Chemosphere 349 (2024) 140852

Contents lists available at ScienceDirect

Chemosphere

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

Environmental chemicals change extracellular lipidome of mature human white adipocytes

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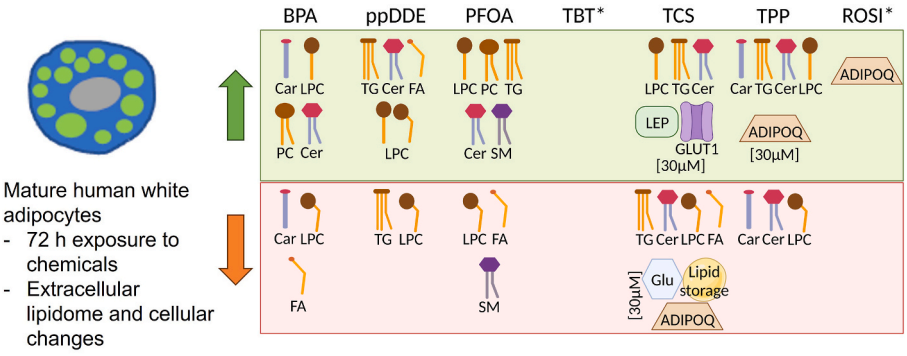
HIGHLIGHTS

- Certain environmental contaminants are metabolism disrupting chemicals (MDCs).
- MDCs strongly change extracellular lipidome of mature white adipocytes *in vitro*.
- Changes indicate possible systemic effects and implication in metabolic diseases.
- In addition, triclosan affects energy balance, and endocrine function of adipocytes.
- MDCs could cause adverse effects in the white adipose tissue of adults.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Mature human white adipocytes
Endocrine disrupting chemical (EDC)
Lipidomic
Energy homeostasis
Metabolic diseases
Metabolism disrupting chemical (MDC)

ABSTRACT

Certain environmental chemicals affect the body's energy balance and are known as metabolism disrupting chemicals (MDCs). MDCs have been implicated in the development of metabolic diseases, such as obesity and type 2 diabetes. In contrast to their well-known impact on developing adipocytes, MDC effects leading to altered energy balance and development of insulin resistance in mature white adipocytes, constituents of adult adipose tissue, are largely unclear.

Here, we investigated the effects of six well-established environmental MDCs (bisphenol A (BPA), perfluorooctanoic acid (PFOA), triclosan (TCS), p,p-dichlorodiphenyl-dichloroethylene (ppDDE), tributyltin chloride (TBT) and triphenyl phosphate (TPP)) on mature human white adipocytes derived from mesenchymal stem cells *in vitro*. We aimed to identify biomarkers and sensitive endpoints of their metabolism disrupting effects. While most of the tested exposures had no effect on adipocyte glucose consumption, lipid storage and assessed gene expression endpoints, the highest concentration of triclosan affected the total lipid storage and adipocyte

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https://doi.org/10.1016/j.chemosphere.2023.140852
Received 28 March 2023; Received in revised form 25 October 2023; Accepted 28 November 2023
Available online 2 December 2023

size, as well as glucose consumption and mRNA expression of the glucose transporter GLUT1, leptin and adiponectin. Additionally, an increased expression of adiponectin was observed with TPP and the positive control PPARγ agonist rosiglitazone.

In contrast, the lipidomic analysis of the cell culture medium after a 3-day exposure was extremely sensitive and revealed concentration-dependent changes in the extracellular lipidome of adipocytes exposed to nearly all studied chemicals. While some of the extracellular lipidome changes were specific for the MDC used, some effects were found common to several tested chemicals and included increases in lysophosphatidylcholines, glycerophospholipids and ceramides and a decrease in fatty acids, with possible implications in inflammation, lipid and glucose uptake.

This study points to early signs of metabolic disruption and likely systemic effects of mature adipocyte exposure to environmental chemicals, as well as to the need to include lipidomic endpoints in the assessment of adverse effects of MDCs.

Abbreviations

Acsl1	acyl-CoA synthetase long-chain family member 1
ADIPOQ	adiponectin
ANOVA	analysis of variance
BPA	bisphenol A
BPS	bisphenol S
Car	carnitine
Cd36	also known as Fat; fatty acid translocase
Cer	ceramide
CE	cholesteryl ester
Cpt1	carnitine palmitoyltransferase 1
CSS	charcoal stripped serum
DDE	p,p-dichlorodiphenyl-dichloroethylene
DDT	p,p'-dichlorodiphenyltrichloroethane
Dgat	diacylglycerol O-acyl- transferase
DM	differentiation medium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxid
ED0	exposure day 0
EDC	endocrine disrupting chemical
FA	fatty acid
Fabp4	fatty acid binding protein 4
GLUT1	glucose transporter 1
GLUT4	glucose transporter 4
GP	glycerophospholipids
HI FBS	heat inactivated fetal bovine serum
hMSC	human mesenchymal stem cell
INSR	insulin receptor
LEP	leptin
LPC	lysophosphatidylcholine
Lpcat	lysophosphatidylcholine acyltransferase
MDC	metabolism disrupting chemicals
MSC	mesenchymal stem cells
OPLS-DA	orthogonal partial least square discrimination analysis
PBS	phosphate buffer saline
PC	phosphatidylcholine
PCA	principal component analysis
PE	phosphatidylethanolamine
PFAS	perfluoroalkyl substances
PFOA	perfluorooctaonoic acid
PI	phosphatidylinositol
Pla2	phospholipase A2
PLS-DA	partial least square
PM	plating medium
Ppap2a	phosphatidic acid phosphatase type 2A
PPARγ	peroxisome proliferator activated receptor gamma
ppDDE	p,p-dichlorodiphenyl-dichloroethylene
ROS	reactive oxygen species
ROSI	rosiglitazone
RT	room temperature
RT-PCR	real time polymerase chain reaction
SD	standard deviation
SM	sphingomyelin
SP	sphingolipid
SphK	sphingosine kinase
TAG	triglyceride
TBT	tributyltin
TCS	triclosan
TPP	triphenyl phosphate
UHPLC-DTIM-QToF	Ultra High Performance Liquid Chromatography Drift Tube Ion Mobility Quadrupole Time of Flight

1. Introduction

In recent decades a substantial rise in metabolic diseases has been recorded, in particular obesity and type 2 diabetes (Chew et al., 2023). While major factors, including unhealthy diets, lack of physical activity and genetic predisposition, have been identified, it has become evident that various environmental chemicals, that humans are exposed to in everyday life, could also contribute to the development of metabolic diseases (Heindel et al., 2017). Such chemicals were most often found to interfere with nuclear hormone receptors and are therefore known as endocrine disrupting chemicals (EDCs). Exposures to EDCs have been associated with various health conditions regarding reproductive, nervous, thyroid, and other systems. Some chemicals have also been reported to have distinct metabolic effects, affecting energy handling, function of pancreatic and hepatic cells, insulin sensitivity, and the development of adipose tissue, contributing to the development of metabolic diseases, such as obesity, insulin resistance, type 2 diabetes, and non-alcoholic fatty liver disease (Sargis and Simmons, 2019). Since it is not yet clear whether all of these effects are of endocrine origin, the subset of chemicals causing these changes were named metabolism disrupting chemicals (MDCs) to account for all of their metabolic effects (Heindel et al., 2017).

Due to its complexity and interconnection of several tissues in metabolic processes ensuring energy balance, many of the effects of MDCs have so far mainly been detected at the organism level, either in *in vivo* experiments or in epidemiological studies on human subjects. This poses a great challenge for the regulation and toxicological assessment of new chemicals that could also have metabolic effects. There are currently no regulatory *in vitro* or *in vivo* tests for identifying MDCs which limits the possibility of regulation. Therefore, one of the aims of the EU Horizon 2020 project GOLIATH is to find suitable *in vitro*

methods that could detect metabolic disruption by MDCs (Legler et al., 2020). For that purpose, six known environmental MDCs have been selected and tested in various cellular systems representing metabolic processes crucial for maintaining a healthy energy balance in the body (Legler et al., 2020).

As a part of the GOLIATH project, in the present study we investigated the impact of MDCs on the function of mature human white adipocytes, which are the main component of adult white adipose tissue. Selected environmental MDCs include: a plasticizer bisphenol A (BPA), a surfactant perfluorooctanoic acid (PFOA), an antimicrobial triclosan (TCS), a metabolite of the insecticide DDT p,p-dichlorodiphenyl-dichloroethylene (ppDDE), organotin used in boat paint tributyltin chloride (TBT) and a plasticizer and flame retardant triphenyl phosphate (TPP). Humans are commonly exposed to all six selected compounds as reported in many biomonitoring studies (summarized by Legler et al. (2020)). An antidiabetic pharmaceutical, and a PPARγ agonist rosiglitazone was added to the study as a compound which markedly affects adipocyte metabolism in many ways, and can, therefore, be considered a pharmaceutical MDC. Most of the selected compounds have well-documented effects on developing adipocytes, in the process of adipogenesis (Norgren et al., 2022; Kim et al., 2016; Watkins et al., 2015; Modaresi et al., 2022; Shoucri et al., 2018; Pereira-Fernandes et al., 2013; Schmid et al., 2005; Guo et al., 2012; Tung et al., 2017). However, little is known about their possible effects on fully developed white adipocytes as found in adult adipose tissue.

