) number of cells at day 10. Therefore, 100 μM BPC and BPAF were excluded from the further analysis of adipocyte number and size.

3.3.2. Bisphenol effects on adipogenesis

Selected eight bisphenols were tested in the developed high content analysis method for detecting adipogenesis in hMSCs. Chemicals were applied in non-cytotoxic concentrations ranging from 0.1 to 100 μM throughout the 10 day adipocyte differentiation, and their effects on endpoints, measured by high content analysis, are shown in Fig. 6. All tested compounds increased the number of adipocytes in the culture of hMSCs, as shown by the increase in % adipocytes (Fig. 6a), while the total number of cells remained unchanged (Fig. 6d). The strongest

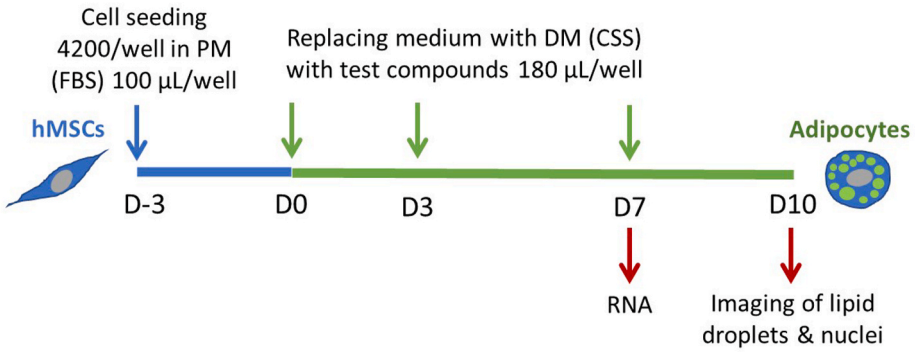

Fig. 4. Schematic representation of experiment workflow for the developed assay of high content screening of adipogenic compounds in conditions of low-intensity adipogenesis of human mesenchymal stem cells (hMSCs). The exact composition of the plating medium (PM) and differentiation medium (DM) is given in section 2.2. Given cell number and medium volumes apply for 96-well plate. In experiments for collecting RNA for gene expression analyses, cells were seeded in a bigger format in 12-well plates (see section 2.7).

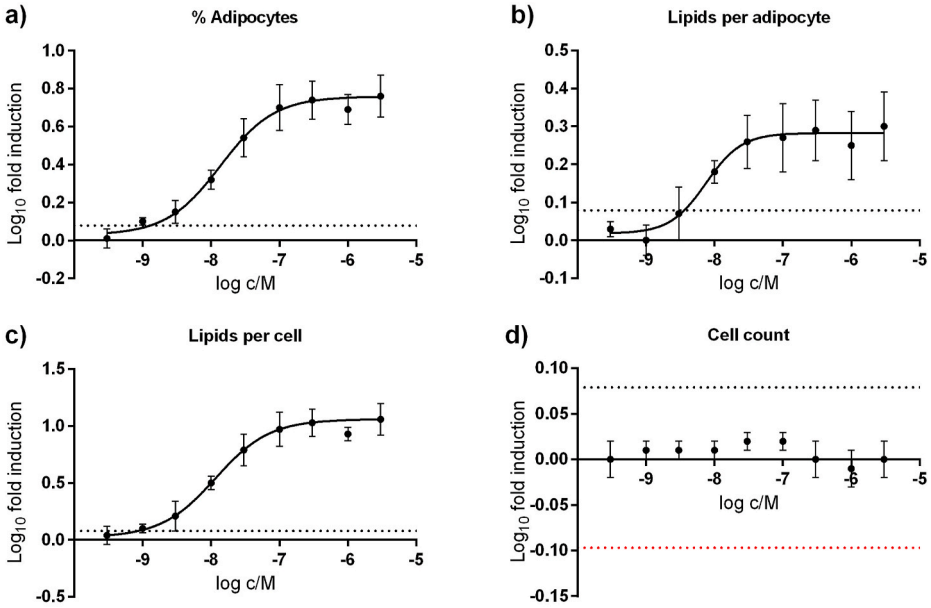

Fig. 5. The activity of positive control compound rosiglitazone in the developed high content screening assay of adipogenesis. MSCs were seeded in 96-well plates at the density of 4200 per well 3 days before experiment and then differentiated for 10 days in 180 μL per well differentiation medium containing CSS. Results are expressed as $\log_{10}$ transformed values of fold induction. Mean values of 4 independent experiments ± SD are shown. Experiments were conducted on cells from 2 donors (1 male and 1 female), with 2 experiments performed on each. Experiments were performed on cells in cell passages: p5, p6, p7 and p8. The black dotted line represents the lowest detectable effect of 1.2-fold over negative control. The red dotted line represents an effect of 0.8-fold over negative control.