Numerous, possibly adipocyte-related, metabolic effects have been observed for all six selected environmental MDCs in humans and animals (Legler et al., 2020). An extensive summary of the epidemiological evidence of the association of exposure to the selected 6 environmental chemicals with metabolism disruption has been provided by Legler et al. justifying their classification into MDCs and the need to understand their effects (Legler et al., 2020). For instance, exposure to BPA has been associated with type 2 diabetes and obesity in humans, and high adipogenicity and increased insulin resistance in liver and muscle in animal experiments. Several modes of action have been proposed from *in vitro* experiments, including the involvement of nuclear receptors, generation of reactive oxygen species (ROS) and effects on glucose uptake and cytokine production (Legler et al., 2020; Hinault et al., 2023; Heindel et al., 2022). Furthermore, strong evidence of association with either type 2 diabetes, gestational diabetes or obesity has been reported for BPA, TBT, PFOA and ppDDE from epidemiological studies on human subjects, as well as from *in vivo* studies as recently reviewed by Hinault et al. (2023).

The focus of MDC impact on adipose tissue has, so far, mainly lied on adipocyte progenitors in the context of fetal development. Considering that the metabolic effects of chemicals were often observed in adult organisms, it is necessary to also investigate mature fat cells, as present in adult adipose tissue, as possible target cells of MDCs. Mature adipocytes account for about 50% of adipose tissue total cells and about 90% of its total volume (Corvera, 2021). Apart from storing lipids, mature adipocytes have an important endocrine function and help maintain the energy homeostasis on the body (Coelho et al., 2013), the function rather distinct from their progenitors. It is, therefore, essential to also consider mature cells in the investigation of metabolism disruption by chemicals.

To investigate the nature of the effects of MDCs on mature adipocytes, we have studied the impact of selected environmental chemicals on glucose consumption, lipid storage and the expression of genes relevant for glucose handling, insulin sensitivity as well as for adipocyte endocrine function. Furthermore, we have conducted a lipidomic analysis of adipocyte extracellular medium using liquid chromatography-drift tube ion mobility coupled with high-resolution mass spectrometry (UHPLC-DTIM-QToF) to further investigate how the exposed adipocytes could induce systemic metabolic effects. To our knowledge, this is the first study of this kind, investigating effects of multiple MDCs on the function of mature human white adipocytes with potential systemic effects.

2. Materials and Methods

2.1. Compounds

Test chemicals: bisphenol A (BPA), perfluorooctanoic acid (PFOA), triclosan (TCS), p,p-dichlorodiphenyl-dichloroethylene (ppDDE), tributyltin chloride (TBT), triphenyl phosphate (TPP) and rosiglitazone (ROSI) were purchased from Sigma, and CAS and product numbers, and purity are available in the Supplementary Information Table S1. Compounds were dissolved in DMSO (D2650, Sigma) at 1000x higher concentration than the final test concentration. Stock solutions in DMSO were stored at −20 °C.

2.2. Cell culture and adipocyte differentiation

Human mesenchymal stem cells (hMSCs) were purchased from PromoCell (C-12974) in passage 2. The cells were cultured as described previously (Norgren et al., 2022) and used in experiments until passage 6. The cells originate from two adult human donors, one male (61 years old) and one female (66 years old). In gene expression and lipidomics experiments, only the cells from the male donor were used. These batches of cells were extensively characterized on adipogenesis in our previous study (Norgren et al., 2022) and here, gene expression of early and late adipocyte markers was investigated using the same primer sequences as previously.

For the assays of cytotoxicity, glucose consumption and lipid storage the cells were seeded in black-walled 96-well plates with μclear bottom (Cellstar, Greiner Bio One), and for RNA isolation and lipidomics in 12-well plates (Thermo Scientific Nunc™). Cells were seeded in the plating medium (PM) consisting of Dulbecco's Modified Eagle Medium (DMEM) with high glucose, GlutaMAX and phenol red and without pyruvate (Gibco, 61965) and 10% heat-inactivated fetal bovine serum (HI FBS) (Gibco). In 96-well plates, the cells were seeded on day −3, at the concentration of 4200 cells in 100 μL per well, with outer wells filled with 200 μL sterile phosphate-buffered saline (PBS) (Gibco). In 12-well plates the cells were seeded at a concentration of 44400 cells in 1.2 mL per well in PM. The plates were then incubated at 37 °C, 5% CO_2 for 3 days for the cells to create a confluent monolayer.

Differentiation was started (day 0) by replacing the medium in the wells with the same volume of differentiation medium (DM) consisting of DMEM, 10% HI FBS, 1 μg/mL insulin (Sigma), 0.25 μM dexamethasone (Sigma) and 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma). The cells were incubated at 37 °C, 5% CO_2 for 14 days and DM was refreshed on day 3, 7 and 10.

2.3. Exposure of adipocytes to MDCs

On day 14 of differentiation, the cells were exposed to test compounds. Experimental setup is shown in Fig. 1. Exposure concentration ranges were selected to cover the range of effective concentrations on hormonal/nuclear receptors and on the development of adipocytes as well as to reach as low as concentrations measured in human serum (Table S2). Compound solutions were prepared at final concentrations in the treatment medium (TM) consisting of DMEM without additives (Gibco, A14430-01), with 10% HI FBS, 1 gL^{-1} D-glucose (Gibco) and 2 mM glutamine (Gibco). Solubility of test compounds was monitored by macroscopic and microscopic visual examination and only in the case of TPP at 100 μM, small crystals have been detected by microscopic assessment using an inverted widefield microscope with 10x objective. The crystals did not seem to affect any other measured cell parameter.

Cells were washed with warm TM and exposed to compounds for 3 days at 37 °C, 5% CO_2. For the assays of cytotoxicity, glucose consumption and lipid storage, test compound samples in 96-well plates were performed in triplicates, and vehicle treated (0.1% DMSO)

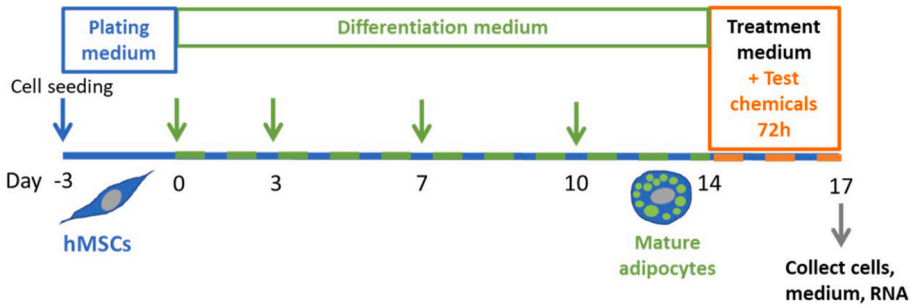

Fig. 1. Experimental setup used in this study. Human mesenchymal stem cells were differentiated into adipocytes for 14 days, and then exposed to test chemicals for three days.

negative control in hexaplicates (distributed evenly on the left and right side of the plate). In the 12-well plate (for RNA isolation and lipidomics), cells were exposed to chemicals in monoplicates, and negative control (DMSO) was done in duplicates (distributed on different plates). All experiments were performed independently three times unless stated differently.

At the end of incubation, supernatant from the 96-well plate was collected for the measurement of glucose concentration and membrane integrity/permeability (cytotoxicity). The cells were used for the measurement of lipid storage. From the samples in 12-well plates supernatants were collected and stored at −80 °C for lipidomic analysis and the cells were washed with PBS and lysed for RNA isolation.

2.4. Cytotoxicity

Membrane integrity/permeability was measured using Toxilight kit (Lonza) according to the manufacturer's protocol. Briefly, 10 μL of supernatant was mixed with 50 μL reconstituted AK reagent in an opaque 384 well plate (Lumitrac) and luminescence was read on plate reader after 10 min incubation.

2.5. Lipid storage

The cells were stained with BODIPY493/503 for lipid droplets, and Hoechst 33342 for cell nuclei, and analysed on a high content imaging system ImageXpress Micro (Molecular Devices) with MetaXpress software, as described previously (Norgren et al., 2022). Image acquisition and analysis setups are provided in the supplementary information (Table S3). Four imaging endpoints were assessed, as described previously (Norgren et al., 2022): number of cells measured by counting nuclei, total lipid content in the cell culture measured as total lipids (BODIPY493/503 fluorescence) per cell, number of adipocytes calculated from % BODIPY493/503 positive cells and nuclei count, and adipocyte size determined as lipids (BODIPY493/503 fluorescence) per adipocyte.

2.6. Glucose consumption

Glucose concentration in the medium was measured using Glucose-Glo™ assay (Promega, J6022) according to the manufacturer's instructions. Briefly, 20 μL of 200x diluted samples (in PBS) was mixed with 20 μL of the reaction mix in 384-well white opaque plates (Lumitrac). The plate was shortly spun (at 70 rcf, 1 min), shaken for 1 min and incubated for 1 h at RT in dark, before reading the luminescence on a plate reader. The concentration of glucose in samples was calculated by extrapolating the concentration from the glucose standard curve.

2.7. Quantitative RT-PCR

The RNA was isolated, and quantitative RT-PCR performed as described previously (Norgren et al., 2022) and the expression of adiponectin (ADIPOQ), leptin (LEP), glucose transporter 1 (GLUT1), glucose transporter 4 (GLUT4) and insulin receptor (INSR) was measured. Primers of selected genes were designed in PrimerExpress software (Applied Biosystems) and are listed in Supplementary Information Table S4. EF1A was used as the housekeeping gene.

2.8. Data analysis of cell based assays

For statistical analysis, fold change or fold induction values have been $\log_{10}$-transformed and analysed in one-way ANOVA with Dunnet's post-test using GraphPad Prism version 6.07 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

2.9. Lipid extraction of cell culture media

Lipids were extracted using the Folch method (FOLCH et al., 1957). Chilled chloroform/methanol (1:2, v/v, 1 mL) was added to the cell media (300 μL) and vortexed. Deionized water (100 μL) was added to the media. The sample was vortexed and centrifuged (10 min, 4 °C, 900 g), and the lower (organic) fraction was transferred to a new glass tube. The remaining portion of the sample was re-extracted by adding an equal amount of chloroform/methanol. The organic layers were combined, dried under nitrogen, and stored at −80 °C until analysis. Samples were resuspended in methanol/chloroform (1:1, v/v, 50 μL), diluted (1:5, isopropanol/acetonitrile/water, 2:1:1, v/v/v) and spiked with SPLASH lipidoMix (Avanti 330707, diluted 1:20) for LC-MS analysis.