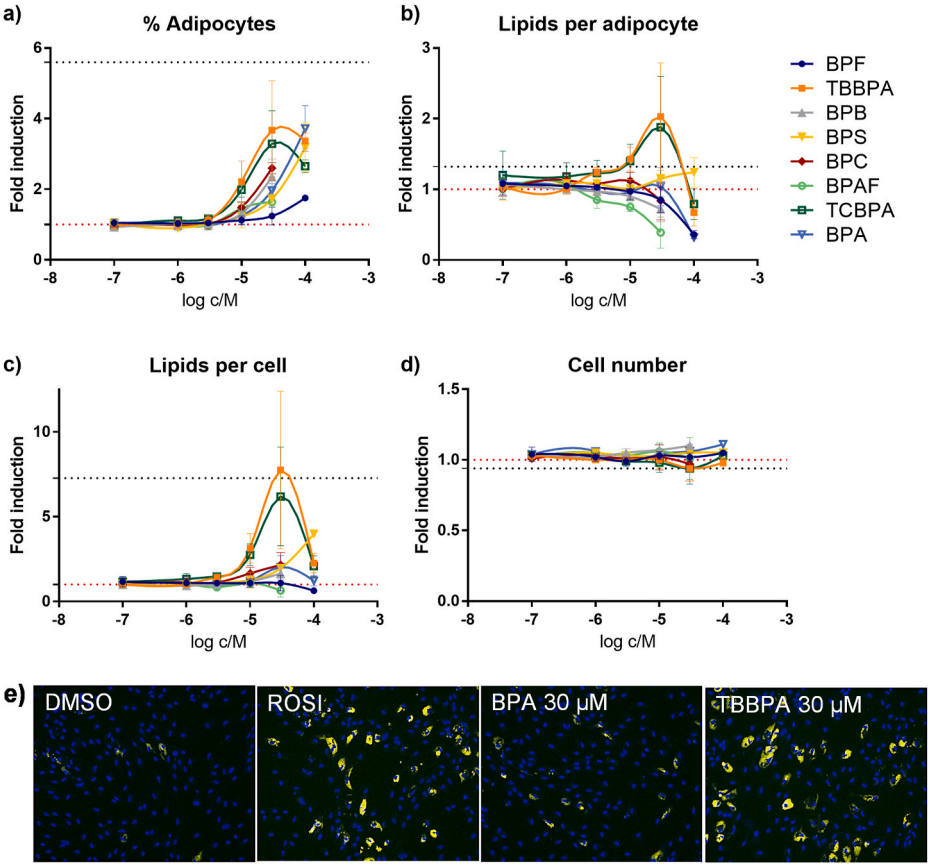

Fig. 6. Impact of 8 bisphenols on the differentiation of adipocytes from human MSCs as measured by high content screening. a) % adipocytes; b) lipids per adipocyte; c) lipids per cell; d) cell number. For the description of endpoints, see Fig. 3. Experiments were conducted according to the setup shown in Fig. 4. BPF – closed dark blue circles; TBBPA – closed orange squares; BPB – closed grey triangles; BPS – inverted closed yellow triangles; BPC – closed red diamonds; BPAF – open light green circles; TCBPA – open dark green squares; BPA – inverted open blue triangles. Fold induction over DMSO-exposed control (vehicle – red dotted line) is shown ± SD from 3 to 5 independent experiments. Connecting lines in graphs were obtained using the fit spline/LOWESS function in GraphPad Prism. The black dotted line represents the mean response of the positive control – 1 μM rosiglitazone. e) Representative images of cells exposed to DMSO, 1 μM rosiglitazone, 30 μM BPA, 30 μM TBBPA – blue – nuclei stained with Hoechst33342; yellow – lipid droplets stained with BODIPY493/503. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

induction of adipogenesis in terms of adipocyte recruitment (percent adipocytes) was observed with TBBPA and TCBPA; however, their effects were markedly lower than the maximum induction observed with rosiglitazone.

Adipocyte size, expressed as lipids per adipocyte, was significantly increased with 30 μM TBBPA and TCBPA and significantly decreased with 30 μM BPAF according to One-way ANOVA with Dunnett's post-test (Fig. 6b and Supplementary Information Fig. S8a). In a similar analysis on % adipocytes, at a single concentration of 30 μM, TBBPA, TCBPA, BPC, BPB and BPA display significantly increased values (Fig. S8b). As shown in Fig. 6c, lipids per cell, representing the total accumulation of lipids in this cell culture, were induced by all tested compounds except BPAF and BPF, where they remained unchanged. Representative images of cells at the end of 10 day differentiation and

exposure, shown in Fig. 6e, show good separation among individual adipocytes enabling precise measurement of adipocyte number and size (Supplementary Information Figs. S3–S6). It is visible that both endpoints were clearly induced in samples exposed to 1 μM ROSI and 30 μM TBBPA, and % adipocytes was induced even with 30 μM BPA.

The data in Fig. 6 originate from experiments performed on hMSCs in passages 5, 6, 7 and 8, and indicate a robust response across these 4 passages. The calculated Z' factor for % adipocytes, as the measure of separation between the positive and negative control on each successful plate, was in the range 0.25–0.58 (median value 0.4) indicating a good separation in the assay and suitability for compound screening in dose response experiments. The sensitivity control, 1 nM TBT, exhibited a rather robust induction of % adipocytes with a mean value ± SD of 1.285 ± 0.095 (median 1.29).

To investigate the impact of different cell batches coming from different donors, selected eight bisphenols at 10 and 30 μM concentration and control compounds, were tested on the MSCs from two male (M1 and M2) and one female donor (F1) (Fig. 7). Even though the experiments on different donors were also separated in time (by several months), the fold induction values of % adipocytes obtained on cells from all three donors were highly comparable and significantly correlated, with Pearson's coefficients of 0.89, 0.95 and 0.93 ($P < 0.0001$ for all) for M1 vs. M2, M1 vs. F1 and M2 vs. F1, respectively. Comparable results on the three tested batches of cells were also obtained on the other two endpoints: lipids per adipocyte and lipids per cell (Supplementary information, Fig. S9).

However, MSCs from one additional female donor (F2) were tested and noticed to differentiate unusually slowly, giving only 0.36% adipocytes in the negative control on day 10 (compared to 2–10% obtained with other 3 donors (Fig. S2a)) and an average of less than one adipocyte per image (0.84), which made these samples unsuitable for reliable quantification of fold induction. Even though, in this case, this resulted in higher fold induction values than those obtained for the other three donors (2 males and 1 female, Fig. S10), the results of tested compound treatments in the F2 cell batch correlated well with the mean results obtained on other three batches (with Pearson's correlation coefficient of 0.8985, $P < 0.0001$). Despite this similarity in relative assessment of chemicals, this information shows the importance of developing clear acceptance criteria for new cell batches used in this method in future to ensure comparable data even on fold induction values. Based on our experience, a possible acceptance criterion could be the morphological assessment of adipocytes under a light microscope on day 7 of differentiation. At that time point, developing adipocytes should be visible in positive (1 μM rosiglitazone) and negative (DMSO) control samples, with a clearly stronger adipogenic effect of the positive control (for images see Supplementary Information Fig. S11).

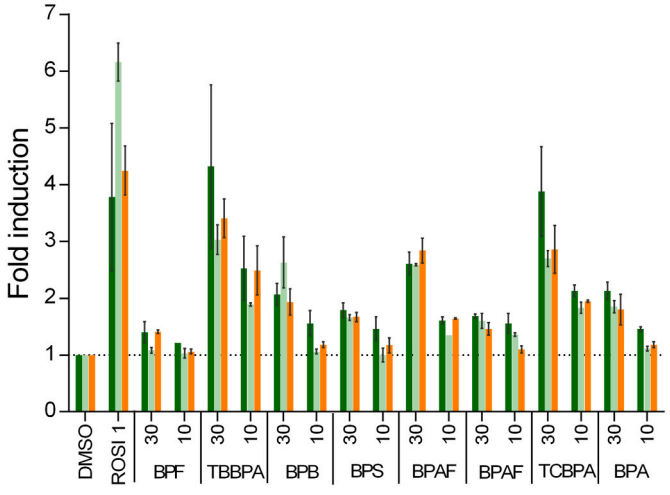

Fig. 7. Assay performance in hMSCs from three donors: two male (M1 - dark green and M2 - light green bars) and one female (F1, orange bars), exposed to 30 and 10 μM bisphenols or 1 μM rosiglitazone (according to experimental setup in Fig. 4). Mean fold induction of % adipocytes is shown from two independent experiments on cells from each donor ± value range. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3.3. *Benchmark concentration modelling of bisphenol effects on adipocyte number*

In order to quantitatively compare the tested bisphenols, a benchmark concentration (BMC) analysis was performed on the data on the induction of % adipocytes. The applied critical effect size used for determining BMCs was 20% or 1.2-fold induction over negative control (DMSO), determined empirically as the lowest detectable effect by the high content analysis method. This was further confirmed by calculating the within-group SD-adjusted CES suggestion, which equalled 16.7% (Slob, 2017, EFSA et al., 2017).

The resulting model fit for the exponential model is shown in Fig. 8a, and the BMCLs, BMCs, and BMCUs obtained after model averaging are shown in Fig. 8b. Obtained BMCs for all tested bisphenols, except BPF, were in the lower micromolar range. For the model averaging step, the AIC-weighed model weights were 0.9657 for the exponential model, 0.0232 for the Hill model, 0.0112 for the lognormal and 0 for the inverse exponential model (the latter was 33 AIC units away from the best fit exponential model). The iterations for model averaging are presented in the supplementary information (Fig. S12).

The obtained relative potency factors of tested bisphenol derivatives compared to bisphenol A are shown in Table 1, resulting in the potency order of: TCBPA ≈ TBBPA > BPC ≈ BPB ≥ BPA ≈ BPS ≈ BPAF ≫ BPF.

3.3.4. *Effects of bisphenol mixtures on adipogenesis*

So far, we have shown that the developed high content screening method for evaluating adipogenesis in hMSCs is quantitative, robust and very sensitive. Since, in reality, humans are concomitantly exposed to a large number of chemicals, we next evaluated the adipogenic potential of the mixture of the six most potent bisphenols in their sub-active concentrations using the developed HCS method. MSCs were exposed during differentiation either to a single bisphenol at 3 μM concentration or to their mixture containing individual bisphenols at either 3, 1.5, 0.75 or 0.375 μM concentration and their measured fold induction in % adipocytes is shown in Fig. 9. The mixture of bisphenols showed a clear dose-dependent effect markedly stronger than the one observed with individual compounds. Even though all individual bisphenols at 3 μM concentration did not reach the threshold of activity of 1.2-fold increase, their mixture at 3 μM and 1.5 μM induced a significant increase in % adipocytes of 1.90-fold and 1.53-fold, respectively. The mixture of six bisphenols at 0.75 μM concentration nearly reached the threshold of detectable activity on % adipocytes (1.18-fold increase, although not statistically significant). These data clearly show that compounds in concentrations too low to detect effects on an individual basis can have adipogenic effects if in a mixture.

To calculate the expected effect level for the mixture of 6 bisphenols, assuming that the effect is additive, we have used the relative potency factors (RPFs) obtained from the BMC model (Table 1) (van Ede, van Duursen and van den Berg, 2016; Bil et al., 2021; Barcelo Culleres et al., 2008). We calculated that an equimolar mixture of the 6 selected bisphenols has a potency of 12.57 BPA equivalents (obtained as the sum of RPFs for 6 selected compounds, Supplementary Information Table S4), meaning that the mixture of 6 selected bisphenols at 3 μM concentration each, would have an effect on adipogenesis corresponding to 37.7 μM BPA alone (3 μM x 12.57 BPA equivalents). Similarly, the mixture of 1.5 μM of each of 6 bisphenols would have an effect corresponding to 18.9 μM of BPA. From the exponential curve that had the highest weight in the BMC model (Fig. 8a), it was obtained that 37.7 μM and 18.9 μM BPA alone increased % adipocytes 2.22-fold and 1.54-fold, respectively. This means that the mixture of these particular bisphenols