2.10. Open profiling by LC-DTIM-MS

Samples were analysed using an Infinity II LC coupled to an 6560 IM-QToF MS (Agilent technologies, Santa Clara, USA) as previously described (Murgia et al., 2018). Lipid fraction was separated using an Acquity CSH C18 column (100 × 2.1 mm, 1.7 μm, Waters) set at 55 °C with water/acetonitrile (6:4, v/v) as solvent A and isopropanol/acetonitrile (9:1, v/v) as solvent B, both with 10 mM ammonium formate (positive mode) or 10 mM ammonium acetate (negative mode) as modifiers. Both ionization modes (positive and negative) were used to improve the formation of adducts of each lipid species, with glycerophospholipids, ceramides and phosphosphingolipids detected in both ionization modes, glycerolipids, cholesteryl esters and carnitines (Car) in positive mode, and fatty acids and sterols in negative mode. Mobile phase was pumped at 0.4 mL/min, with 40% solvent B which was increased to 43% at 2 min, to 50% at 2.1 min, followed by a further increase to 54% at 12 min, and to 70% B at 12.1 min. At 18 min, solvent B reached 99%, followed by a decrease to 40% at 18.1 min and a column re-equilibration for 4.9 min. The MS parameters were set at m/z range of 100–1700, maximum drift time of 60 ms using nitrogen (99.999% purity), trap fill time of 20 ms, trap release time of 0.5 ms, with frame rate of 0.9 frames/s. Other MS parameters included Dual AJS ESI ion source operated at 325 °C gas temperature, 5 L min^{-1} gas flow, 275 °C sheath gas temperature and 12 L min^{-1} sheath gas flow.

2.10.1. LC-DTIM-MS & MS/MS data acquisition

Agilent MassHunter Workstation Data Acquisition B.09.00 (9.0.9044.0) from The MassHunter suite was used for data acquisition. Agilent tuning mix solution was injected before the analysis to tune the instrument in the *m/z* range of 100–1700, and every ~ twenty samples to perform $^{DT}CCS_{N2}$ re-calibration. Additionally, Agilent reference mix was continuously injected alongside the samples using the secondary sprayer from the Dual Agilent Jet Stream electrospray to perform mass re-calibration. Pooled samples (QCs) were used for fragmentation identification using both iterative MS/MS mode and all ions IM-MS/MS mode, with the collision energy frame two fixed to 30V.

2.10.2. Data processing

IM-MS Reprocessor, IM-MS Browser, lipid annotator, PCDL (personal compound database and libraries), and Mass Profiler packages (B.09.00, Agilent Technologies, Santa Clara, USA) were used for mass recalibration, $DTCCS_{N2}$ re-calibration, lipid identification, lipid annotations managing and feature finding, respectively. Data pre-processing was performed following the Lipidomics Workflow Guide for Agilent 6560 Ion Mobility LC/Q-ToF. Lipid annotator (Koelmel et al., 2020) was set at Q score >50, mass deviation ≤10 ppm, fragment score ≥30 and total score >60, for both iterative and all ion mode. Feature finding was fixed at minimum ion count of 100 for maximum ion volume for IM-MS features, using isotope model for common organic molecules without halogens and limited to 1–2 charge species. Features were aligned for retention time (0.30 min), mass (5 ppm ± 2 mDa) and drift time (1.5%), and filtered for Q-Score ≥70 and abundance ≥1000. KniMet (Liggi et al., 2018), an MS-based metabolomics data processing pipeline, was used to retain features based on their presence in blanks (2-fold higher in samples, 55% threshold) and dilution series of QC samples (20% RSD, 55% threshold, 0.7 Pearson correlation). Missing values were imputed in the remaining features using the Key-nearest neighbour (KNN) approach. Peak intensities were normalized to an appropriate internal standard from the SPLASH lipidoMix. Additionally, features were also annotated based on accurate mass match with the LIPID MAPS database (10 ppm).

2.11. Multivariate statistical analysis

Principal components analysis (PCA), partial least square (PLS-DA) and orthogonal partial least square discrimination analysis (OPLS-DA) models were constructed using SIMCA 17.0 (Santorius, Umetrics®) to log base 10 transformed and Pareto scaled X variables. Statistical power of the models was evaluated by R2X, R2Y and Q2 parameters and cross-validation analysis (CV-ANOVA). Chemical concentration was used as a Y variable (PLS-DA models) and to set classes (OPLS-DA), with DMSO as control.

Models were used to explore the distribution of the samples (PCA), evaluate the differences in the lipidome between MDCs and controls, identify and visualize the individual discriminatory lipids (and features) driving the differences (PLS and OPLS-DA). Multivariate approaches are more robust when considering datasets where there are more variables than samples, avoiding multiple testing issues that impact univariate approaches. These approaches are also useful in identifying hidden (latent) relationships between variables.

Additionally, the lipid ratios were calculated by dividing the sum of each lipid within a class (normalized abundance) for each exposure (MDCs) with the sum obtained for the relevant control (DMSO or ED0). Unknown features were excluded, while those with ≥1 hit for annotation (glycerophospholipids, GP) were included in the corresponding class. The ratios were calculated to observe the total changes in the lipid classes as a whole after exposure to MDCs compared to the control. Such relative changes were easier to interpret biologically than some of the multivariate models that were built.

3. Results

3.1. Characterization of the adipocyte model

The differentiation of adipocytes was evaluated at the day 14 of differentiation, before the beginning of exposure to chemicals. The mRNA expression of adipocyte differentiation markers was measured by quantitative RT-PCR and morphologically. Both early and late markers were markedly induced relative to non-differentiated cells (Fig. 2a), and morphologically, adipocytes had well developed and many lipid droplets (Fig. 2b). The remaining non-differentiated cells in the adipocyte culture did not show any sign of lipid accumulation.

3.2. Cytotoxicity of test compounds

In this study, we examined the effects of six environmental MDCs and one pharmaceutical MDC (ROSI) in a 3-day exposure on mature human white adipocytes derived from mesenchymal stem cells. To confirm that tested chemicals are not cytotoxic in this experimental setup, we measured the release of the cytosolic enzyme adenylate kinase into the cell culture medium, as well as the number of cells by high content imaging (nuclei count) at the end of the exposure. The examined exposures to MDCs did not show any cytotoxic effects (Supplementary information, Fig. S1a and Fig. S1b).

3.3. Impact of MDCs on adipocyte glucose consumption

Next, we investigated if the tested exposures to MDCs had any effect on the extent of adipocyte glucose consumption from the cell culture medium (Fig. 3a). Glucose was measured in the medium collected after the 3-day exposure and compared to the initial glucose concentration in the medium (exposure day 0 - ED0). Nearly half of the initial amount of glucose in the culture medium was consumed after three days and all MDCs in all tested concentrations had no effect on the extent of glucose consumption by adipocytes, except for triclosan (TCS). At the concentration of 30 μM, TCS significantly increased glucose consumption, resulting in a rather low final glucose concentration.

3.4. Impact of MDCs on adipocyte lipid storage

To investigate if the tested MDCs affect the total lipid storage and distribution within adipocytes, at the end of the exposure cells were stained for lipid droplets and nuclei and analysed by high content imaging to measure the total lipids, adipocyte size and number as described previously (Norgren et al., 2022). None of the tested exposures to MDCs had any effect on the total lipids (measured as total lipids per cell, Fig. 3b), and adipocyte size (measured as total lipids per adipocyte, Fig. 3c), except for triclosan, which at the concentration of 30 μM significantly reduced both total lipids and adipocyte size. The percentage of adipocytes at the end of the experiment was constant across different exposures within each experiment (Supplementary information, Fig. S1c) and ranged 10–30% between experiments. The total number of adipocytes seemed significantly affected only by ROSI at 0.3 μM (Supplementary information, Fig. S1c), but since the effect did not show any concentration dependence, it is possible that it was an error in the measurement of multiple samples.

3.5. MDC effects on adipocyte gene expression

Impact of MDC exposures on adipocytes was also monitored at the level of mRNA expression of genes for glucose transporters GLUT1 and GLUT4, insulin receptor as well as two adipokines: adiponectin and leptin. GLUT1 expression was increased only by 30 μM TCS (Fig. 4a). The same exposure showed a trend to decrease GLUT4 in two experiments (Fig. 4b). Insulin receptor expression was not affected by any of the MDC exposures (Fig. 4c). Adiponectin expression was significantly