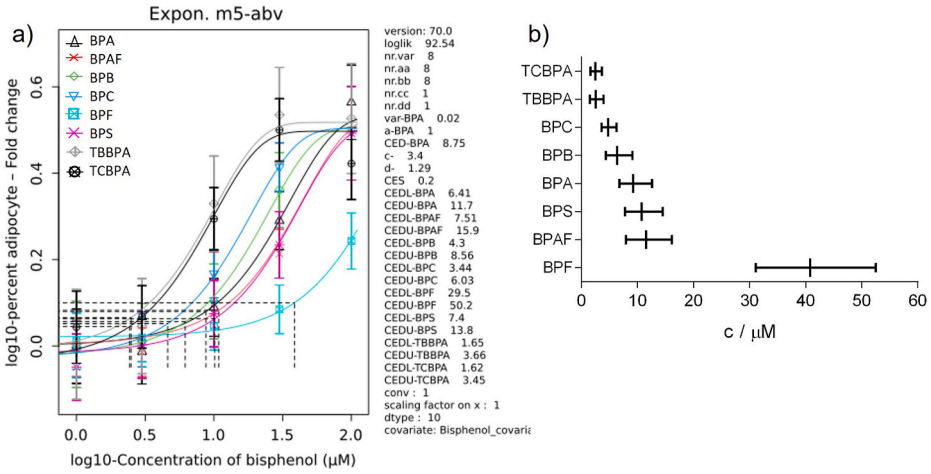

Fig. 8. Benchmark concentration (BMC) modelling of the increase in % adipocytes in hMSCs exposed to bisphenols during differentiation. a) The BMC-modelling results for the exponential model 5-bv of % adipocytes. The shown model is representative of 1000 iterations of model development. Colour code: BPA, black upward triangle; BPAF, red cross; BPB, green diamond; BPC, dark blue downward triangle; BPF, light blue cross-square; BPS, pink cross-star; TBBPA, grey diamond-plus; TCBPA, black cross-star. b) Benchmark concentration ranges for the 1.2-fold increase in % adipocytes for the eight bisphenols. The tick represents the median BMC, and the error bars represent 90% confidence intervals (BMCL-BMCU). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Benchmark concentrations and the estimated relative potency factors (RPF), using bisphenol A as an index chemical (RPF = 1). The critical effect size was a 20% (1.2-fold) increase in % adipocytes relative to the negative control (DMSO) as a function of increasing the dose of the different bisphenols. Results were based using 1000 iterations for model averaging purposes.

Compound	BPA	BPAF	BPB	BPC	BPF	BPS	TBBPA	TCBPA
BMC median/µM	9.25	11.55	6.40	4.76	40.83	10.74	2.56	2.49
BMC CI/µM	6.80–12.60	7.99–16.10	4.35–9.13	3.58–6.24	31.10–52.50	7.79–14.50	1.56–3.95	1.66–3.66
RPF	**1.00**	**0.80**	**1.45**	**1.94**	**0.23**	**0.86**	**3.61**	**3.71**

at an equimolar concentration of 3 µM or 1.5 µM is expected to increase % adipocytes 2.22-fold or 1.54-fold, respectively, provided their effect is additive. Further comparison of the experimental values of mixtures to those calculated based on RPFs, revealed that experimental values at 4 test concentrations examined are not significantly different from the values for their mixtures calculated using RPFs under the assumption of their additive effect (Fig. 9). This indicates that the examined 6 bisphenols have an additive effect on the induction of adipogenesis.

3.3.5. *Bisphenol effects on adipocyte marker expression*

The results shown so far were based on image analysis of lipid accumulation in hMSC derived adipocytes using high content screening. To confirm the obtained effects further, adipocyte marker gene expression was measured by quantitative RT-PCR (Table 2). All measured adipocyte marker genes were significantly lower in non-differentiated hMSCs compared to adipocytes on day 7 (CCAAT enhancer binding protein alpha (CEBPα) 10-fold, peroxisome proliferator activated receptor gamma (PPARγ) 3.3-fold, fatty acid binding protein 4 (FABP4) 2000-fold, lipoprotein lipase (LPL) 14000-fold and adiponectin (ADIPOQ) 50-fold), indicating the induction of adipogenesis. Similar to lipid accumulation data, the strongest induction of adipocyte marker genes above negative control was observed with ROSI, followed by TBBPA and TCBPA. The remaining compounds show a trend of low to medium induction; however, not statistically significant. CEBPα and adiponectin were the most strongly induced genes by most compounds, and PPARγ was the least affected.

4. Discussion

One of the main aims of the EU funded GOLIATH project is to develop *in vitro* methods for the toxicological assessment of chemicals that can disrupt metabolic processes in the organism, contributing to the development of obesity, insulin resistance, and type 2 diabetes (Legler et al., 2020). Here, we show a high content screening method for the detection of adipogenic effects of chemicals in primary human mesenchymal stem cells in conditions of low-intensity basal adipogenesis in a ten-day differentiation protocol. Finally, we successfully applied the method to evaluate the adipogenic effects of eight bisphenol A derivatives and their mixture, showing the first experimental evidence of their additive effect. The applied high content screening method was shown to be precise enough to detect, quantify and even investigate the nature of the bisphenol mixture effect.

4.1. *Optimization of experimental conditions*

Selecting the right conditions for *in vitro* cell culture may be challenging when trying to mimic long-term exposures and processes such as cell differentiation, while aiming for practical and robust solutions. Differentiation of mesenchymal stem cells into adipocytes in a monoculture induced by chemical stimuli *in vitro* is a model that is in many aspects different from the physiological process of adipocyte development in the organism, but with high content screening readouts and optimized cell culture conditions represents a good balance between the relevant physiology and a robust throughput for screening of chemicals. It is, therefore, of high importance to select the right conditions for the *in vitro* culture to ensure high sensitivity of the cell-based assay. Often, culture conditions that are considered common or irrelevant may be critical for detecting a certain response in cells.