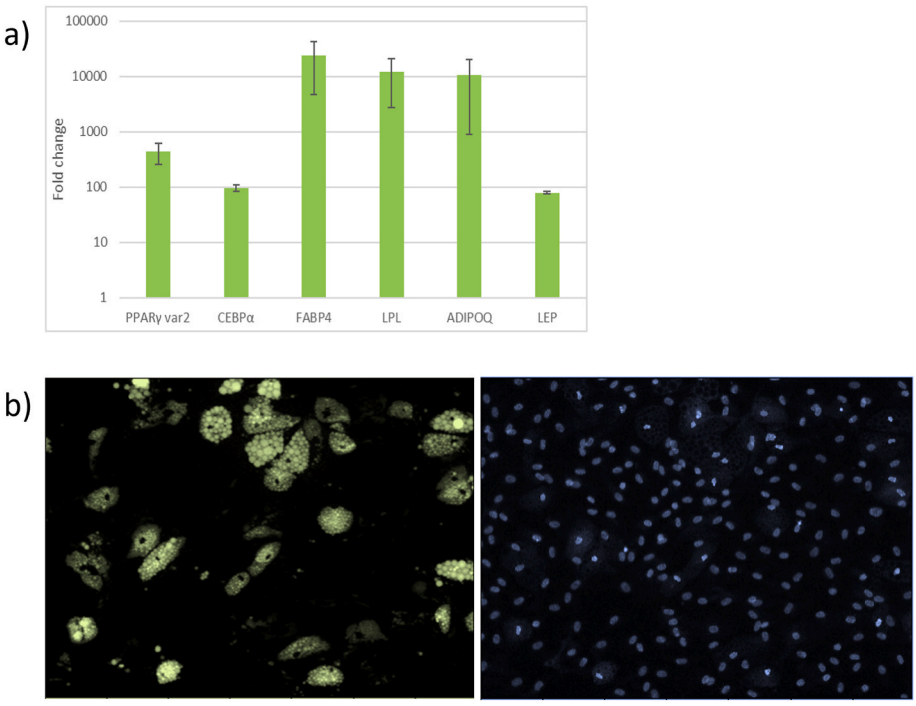

Fig. 2. Adipocyte characteristics after the 14-day differentiation protocol in a medium containing dexamethasone, insulin and IBMX as described in the Materials and Methods: a) increase in the mRNA expression of adipocyte differentiation markers determined by qPCR relative to non-differentiated hMSCs. Mean values of two independent experiments ± value range is shown. b) Representative images of adipocytes after 14-day-long differentiation (left: BODIPY493/503 stained lipid droplets, right: Hoechst 33342 stained nuclei).

increased by all tested concentrations of ROSI and 30 μM TPP, and decreased by 30 μM TCS (Fig. 4d). Leptin expression was the only one that increased over the 3-day exposure time even with the vehicle. The highest concentration of TCS significantly induced leptin expression further (Fig. 4e). BPA at concentration of 100 μM showed a trend to decrease GLUT4, ADIPOQ and LEP, albeit not statistically significant.

3.6. Lipidomic analysis of cell culture medium

To further investigate possible effects of MDCs on mature human adipocytes, lipidomic analysis was performed on cell culture medium taken before and after exposure to MDCs for 3 days. The lipid profile of adipocyte culture media was analysed by LC-DTIM-MS, with 18,545 (540 retained following exact mass database search and 75 MS^2 annotated) and 13,275 (245 retained following exact mass database search and 67 MS^2 annotated) features in positive and negative ionization modes detected, respectively. Note, two ionisations modes were used with the mass spectrometry to maximise the number of lipid species detected, as not all lipids will ionise in a given ionization mode (e.g. TAGs are only detected in positive ionization mode as ammonium ion adducts and free fatty acids are only detected as anions in negative ionization mode). Where lipids are detected in both positive and negative ionization mode we looked for consensus between the two modes of MS. Lipids identified (for both ionization modes) from 15 classes, included mostly triglycerides (TAGs), lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs), cholesteryl esters (CEs), ceramides (Cer), carnitines (Car), phosphatidylethanolamines (PEs), phosphatidylinositol (PIs), sphingomyelins (SMs) and fatty acids (FAs). Unknown features and those with more than one possible hit for annotation (glycerophospholipids, GP, sterols, and sphingolipids, SP) were kept for multivariate statistical analysis.

3.6.1. Extracellular lipidome baseline changes of mature adipocytes after 72 h cell culture

During the course of adipocyte culture of 72 h without any MDC exposure, there was a significant increase in PCs and TGs and decrease of LPCs and FAs compared to medium before exposure (ED0) (Fig. 5 and Table 1 for the discriminatory lipids, and Table S5 for the summary on the OPLS models). Accordingly, total PCs and TGs were upregulated while total LPCs, FAs, CEs and Car were downregulated in the supernatant after 72 h (Table S6 for the changes according to total lipid classes) (see Fig. 6).

3.6.2. Extracellular lipidome remodelling upon MDC exposure on mature adipocytes

Extracellular lipidome of adipocytes treated with MDCs for 72 h was compared to negative (vehicle) control DMSO-exposed cells for 72 h. The lipid profile was strongly affected by the studied chemicals and often in a concentration dependent manner. PLS models were constructed to assess the dose response to MDCs. Exposure to BPA, PFOA and TPP showed a concentration-dependent change in the extracellular lipidome, for lipids detected in both positive and negative mode (Fig. 6), while ppDDE and TCS showed a concentration-dependent change for lipids detected in negative mode only (Table S4). When OPLS-DA models were constructed to discriminate between control (DMSO, no MDCs exposure) and individual MDCs (all concentrations included), differences for ppDDE (positive mode), PFOA (negative mode), and TCS (both modes) exposure were identified. In contrast to PLS, the OPLS-DA models for BPA and TPP models did not reach significance. Furthermore, TBT and ROSI did not show separation between classes (OPLS-DA, DMSO vs. all concentrations), nor according to concentration (PLS, dose response) for both ionization modes (Table S5). See Table S6 for the summary of all the models.

The lipid remodelling in the supernatant (cell medium) varied according to the chemical the adipocytes were exposed to after differentiation. The classes of individual discriminatory lipids, found significantly affected in the developed statistical models (Table S5), are summarized in Table 1. Detailed information on the individual discriminatory lipids is provided in Table S7 (positive mode) and Table S8 (negative mode). Since the models for ROSI and TBT did not

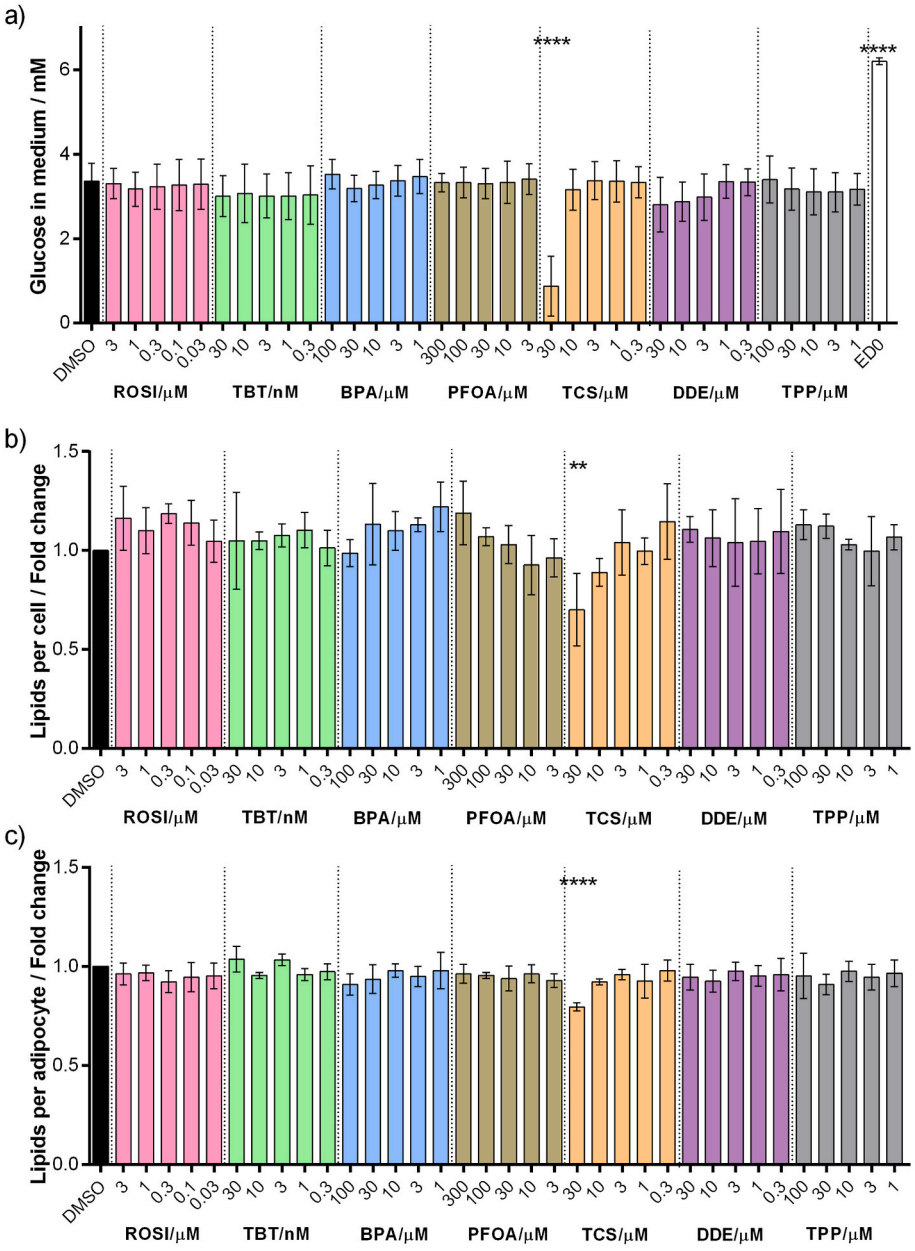

Fig. 3. Impact of MDCs on mature human white adipocytes. a) glucose consumption from cell culture medium, b) total lipid content and c) adipocyte size, after a 3-day exposure. In b) and c) results are expressed relative to the negative control (DMSO). Mean values of 3–4 independent experiments ± SD are shown (N = 3–4). ED0 – exposure day 0, sampled before the start of exposure. Statistical analysis was performed on log-transformed values. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 according to the one-way ANOVA with Dunnet's post-test, compared to DMSO.

reach statistical significance, no discriminatory lipids could be reported for these two compounds. From discrimination analysis, lipid species belonging to glycerophospholipids (detected in positive mode) were upregulated when cells were exposed to BPA, ppDDE, PFOA, TCS and TPP, compared to control (DMSO). In positive mode, discriminatory LPCs increased with BPA, ppDDE, TCS, TPP, but decreased with PFOA. In negative mode, saturated LPCs increased and unsaturated LPCs decreased with exposure to all chemicals. Discriminatory ceramides, detected in negative mode, were upregulated with BPA, ppDDE and PFOA, as well as upregulated with PFOA in positive mode. Interestingly, discriminatory FAs decreased with BPA, PFOA and TCS, but increased with ppDDE (Table 1, Table S7, Table S8).