In our prescreening and method optimization phase, we have identified serum stripping and volume of medium per well as critical factors that can affect the overall sensitivity of high content analysis readouts of hMSC adipogenesis assay for adipogenic MDCs. Charcoal stripped serum is often used in endocrine disrupting chemicals (EDC) research as it contains less lipophilic molecules, especially hormones, which can mask the effects of test chemicals acting upon the same nuclear receptors in cells (NICEATM, 2003). Therefore, the usage of stripped serum often increases the sensitivity of an *in vitro* model to endocrine disruption.

The impact of medium volume per well may be less intuitive to understand. It was, however, shown previously that the height of the medium in *in vitro* cell culture can affect the differentiation of chondrocytes by changing the pericellular oxygen level (Oze et al., 2012). It is, therefore, conceivable that in our experiments, higher medium

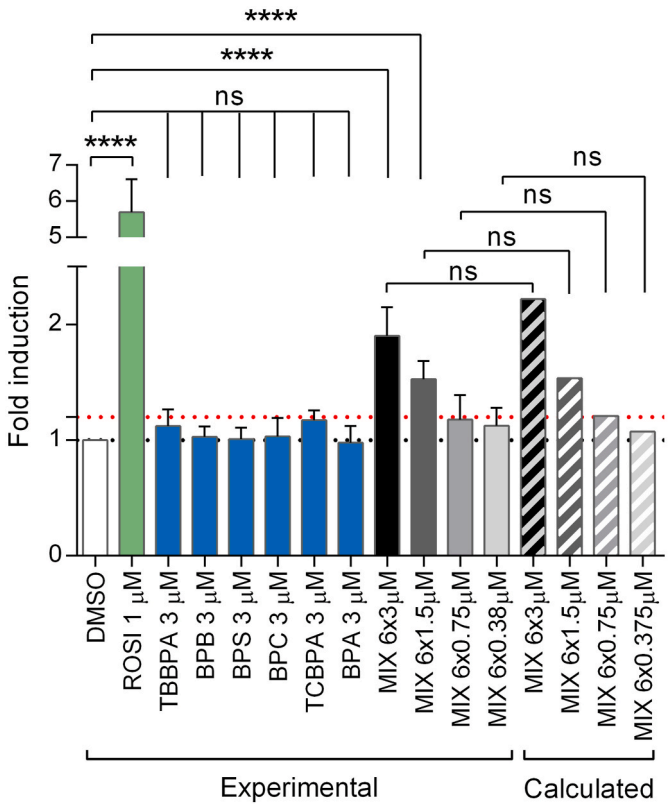

Fig. 9. Additive effect of 6 most potent bisphenols in mixtures of sub-active concentrations on the induction of % adipocytes in hMSCs. Experiments were conducted according to the experimental setup shown in Fig. 4. Mean values ± SD from 4 independent experiments are shown. Striped bars denote values calculated using each bisphenol's relative potency factors (RPF) compared to BPA, obtained from the BMC model (Table S4, Fig. 8a). Black dotted line - the level of the negative control (DMSO); red dotted line - the minimum detectable effect of 1.2-fold induction. Stars denote statistically different treatments compared to DMSO according to one-way ANOVA with Sidak's multiple comparisons test performed on log-normalized values (****P < 0.0001, ns – not significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

volume also reduced the pericellular oxygen concentration, reducing the extent of basal hMSC differentiation into adipocytes.

Cell density ranging from 100 to 250 cells per image (corresponding to 16600-41500 cells/cm^2 plate well surface during imaging) gave consistent results and was not significantly affecting the extent of compound effects. This optimal cell density was most robustly obtained when adjusting the seeding concentration to 4200 cells per well, 72 h before starting the differentiation. The extent of adipogenic MDC effects was the strongest on day 7 and decreasing until the last day measured - day 17. Since on day 7 lipid droplets were not fully developed and can lead to bigger errors in the quantification of adipocyte number, we have selected differentiation day 10 as an optimal time point to obtain adipocytes with well-developed lipid droplets, but still in the growth phase. In addition, using a 10-day long differentiation protocol significantly reduced the resource demand of these experiments which in most previous reports require 2–3 weeks of differentiation.

Additionally, the chosen experimental conditions with lower number of adipocytes gave perfect conditions for image analysis as adipocytes were well separated and present in not very dense cell monolayer, that was also not detaching from the well bottom, which may have been the case in previous attempts to run an *in vitro* adipogenesis assay in 96-well format. Thus, this improvement has a significant positive impact on the overall throughput of this assay.

Comparison of the dose-response curves of the positive control, rosiglitazone, with previously reported ones, indicate superior sensitivity of the presented HCS method. EC_{50} values of rosiglitazone determined on lipid accumulation in 3T3-L1 cells, mouse bone marrow-derived MSCs, and human adipose tissue-derived stem cells ranged from 40 to 60 nM, and LOEC values range from 15 to 50 nM (Foley et al., 2017; Pereira-Fernandes et al., 2013; Yanik et al., 2011). In the presented HCS method, rosiglitazone had EC_{50} values 7.5–13.8 nM and LOEC of 1 nM for % adipocytes and lipids per cell. Most importantly, some compounds that did not appear to have any detectable adipogenic effect in some reported studies (Andrews et al., 2020), as well as in some of our tested experimental conditions, such as BPA, had a clearly measurable effect in the developed HCS method with low intensity adipogenesis.