Apart from the developed statistical models, we also calculated the total changes for each lipid class, as the sum of all lipids in a class (normalized abundance) relative to control (total lipid per class), to observe the changes within lipid classes, shown in Table S6. Particularly, total Car, Cer, LPCs, PCs, and TGs were higher, and FAs were lower after BPA exposure. In the case of TPP, total Cer, DGs and LPCs increased compared to control. While ppDDE exposure increased total LPCs, and TCS exposure decreased total FAs and PCs. Considering the total lipid per class changes, exposure to ROSI decreased the total FAs in the medium.

4. Discussion

In the present study, we investigated the impact of seven known MDCs on the function of mature human white adipocytes. In contrast to their well-documented effects on developing adipocytes in the process of adipogenesis, little is known about MDC impact on adipocyte energy handling and endocrine function when exposure is in the mature state.

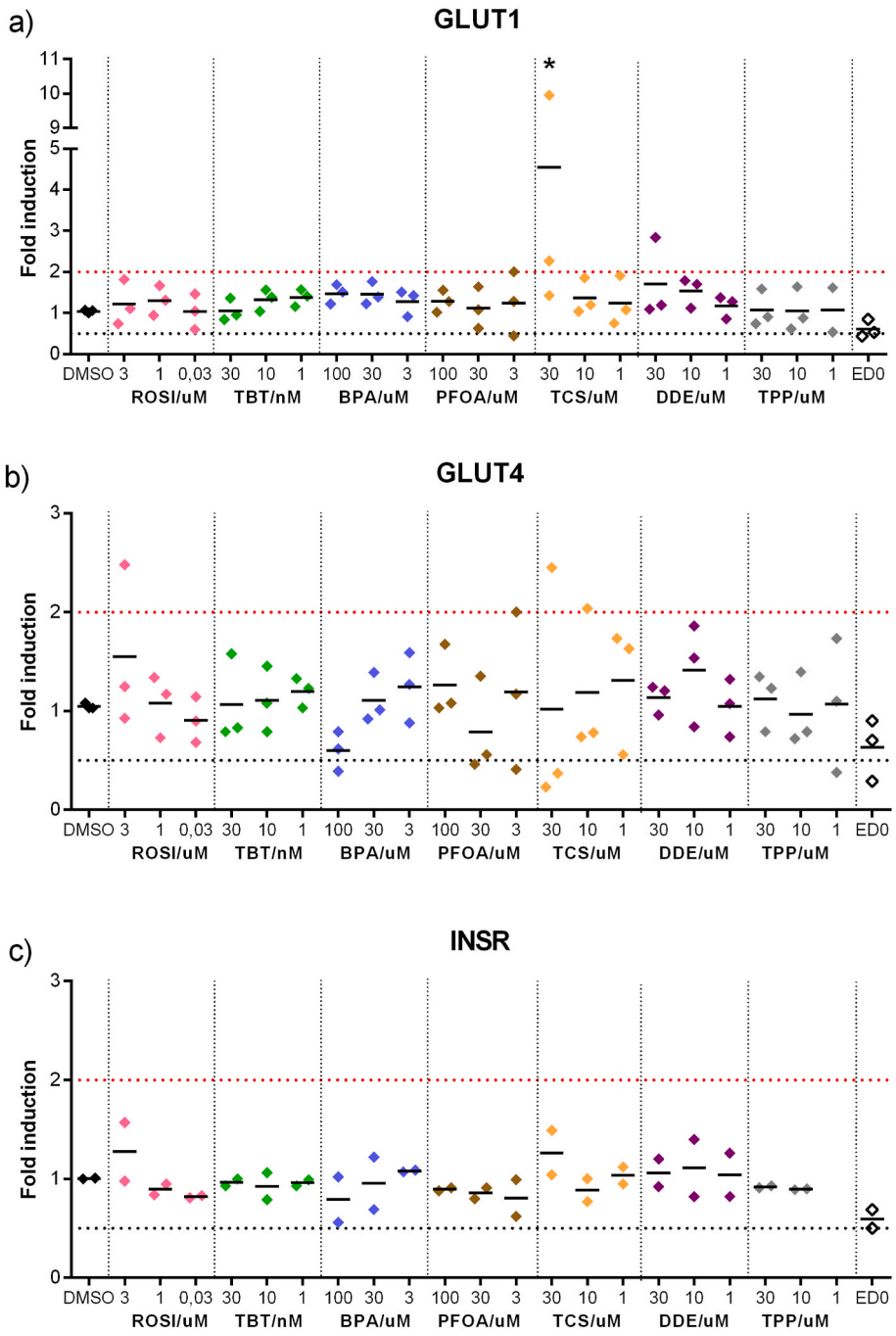

Fig. 4. Gene expression of a) glucose transporter GLUT1, b) glucose transporter GLUT4, c) insulin receptor, d) adiponectin and e) leptin in mature human adipocytes exposed to MDCs for 3 days. Gene expression is expressed relative to negative control (vehicle DMSO treated cells). Data points are from independent experiments, and black horizontal lines represent mean values of 2–3 experiments (N = 3, except c) N = 2). ED0 – exposure day 0, sampled before the start of exposure. Red and black dotted lines represent 2-fold increase or decrease in expression, respectively. Statistical analysis was performed on log-transformed values. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 according to one-way ANOVA with Dunnet's post-test, compared to DMSO.

Here, we report evidence of strong metabolism disruption in mature adipocytes by triclosan (TCS) detectable with applied cell-based endpoints, as well as profound effects in a concentration dependent manner, of nearly all tested MDCs identified by lipidomic analysis. The fact that MDC-exposed mature adipocytes induced the changes in the lipids outside the cells indicates a possible origin of systemic effects of MDCs, even in adult organisms.

4.1. *Mature adipocytes are resistant to the effects of MDC exposure*

Surprisingly, most of the test chemicals had no effect on either overall glucose consumption, production of adipokines adiponectin and leptin, or morphologically assessed lipid storage in adipocytes including number of adipocytes, their size, and total lipid content in the cell culture. The only MDC exposure that affected these endpoints was 30 μM triclosan (TCS). TCS was found to increase glucose consumption by mature white adipocytes, which is in agreement with the observed

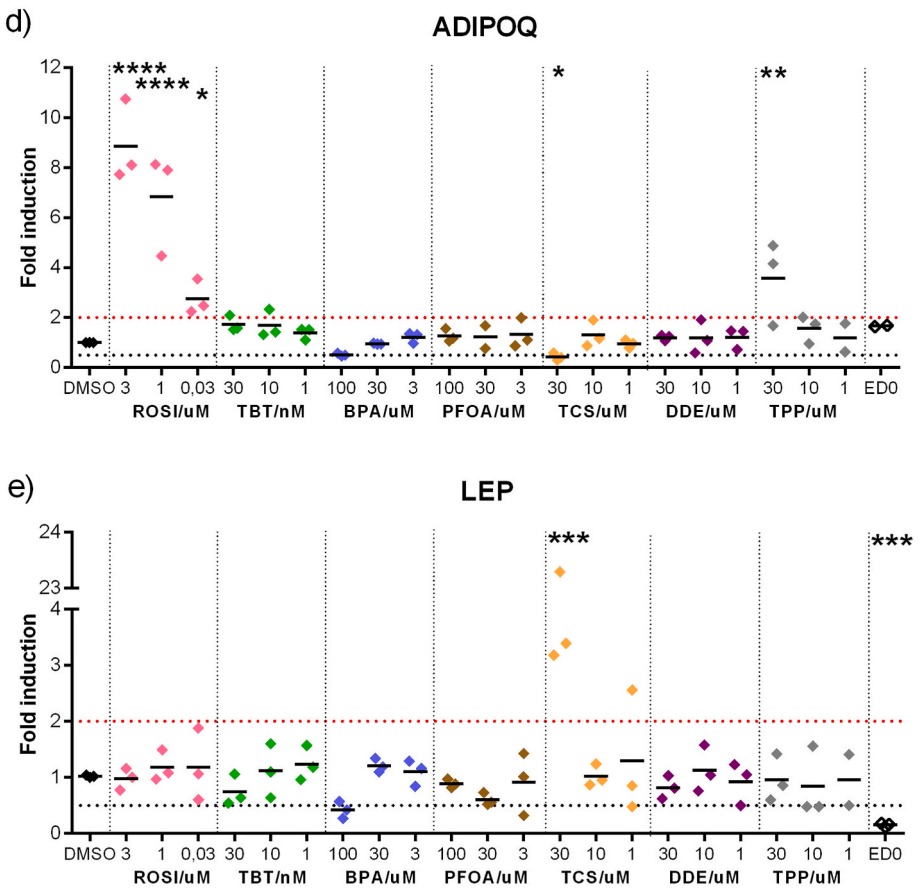

Fig. 4. (*continued*).

increase in GLUT1 glucose transporter expression by this chemical. In addition, adiponectin expression was reduced, whereas the expression of leptin was increased in the cells exposed to 30 μM TCS. Adiponectin and leptin are known to mostly act in antagonism, regulating energy balance, hunger and body weight. Moreover, adiponectin is connected with insulin sensitivity and normal body weight, whereas leptin is more often linked to inflammation and, thus indirectly, with insulin resistance (Finucane et al., 2009; Taylor, 2021). In this sense, effects of TCS on the expression of these two adipokines indicate metabolism disruption with possible contribution to insulin resistance at the organism level. Furthermore, 30 μM TCS was found to reduce the total amount of lipids per cell and adipocyte size, while leaving the adipocyte number unchanged. This indicates the activation of lipolysis and, together with the rise in glucose consumption, an increased energy expenditure in TCS-exposed adipocytes. To our knowledge, this profile of activity most resembles LXRα agonists, which were found to increase basal lipolysis in human (Stenson et al., 2011) and trout (Cruz-Garcia et al., 2012) adipocytes, decrease adipocyte size in white adipose tissue *in vivo* and *in vitro* (Dib et al., 2014), increase adipocyte GLUT1 expression (Pettersson et al., 2013) and basal glucose uptake (Laurencikiene and Rydén, 2012), as well as decrease adiponectin expression (Zheng et al., 2014). TCS is indeed predicted to bind to LXR at concentrations of around 30 μM by VirtualToxLab™ (Kenda et al., 2020) and shows a trend of LXRα activation at 10 μM in a reporter gene assay (Yueh et al., 2014). Our preliminary experiments indicate that LXRα is markedly expressed in our adipocyte model (with ΔCt of 9.2, normalized to EF1A housekeeping gene). Taken together, it is conceivable that 30 μM TCS affected mature adipocytes in our study via LXRα.