4.2. Adipogenic effects of bisphenols and their mixtures

Comparing eight bisphenols on their adipogenic effects revealed a separation of compounds into three groups, one comprising TCBPA and TBBPA (brominated flame retardants, structurally related to BPA), which increase both adipocyte number and size. The second group comprises BPC, BPB, BPA, BPAF and BPF (replacement chemicals for BPA), which all increase the number of adipocytes and tend to decrease the adipocyte size. The last group contains only BPS (also replacement for BPA), which increased the number of adipocytes but had no marked effect on the adipocyte size. Since an increase in adipocyte number is a process that also predisposes an individual to obesity, based on our results, all eight tested bisphenol A derivatives can be classified as adipogens and potential obesogens. Considering that a significant increase in adipocyte size is an early marker of insulin resistance in adipocytes (Longo et al., 2019; Stenkula and Erlanson-Albertsson, 2018), TBBPA and TCBPA can also be considered potential diabetogens. In conclusion, from the aspect of metabolism disruption on developing adipocytes, all the tested bisphenols do not seem to have a markedly better toxicity profile on adipocytes then BPA. Therefore, their applicability as safe replacement chemicals for BPA is questionable.

In our study, the test bisphenols were first screened on their adipogenic effects in the concentration range from 100 nM to 300 μM, covering the active concentrations for tested bisphenols on various nuclear receptors (Liu et al., 2019). Cytotoxic treatments were then removed from further study, and it was noticed that all compounds exerted adipogenic activity in the range of 3 μM–100 μM. At the concentration of 30 μM tested bisphenols were well separated according to their potency on the most sensitive readout, % adipocytes.

To our knowledge, the effects of bisphenols on bone marrow-derived MSC (BM-MSCs) were reported in only three studies so far. In mouse BM-MSCs, BPA failed to increase adipogenesis at 20 μM (Andrews et al., 2020) and was found inactive in human BM-MSCs at 1 μM concentration and lower (Chamorro-García et al., 2012). TBBPA, on the other hand, was found to increase adipogenesis from 1 μM in human BM-MSCs (Kakutani et al., 2018) and at 20 μM in mouse BM-MSCs (Andrews et al., 2020). Our study corroborates the previous findings in sense that TBBPA is much stronger inducer of adipogenesis than BPA and active in low to medium micromolar concentrations. However, the adipogenic effect of BPA in these studies was possibly missed due to the lower sensitivity of the applied methods.

On MSCs derived from adipose tissue, reported effects of bisphenol A vary much more across studies, including adipogenic effects in the medium micromolar range (Junge et al., 2018; Foley et al., 2017), lower micromolar range (Ohlstein et al., 2014), lower nanomolar range (Dong et al., 2018), and even inhibitory effect on adipogenesis in the medium micromolar range (Linehan et al., 2012). TBBPA and BPS were found to increase adipogenesis at 1 μM and BPF at 10 μM (Reina-Pérez et al., 2021; Woeller et al., 2017). It is unclear the cause of such big differences in active concentrations in adipose-derived MSCs, especially for BPA. Nevertheless, it seems likely that bone marrow represents a more uniform cell source of MSCs, as it is taken from healthy tissue during hip

Table 2
Induction of adipocyte marker gene expression on days 0 and 7 of differentiation of hMSCs into adipocytes during exposure to test compounds in concentration of 30 μM (or ROSI 1 μM), as measured by quantitative RT-PCR, with EF1A as the housekeeping gene. Experiments were conducted according to the setup shown in Fig. 4. DMSO treated cells on day 7 were used as a comparator. Mean values of fold induction ± SD are shown of 3 independent experiments conducted on cells from 2 different donors (1 male and 1 female), Statistically significant higher (dark orange), or lower expression (blue) compared to DMSO day 7 according to one-way ANOVA with Dunnett's multiple comparisons test (****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05). Fold induction >1.5 (light orange).

		CEBPα	PPARγ	FABP4	LPL	ADIPOQ
Day 0		0.1 ± 0.1***	0.3 ± 0.1***	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****
Day 7	DMSO	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
	ROSI 1	8.2 ± 5.0**	2.4 ± 1.1*	17.2 ± 8.9****	21.6 ± 12.7***	170.7 ± 128.5****
	BPF 30	1.4 ± 0.4	0.9 ± 0.2	1.3 ± 0.2	0.8 ± 0.2	1.6 ± 0.6
	TBBPA 30	4.1 ± 2.7	1.7 ± 0.4	6.1 ± 1.5**	8.6 ± 3.4**	30.9 ± 16.0**
	BPB 30	2.1 ± 1.4	1.1 ± 0.3	0.8 ± 0.1	1.3 ± 0.2	1.5 ± 0.1
	BPS 30	1.7 ± 0.5	1.2 ± 0.2	2.4 ± 0.5	3.1 ± 0.8	5.1 ± 1.9
	BPC 30	3.1 ± 1.3	1.5 ± 0.3	1.9 ± 0.6	2.4 ± 0.3	2.8 ± 0.6
	BPAF 30	1.9 ± 0.7	1.2 ± 0.4	1.2 ± 0.2	1.2 ± 0.4	1.4 ± 0.7
	TCBPA 30	3.8 ± 2.2	1.7 ± 0.4	5.8 ± 1.6*	6.3 ± 1.7*	14.8 ± 4.4*
	BPA 30	4.2 ± 2.0	1.2 ± 0.2	1.0 ± 0.2	1.5 ± 0.4	1.3 ± 0.6

surgery, in contrast to adipose-derived MSCs obtained from liposuction of individuals at different stages of obesity and metabolic syndrome. In our hands, BM-MSCs gave a very stable response across experiments and tested batches (donors), which altogether confirms this cell model is more suitable for long time screening. Although the presented data on only four donors are not sufficient to exclude any sex-specific differences, it is noteworthy that the assessment of compounds gave highly comparable results in cells from the tested two male and two female donors, with three donors even having directly comparable fold induction values. Even though some level of diversity in expressed nuclear receptors important for adipogenesis is expected between the two sexes (Le Magueresse-Battistoni, 2020), it should be kept in mind that female donors in the present study were postmenopausal women, who may have reduced expression of sex-specific hormone receptors. We may speculate that this may have contributed to a more uniform and robust response observed in these cells so far. A more extensive analysis on a larger number of donors is needed to fully assess the interindividual variability in this system and possible sex-related differences.