Apart from TCS effects, ROSI and TPP increased the expression of adiponectin, which is a known consequence of the activation of nuclear receptor PPARγ (Banga et al., 2009), which both compounds activate (Belcher et al., 2014). For the tested MDCs, the exposure concentrations used in our study were selected to cover the range of concentrations that were found to affect the activity of hormonal receptors, the activity on the development of adipocytes as well as to reach as low as measured concentrations in human serum (Table S2). However, the overall lack of observed effects (apart from TCS) on cell-based endpoints, such as glucose consumption, lipid storage and expression of relevant genes, indicates that mature adipocytes are more resistant to MDC exposure than developing adipocytes. This could possibly be explained by the fact that tested environmental MDCs are highly lipophilic molecules with $\log K_{ow}$ (logP) values higher than 3 (Table S2) and may be, at least partially, sequestered into and accumulated in lipid droplets, which are present and well developed in mature, but not in developing adipocytes. For polychlorinated biphenyls (PCBs), for example, it has been reported that the intensity of their uptake into different models of adipocytes is dependent on the amount of lipids present in the cells (Bourez et al., 2013). Furthermore, $\log K_{OW}$ was highly correlated with the extent of uptake for various compounds (Bourez et al., 2013). This corroborates the fact that compounds with high $\log K_{OW}$ accumulate in adipose tissue (Levitt, 2010; Jackson et al., 2017).

In line with our findings, TCS has been previously reported to reduce adiponectin gene expression, as well as lipid formation in developing adipocytes derived from hMSCs (Guo et al., 2012; Biemann et al., 2012). The effects of TCS in these studies were found at much lower concentrations than in our study. This further supports the conclusion that mature adipocytes are more resistant to metabolism disrupting effects of chemicals.

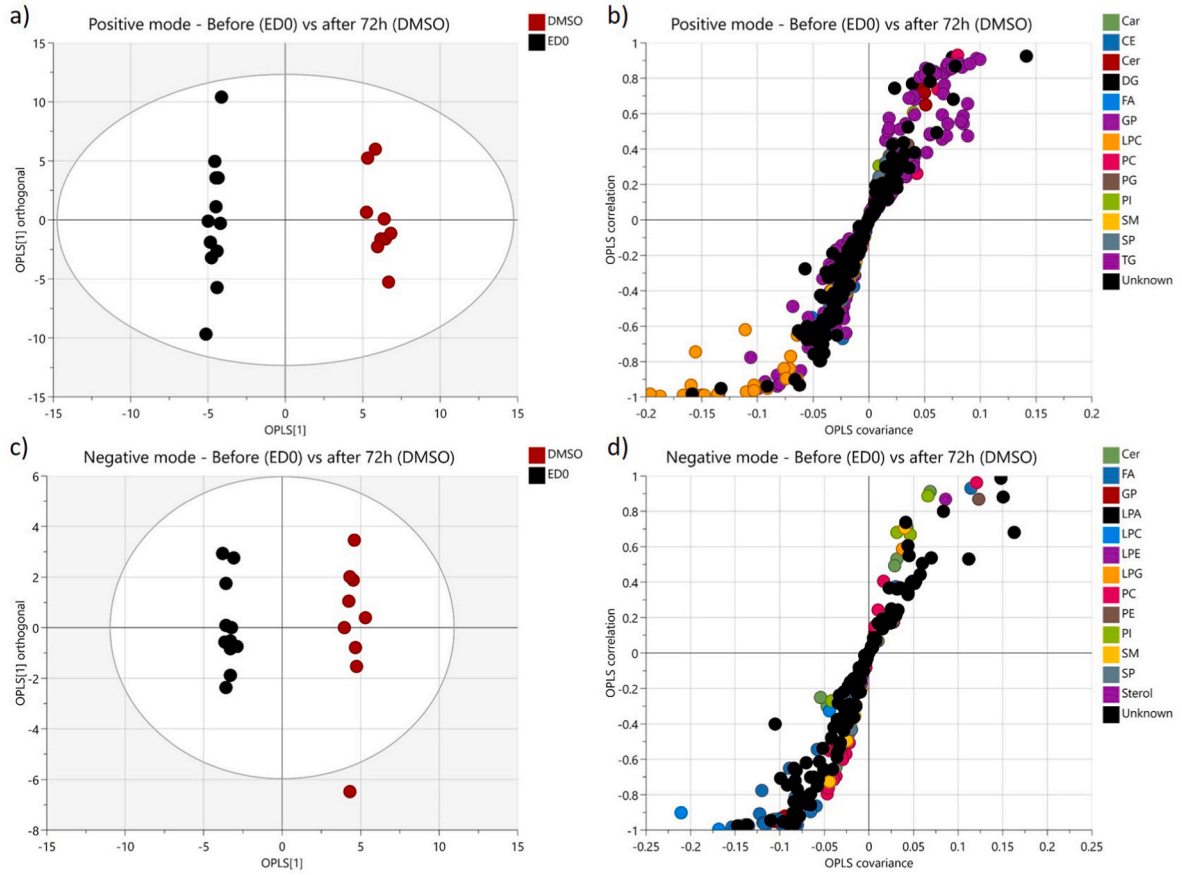

Fig. 5. Baseline lipid profile of culture medium of mature human adipocyte before (ED0) and after vehicle exposure for 3 days (72 h) without any MDC exposure. Principal component analysis score plots for a) positive and c) negative mode with the corresponding loading plots (b & d, respectively) showing the lipids detected in mature human adipocytes by LC-DTIM-MS. ED0 – exposure day 0, sampled before the start of exposure. Shown data points are from 3 independent experiments.

4.2. MDC-exposed mature adipocytes show strong changes in the extracellular lipidome

To further explore the effects of selected MDCs on adipocytes, especially on their potential systemic effects, we have analysed the extracellular lipidome of mature adipocytes exposed for 72 h. Since the exposure medium must contain 10% serum (FBS) to sustain adipocytes in culture, we have compared cell culture medium taken from adipocytes after 72 h of exposure to the fresh medium not applied on cells. This way we could, at the same time, monitor the baseline changes in serum lipids during 72 h of contact with cells, as well as detect the lipidome changes due to exposure to MDCs.

Interestingly, the extracellular lipidome of exposed adipocytes showed profound, statistically significant changes with five out of seven applied MDCs: BPA, PFOA, TCS, ppDDE, TPP. It is noteworthy that BPA, PFOA and ppDDE showed no effect whatsoever on any other energy handling and gene expression endpoint measured, yet they showed concentration dependent effects on extracellular lipid profile.

4.2.1. Extracellular lipid analysis of mature adipocytes revealed baseline changes in fatty acids, triglycerides and glycerophosphocholines

A relative increase of total and discriminatory TGs and PCs with a decrease of LPCs and FAs were observed in the supernatant of mature adipocytes after 72 h without any MDC exposure. Likewise, a decline in total carnitines and total CEs was observed (Table S5). These results suggest a cellular uptake of most FAs from the media as part of the production of TGs and PCs, as observed in the media, also indicating limiting cellular lipid storage and lipid droplets. In this context, a decrease of LPCs in the medium could contribute to the glycerophospholipids components of the membrane and the lipid-mediated signalling as part of the regular function of adipocytes. This is in agreement with the metabolic phenotype of differentiated 3T3-L1 adipocytes, characterised by a cellular increase of carnitine, FAs (including short, long and unsaturated chain length FAs), and TGs (unsaturated and odd chain length FAs into TGs), indicating dominant fatty acid metabolism in the cells (Roberts et al., 2009).

4.2.2. Remodelling of extracellular lipidome varied according to the MDC exposure on mature adipocytes

Lipid remodelling varied according to the MDC used, with fatty acids, lysoPCs, PCs and ceramides as the main affected lipids in the supernatant. Notably, these lipids have been largely related with obesity and metabolic diseases. Particularly, our results suggest a continuous cellular uptake of most FAs from the media that could contribute to lipid synthesis and lipotoxicity. Total FAs were further decreased after exposure to BPA, PFOA and TCS (including ROSI) but not ppDDE and TPP, when compared to the DMSO-treated control. Relevant discriminatory FAs were mostly unsaturated, including myristoleic acid, palmitoleic acid, hexadecadienoic acid, oleic acid, linoleic acid, dihomolinoleic acid, arachidonic acid, EPA and DPA. This is in agreement with previous studies, with cellular unsaturated FAs upregulated in 3T3-L1 adipocytes at differentiation upon BPA and BPS exposure (Zeng et al., 2021). Similarly, TCS-exposed mice (100 mg/kg/d) (Huang et al., 2020) showed hepatic FAs and TGs increased, and hepatic expression of genes related to FAs uptake and biosynthesis, which would indicate an increased FAs uptake upon chemical exposure and possible lowering of FA level in serum.