To obtain a quantitative comparison of potencies of tested bisphenols, we applied benchmark concentration (BMC) modelling on adipocyte number, expressed as % adipocytes. The benchmark concentrations capable of inducing a 1.2-fold increase in the number of adipocytes compared to vehicle-treated control resulted in the following potency order: TCBPA ≈ TBBPA > BPC ≈ BPB ≥ BPA ≈ BPS ≈ BPAF ≫ BPF. To our knowledge, there are no comparative adipogenesis studies comparing so many bisphenols, and intrinsic differences in cellular models, cell source, and experimental setups make it difficult to compare even orders of magnitude of active concentration ranges across studies. Most of the studies comparing two to max four bisphenols were performed on 3T3-L1 murine preadipocyte cell line, and even though it is a cell line, a wide variety of potency rankings have been observed. Still, some studies are well in line with our observations. TBBPA and TCBPA were found to be of approximately equal potency in inducing adipogenesis (Riu et al., 2011). In another study on 3T3-L1, BPA and BPS had similar potency to induce adipogenesis, whereas BPF had no effect (Drobna et al., 2019). TBBPA was found stronger inducer than BPA in three cell models of a single study: 3T3-L1, mBM-MSCs and OP9 (Andrews et al., 2020). BPA was also found to be of similar potency to BPB and stronger than BPF (Masuno et al., 2005). Nevertheless, the main weakness of so far published comparative studies is that most were performed at a single concentration of bisphenols, rather than on a broader concentration range. A marked improvement in assay throughput, enabling dose-response studies, should help resolve similar conflicting data in future.

The effects of bisphenols on lipid accumulation have also been confirmed on gene expression level, where tested bisphenols tended to increase the expression of either late (CEBPα, PPARγ) or mature (FABP4, ADIPOQ, LPL) adipocyte markers, or both. Bisphenol effects were most pronounced on adiponectin. Interestingly, the expression of PPARγ, the key regulator of adipogenesis (Lee, 2017), was the least affected of all studied adipocyte markers. Only ROSI, had a low but detectable effect on PPARγ expression. Adipogenesis can be induced by multiple parallel pathways involving CEBPs, PPARg, GR, ER, thyroid hormone receptor (TR), bone morphogenetic proteins (BMPs) and more (Lee, 2017). Furthermore, each bisphenol is known to activate more than one nuclear receptor with different affinities. BPA alone, as the most widely studied bisphenol, is known to activate PPARγ, GR, ERα, ERβ, liver X receptors (LXRα and LXRβ), pregnane X receptor (PXR), CAR, ERRγ and progesterone receptor (PR) (Janesick et al., 2016; Liu et al., 2019). Since classical adipocyte differentiation *in vitro* relies on activation of GR with dexamethasone, which induces PPARγ, the crucial regulator of adipogenesis, it is very likely that at least these two nuclear receptors are involved in bisphenol's effects on adipocyte differentiation. TBBPA and TCBPA induce adipogenesis in 3T3-L1 primarily by activating PPARγ, while the effect of BPA on that receptor is much weaker (Riu et al., 2011). It was also found that the more halogenated the bisphenol, the more potent it is on activating PPARγ and less for ERα & ERβ (Riu et al., 2011).

Altogether, these data clearly confirm that the changes the bisphenols induce in human BM-MSCs are clearly adipogenic and likely a consequence of a complex interaction with multiple pathways. Further mechanistic studies are required to decipher their exact mode of action. However, the advantage of phenotypic assays, such as cell differentiation, is the possibility to detect the net main outcome even when multiple signalling pathways and mechanisms are involved.

The developed HCS method for adipogenesis of human MSCs offers some important advantages over commonly used murine 3T3-L1 cells. Apart from being human and primary cells, therefore more relevant for modelling human toxicological responses, MSCs are non-committed stem cells, which allows monitoring the interference of chemicals with the whole adipogenesis process. In contrast, 3T3-L1 cells are

preadipocytes, therefore already committed cells. Another crucial technical advantage comes from this difference, and that is the fact that only a fraction of all MSCs in culture become adipocytes. In conditions of low adipogenesis of hMSCs, as presented here, this results in well-separated adipocytes (Fig. 6e, Figs. S3–S6), allowing precise quantification of adipocyte number and analysis of the size of individual adipocytes using cell imaging. On the contrary, during the differentiation of 3T3-L1 cells, all cells are becoming adipocytes, differing only in the amount of lipids they have accumulated in a given time. This makes a rather dense monolayer of adipocytes, where individual cells can hardly be separated, and the exact quantification of adipocyte number and size is significantly impaired. Moreover, another considerable disadvantage for a cell line is that 3T3-L1 cells have been shown to significantly differ between batches depending on the cell source (Kassotis et al., 2017). However, even when using the same standardized protocol and the same batch of 3T3-L1 across different labs, considerable differences in adipogen potencies and assessment have been observed, as shown in a recently published study (Kassotis et al., 2021).

Using HCS imaging readouts to evaluate adipogenesis has a clear advantage for the assessment of compounds. The values of total lipids in the culture as used in conventional models of adipogenesis, expressed as lipids per cell, logically include in themselves information on changes in both adipocyte size and number together, and their fold induction represents the multiplication product of fold induction of adipocyte number and the fold induction of adipocyte size. Since the adipocyte size is decreasing with the concentration of some bisphenols, while the number of adipocytes is increasing, it is not surprising that some bisphenol treatments seem not to affect adipogenesis when looking at the total lipids changes, but show profound effects on adipocyte size and number when this endpoint is broken into these two main components. Understanding the impact of adipogens on adipocyte number and size not only brings us closer to their mechanisms of action but also indicates the potential consequences of this exposure on the whole organism, where an increase in adipocyte size is more connected with insulin resistance and an increase in adipocyte number may make the organism prone to gaining weight (Stenkula and Erlanson-Albertsson, 2018; Longo et al., 2019; Spalding et al., 2008). In addition, due to the ability to focus fluorescence measurement directly on lipid droplets and nuclei, the HCS method has a very low background noise, which greatly contributes to the increased sensitivity of the assay compared to conventional total fluorescence measurements.

Finally, for the first time, this study shows a mixture effect of six bisphenols on adipogenesis, indicating their additive nature. Six bisphenols were mixed at concentrations that were too low to induce a detectable effect on adipogenesis in single compound exposure. However, such mixture produced a strong and significant adipogenic effect, in line with the concept of *something from "nothing",* stating that a mixture of chemicals at their NOEC level can produce a marked effect on the same endpoint (Silva, Rajapakse and Kortenkamp, 2002). We also show how calculating relative potency factors from benchmark concentrations (BMCs) can help estimate additive effect of test compounds in a mixture, when tested at sub-active concentrations. The BMC approach has previously been used for testing if dose addition applies in chemical mixtures (Kienhuis et al., 2015) and this approach has been appointed as a useful method for this purpose by EFSA (EFSA et al., 2017). From the toxicological point of view, it is extremely worrisome to see such additive effects from concentrations declared non-active by the same assay. Since the exposure to chemical mixtures much more corresponds to reality than the exposure to a single chemical, it points out the need for chemical risk assessment in real-life mixtures, and even including chemicals that in single exposure induce a detectable adverse effect at concentrations higher than their human-relevant exposure concentrations.

The active concentrations of bisphenols in the present study were mainly in the lower micromolar range and are higher than those measured in human serum. However, for bisphenol A, as the most studied chemical, the median concentration found in the serum in an occupational setting is 0.13 μM, ranging up to 1.4 μM (Heinälä et al., 2017). It is noteworthy that in our study, due to the observed additive effect, the active concentrations of individual bisphenols in the mixture reached below 1 μM, which is markedly below the active concentrations of bisphenols in a single exposure and is in the range of human exposure concentrations in an occupational setting (Heinälä et al., 2017). Thus, we can speculate that mixtures of a multitude of chemicals, rather than just six of them that we are exposed to in our everyday life can induce adipogenesis at markedly lower concentrations, reaching well into the human-relevant exposure concentrations. This result clearly shows the difficulty of properly assessing the toxicological potential of a chemical by applying it in a single compound exposure. Future studies need to address further the identification and potency of adipogen mixtures in real-life exposures.

5. Conclusion

In conclusion, we present here a robust and sensitive quantitative *in vitro* assay with high content analysis readouts on primary human cells that can be used to assess potential obesogens and their mixtures. We present several rather ordinary but critical points in the cell culture of human mesenchymal stem cells that greatly contributed to the increased sensitivity of the model and enabled shortening of the whole differentiation protocol to 10 days. Finally, we show the model's applicability on the screening of bisphenol A derivatives, whose effects were known to be of lower intensity and therefore difficult to detect. All eight tested bisphenols induced adipogenesis, questioning their applicability as safe BPA replacement chemicals or as chemicals leading to significant human exposure. Flame retardants, TBBPA and TCBPA, were the most potent of tested bisphenols, increasing both the adipocyte size and number. BPF was the least potent, and BPC, BPB, BPA, BPS, and BPAF exerted an adipogenic effect of medium potency mainly increasing adipocyte number.

We show that a significant effect on adipogenesis can be obtained by mixing bisphenols at their sub-active concentrations, emphasizing the need to consider possible mixture effects in the risk assessment of chemicals. We present the first evidence indicating that the effects of bisphenols on adipogenesis are additive and can be well predicted from the relative potency factors obtained from the BMC modelling conducted on single exposure experimental data. Furthermore, the active concentrations of bisphenols in tested mixture exposure reached below 1 μM, which is within the relevant exposure levels observed in humans, indicating the need to investigate the toxicological effects of even less potent chemicals in the real-life mixture.

This study confirms the critical role that quantitative *in vitro* assays can play in the investigation and risk assessment of chemicals and chemical mixtures that we are exposed to in our daily lives.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement GOLIATH No. 825489 and Swedish Research Council for Sustainable Development (Formas) grant No. 2019-00375.

Data availability

Additional data are available as supplementary information. The remaining data are available upon request.

CRediT authorship contribution statement

Kalle Norgren: Investigation, Methodology, Formal analysis, Data curation. **Astrud Tuck:** Investigation, Methodology, Formal analysis, Data curation. **Antero Vieira Silva:** Investigation, Methodology, Formal

analysis, Data curation, Visualization, Writing – original draft. **Paula Burkhardt:** Investigation, Formal analysis, Data curation. **Mattias Öberg:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Vesna Munic Kos:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Acknowledgements

The authors wish to thank Hildur Palenius for help with the literature search.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.112842.

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