The effects of MDCs on TG regulation were not straight forward to

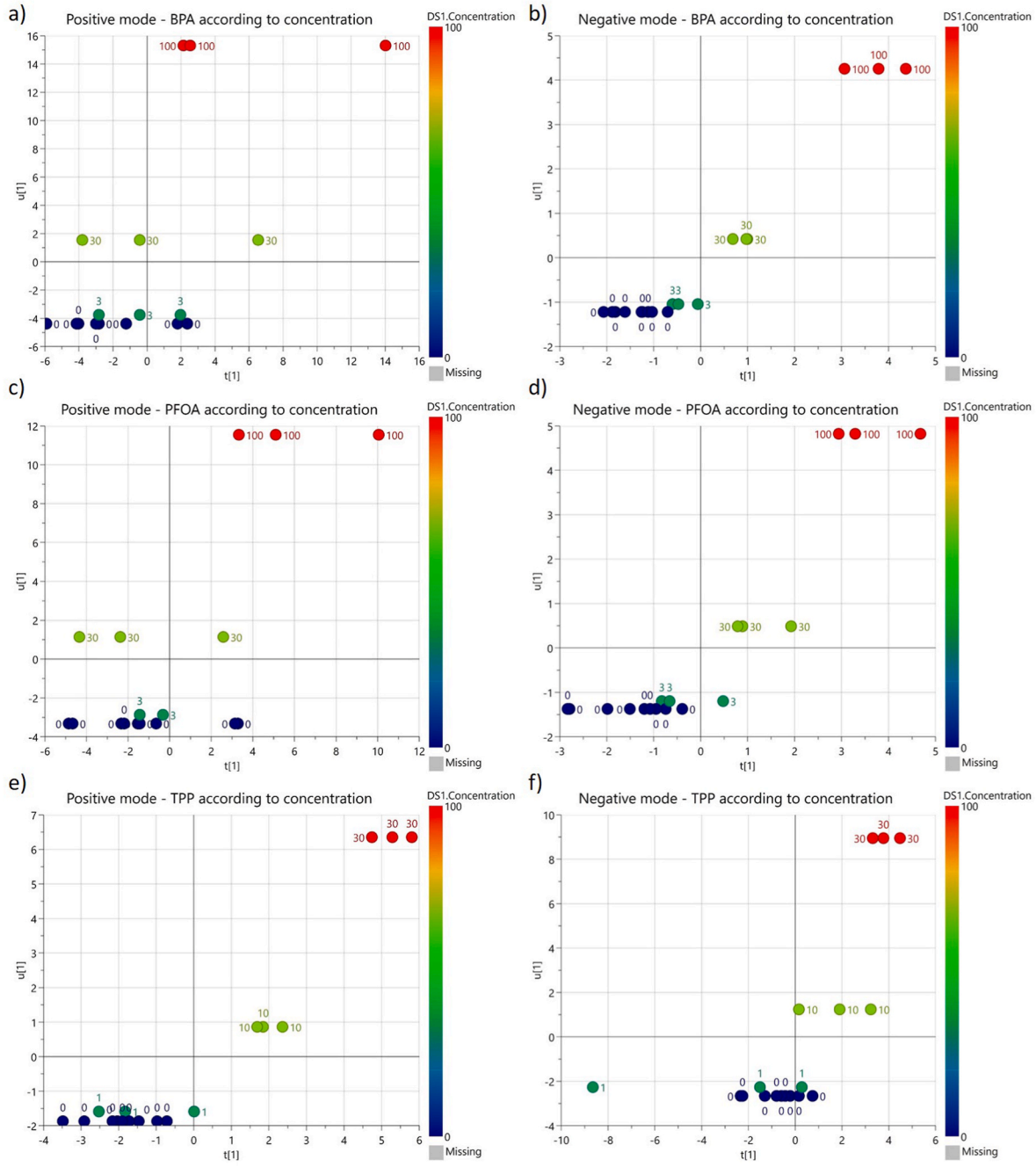

Fig. 6. Lipid profile of cell culture medium of mature human adipocytes after MDC exposure according to concentration. Score plots of BPA (a & b), PFOA (c & d) and TPP (e & f) exposure affected the lipidomic profile in a concentration dependent manner in both positive (a, c, e) and negative mode (b, d, f). Corresponding loading plots are shown in Figure S2. Data points are from 3 independent experiments.

interpret for mature adipocytes, with a relatively minor increase of total TGs in the supernatant observed only for BPA, and an increase of total DGs for TPP. In a previous study, mature HepaRG liver cells presented no changes in total TGs and a decrease in total DGs for BPA, and an increase in total DGs and total TGs for ROSI, PFOA and TBT (Franco et al., 2020) demonstrating that MDCs produce mixed results in other cell lines. Previously, 3T3-L1 pre-adipocytes exposed to BPA and BPS (0.1, 1 & 100 μM) during differentiation (0–8 days), showed evident systemic cellular lipidome changes throughout differentiation but TGs downregulation only at high BPA concentration (day 7), with formation of larger lipid droplets only at a later stage of maturity (day 16) (Zeng et al., 2021). Furthermore, pre-adipocytes have shown to retain 'memory' of prior cellular events that can alter cellular differentiation and the subsequent metabolic function of mature adipocytes (Reyes-Farias et al., 2021). However, in our study, adipocytes were exposed after maturation (day 14) and for a relatively short period (3 days). Therefore, the lack of lipid accumulation in our results could be explained by the capacity of the mature adipocytes to keep a 'normal' TGs biosynthesis levels when exposed to MDCs, comparable to the control.

Recently, ceramides, along with specific PC species, have attracted attention due to their clinical capacity to predict cardiovascular outcomes beyond traditional biomarker such as cholesterol (Carrard et al., 2021), and their association with insulin sensitivity and glucose control (Poss et al., 2022). In our study, total ceramides increased in the supernatant upon TPP and BPA exposure in our multivariate models. Previously defined serum ceramides, positively associated with insulin

Table 1
Summary of the classes of main individual discriminatory lipids affected in the culture medium of mature human adipocytes after the exposure to metabolism disrupting chemicals (MDCs) compared to control DMSO, as determined by multivariate statistical models. Comparison of baseline changes in lipidome was also done by comparing samples exposed to vehicle DMSO for 72 h to the fresh medium before exposure (ED0).

Comparison	ED0	Control (DMSO 72h)						
Ionization mode	DMSO (72h)	BPA	ppDDE	PFOA	ROSI†	TBT†	TCS	TPP
Positive								
Car		↕						↕
Cer				↑				↓
GP		↑	↑	↑			↑	↑
LPC	↓	↑	↑	↓			↑	↑
PC	↑	↑		↑				
SM				↕				
TG	↑		↕	↑			↕	↑
Negative								
Cer		↑	↑	↑			↕	↕
FA	↓	↓	↑	↓			↓	
LPC	↓	↑saturated ↓unsaturated	↑saturated ↓unsaturated	↑saturated ↓unsaturated			↑saturated ↓unsaturated	↑saturated ↓unsaturated

The arrows represent an (↑) increase or (↓) decrease for discriminatory lipids while those with an (↕) arrows represent both an increase and decrease of specific lipids observed within the class as determined by multivariate analyses after DMSO exposure at 72 h compared to before exposure (ED0), or MDC exposure compared to vehicle DMSO (both after 72 h). See Tables S7 and S8 for detailed information on the individual discriminatory lipids. Cells without arrows: presented no discriminatory lipids for that class of lipids compared to the control, or †models were not significant. Unknown features are not included in this table, while those with more than one hit for annotation (glycerophospholipids, GP) and those with more than three discriminatory lipids (VIP list, loadings and S-plot of significant models) in a class are included in this summary table.

resistance (HOMA-IR) (Poss et al., 2022), glycemic control (HbA1c) (Poss et al., 2022) and cardiovascular risk (Carrard et al., 2021), including Cer 18:1; O2/16:0 and Cer 38:1; O2, were detected in our study on cell culture medium, and significantly increased with PFOA and BPA by both multivariate analysis and univariate analysis (*t*-test) of the top dose compared with the control group, respectively. This means that the adipocytes exposed to studied chemicals could induce lipidome changes in the serum, characteristic for insulin resistance in human subjects. However, it should be noted that TPP exposure was associated with decreases of Cer 18:1; O2/16:0 and Cer 42:1; O2. Additionally, the PC species associated with cardiovascular risk (Carrard et al., 2021) (PC 16:0/22:5, PC 14:0/22:6, PC 16:0/16:0) and obesity (Chen et al., 2022) (PC 16:0/18:1, PC 18:0/16:1, PC 18:1/20:3) were not detected in the supernatant nor significantly disturbed (PC 16:0/16:1, PC 16:0/20:3 and PC 18:0/20:3) after the MDC exposure. Previously, upregulation of total cellular ceramides was observed in 3T3-L1 pre-adipocytes exposed to BPA (Zeng et al., 2021). Moreover, ceramides (Cer 34:1; O2, Cer 36:2; O2 and Cer 40:2; O2) upregulated in 3T3-L1 pre-adipocytes at early differentiation stage (day 2–3) exposed to BPA (Zeng et al., 2021) were found to be significantly increased in our study for PFOA, or not altered (Cer 42:2; O2, Cer 42:3; O2) in the supernatant. In mature HepaRG liver cells, on the other hand, TBT at high concentration (1 μM) and PFOS significantly reduced total ceramide levels (Franco et al., 2020).

An increase of total carnitines in the supernatant was observed for BPA, with a significant increment of Car 10:0, and decrease of Car 12:0, Car 14:2 and Car 14:1 in TPP. Similarly, TCS-exposed mice (100 mg/kg/d) showed an increase on long acylcarnitines (18:0, 18:2, 20:4 & 22:1) (Huang et al., 2020). On the contrary, mature HepaRG cells exposed (7 days) to obesogenic compounds (including ROSI, BPA, PFOA & TBT) presented no significant changes of intracellular short acylcarnitines (C2-5) (Franco et al., 2020). Acylcarnitines in human white adipose tissue act as intermediates within lipid metabolism, with long acylCar acting in the transportation of FAs into the mitochondria and cellular membranes (Lange et al., 2021). Considering that a nuclear receptor PPARα is a regulator of (acyl)carnitine metabolism, this further supports the importance of the finding that MDCs can modify these lipid intermediates and subsequently disturb lipid flux (Schooneman et al., 2013).

ROSI is known as a strong PPARγ agonist and hence it was included in our study as a positive control, for its effects on adipocytes. We did observe an increase in the expression of adiponectin, indicating PPARγ activation. In the lipidomic analysis, however, some lipids in samples exposed to ROSI, as well as TBT, seemed to be affected, but the overall multivariate models for these two compounds did not reach statistical significance. For example, ROSI was shown to decrease total FAs in the medium. Hepatic and adipose cell systems have previously shown that ROSI disrupts intracellular lipids. ROSI administration to mice (30 mg/kg/d x 8 w) induced serum adiponectin secretion but not its mRNA expression in inguinal white adipose tissue (iWAT). On the other hand, iWAT accumulation of PCs, PEs, CE, and TGs and no changes in Cer, SM and acylCar has been reported with ROSI exposure (Andrade et al., 2021). Total lipids, DGs, TGs, PCs, but not acylCar, CE, Cer, SM, increased in HepaRG liver cells exposed to a range of concentrations of ROSI after differentiation (0.1–1000 nM) (Franco et al., 2020), yet there are no reports on how this may have affected the lipids in the cell medium. However, the observed intracellular changes in lipids upon ROSI exposure in the mentioned studies is in agreement with our observation of a decrease in total FAs extracellularly.

4.2.3. *Extracellular lipid remodelling and potential metabolic pathways disrupted upon MDC exposure in mature adipocytes*

As mentioned above, supernatant lipidome remodelling varied according to the MDC used for mature human white adipocytes. These differences may impact distinctive metabolic pathways or may influence the capacity to modulate metabolic pathways. MDCs with comparable chemical structure and similar mechanism of action (e.g. BPA & BPS) have been shown to influence the lipidome differently after exposure (Zeng et al., 2021). Moreover, chemical effects varied according to the exposure concentrations, and not all *in vitro* and *in vivo* models have exhibited a dose-dependent response as observed here for some of the MDCs used. Collectively, these data emphasize the complex mode of action that may be involved in lipidome remodelling.

Nevertheless, tested MDCs share several common points in their effects on extracellular lipidome. Even TCS, which was the only tested MDC in this study that induced changes in cell-based endpoints (gene expression (GLUT1, adiponectin, leptin), lipid storage, glucose consumption), shared substantial lipidome changes with BPA, ppDDE and TPP. Glycerophosphocholines, particularly LPCs, were predominantly modulated after MDC exposure. Total LPCs increased for BPA, ppDDE and TPP, while total PCs increased for BPA (with specific PCs also significantly increased for BPA and PFOA) but decreased for TCS (Table S5). GPs are important in maintaining the structure and function

of cell membranes and various lipid-mediated signalling processes. Considering that LPCs have been associated with oxidative stress, inflammation, and mediators of FFA-induced lipoapoptosis (Stenson et al., 2011), our results support that BPA, ppDDE, TCS and TPP can alter the membrane components and the integrity of the cellular membrane. The induced changes on the membrane are likely subtle, early-stage changes, since no increase in the leakage of cytosolic enzyme adenylate kinase was detected (Fig. S1a). Furthermore, the observed decrease in arachidonic, linolenic and linoleic acids (with BPA, PFOA and TCS), and the related LPCs, suggests their possible uptake into cells where they could act as substrates for eicosanoid and other oxylipin production in inflammatory signaling.

Notably, PFOA exhibited particular changes in discriminatory alkyl ether-glycerophosphocholines (LPC O- decrease and PC O- increase), SMs modulation and distinct ceramides increase (Table S7). This is a clear alteration of sphingolipids in the cell medium, with these classes of lipids involved in cell-to cell signalling, cell intracellular processes, cell membrane components and function (Carrard et al., 2021). A distinct lipidome profile observed for PFOA may be linked to the extraordinary affinity of this chemical for phospholipids, that we reported previously (Sanchez Garcia et al., 2018). We may speculate that cell membrane bilayer could even be a real molecular target of PFOA and some other PFAS, leading to specific lipid-mediated adverse effects.

Combining the significant changes we observed in the lipidome remodelling, with previously reported results from *in vivo* and *in vitro* models, we can begin to clarify the potential metabolic mechanisms of MDCs in mature adipocytes. TCS (Huang et al., 2020), BPS (Zhao et al., 2017), BPA (Fang et al., 2022) and TBT (Ceotto Freitas-Lima et al., 2018) have been shown to upregulate the expression of genes associated with lipid synthesis (Dgat1/2, Acsl1, Cpt1, Pla2, Lpcat1/2, SphK, Ppap2a) and uptake (Cd36, Fabp4), and enhance pro-inflammatory cytokine secretion (TNF-α, IL-6, IL-1β). Therefore, exposure to MDCs not only alters the pathway of potent signalling molecules (SM-Cer) that can regulate cytokine secretion and cell fate, but also induces remodelling of fatty acids, enhances *de novo* phospholipids synthesis, TGs synthesis, and the general synthesis and degradation of GPs (The Lands' cycle for LPCs).

While the cellular lipidome has, so far, been the main target used to identify and characterize lipid composition, the extracellular lipidomic profile shown here provides useful insight into the early-stage mechanisms by which MDCs disrupt metabolic pathways, as well as the origin of possible systemic effects. Taken together, MDC-induced lipotoxicity may in part be attributed to inflammation-related metabolic pathways. The significant remodelling of PCs, lysoPCs, ceramides and fatty acid metabolism strengthens the association of MDCs exposure with insulin resistance and increased risk of obesity and diabetes. There are many epidemiological studies showing positive association between the exposure to chemicals, such as PFOA, ppDDE, BPA and TCS, and adverse metabolic effects, such as adiposity, glucose handling, metabolic syndrome, and type 2 or gestational diabetes (reviewed by Legler et al., 2020) with the details on epidemiological studies in Supplementary Information). Our present study points to extracellular lipid-related signals as possible mediators in the adversity of MDCs.

Finally, the impact of MDCs is often studied in relation to the developmental origins of health and disease (DOHaD), presuming that the exposure to chemicals has the strongest impact on persons' health if it happened in certain sensitive periods of organism and tissue development (Hoffman et al., 2021). Here, we show that metabolism disruption in adipocytes with potential systemic effects could also be happening in fully developed cells, as present in adults.

4.3. Limitations and future perspectives

There are several limitations in the presented study that need to be considered before drawing more general conclusions. The study was done on mesenchymal stem cells differentiated into mature adipocytes grown and exposed *in vitro*. Adipose tissue, however, contains a significant number of other cell types, such as immune cells, endothelial cells, fibroblasts, and progenitor cells (Corvera, 2021), which could affect the overall response of mature adipocytes to MDCs. Furthermore, in human MSC differentiation models, the obtained culture of adipocytes still contains some non-adipocyte cells (cells that do not accumulate any lipids), likely fibroblasts. We have controlled for this using high content screening and found that their number remained constant across various exposures (Supplementary information). It is, however, possible that the effects on some lipid species could be due to an effect on these non-adipocytes. In future work, the observed effects would need to be confirmed *in vivo*.

To be able to test multiple chemicals representing diverse chemical space and covering broad concentration range, the study was conducted on cells from two donors. The most prominent effects, mainly coming from lipidomic analyses, would in future need to be studied on cells from larger number of donors, and diverse in terms of sex, race and age, to draw conclusions for broader population. In addition to the extracellular lipidome, intracellular lipidomic profiling could be used to complement metabolic pathway disruption on mature adipocytes and facilitate comparability between studies.

Finally, the test concentration range in this study reaches the concentrations that are sometimes well beyond the ones that the general human population is exposed to in their daily life. Here, we used an approach to assay development focusing on hazard identification, to develop screening assays that are applicable for various kinds of exposures, even accidents. Furthermore, compared to rather long, sometimes even life-long, exposures to MDCs in real life, the *in vitro* models for metabolism disruption, have a quite short exposure period, meaning that they could possibly miss the effects that require longer time and interaction of multiple tissues and organs to develop. We hope that the detected changes in the extracellular lipidome of mature adipocytes reported here will help guide future research of complex MDC effects on the whole organism.

5. Conclusion

The present study investigated the impact of seven known metabolism disrupting chemicals (MDCs) on adipocyte function. When exposed in the mature state, human adipocytes were quite resistant to the effects of MDCs in terms of glucose consumption, expression of relevant genes and morphologically assessed lipid storage. Triclosan (TCS) was the only compound that affected all these endpoints, albeit at a rather high concentration, indicating metabolism disruption in these cells. On the contrary, lipidomic analysis of cell culture medium revealed significant changes in the extracellular lipidome even for chemicals that showed no effects on any other studied endpoint. The observed changes were mostly concentration dependent and indicated a clear disruption of lipid metabolism and/or transport at this level, providing the evidence of the early effects of MDCs on mature human white adipocytes. The total effects of MDCs on mature adipocytes are summarized in Fig. 7. Since the changes in lipid profile were observed outside the adipocyte cell (in culture medium), they could, therefore, have systemic effects, for example to contribute to the development of insulin resistance at the organism level. The fact that the changes were detected on fully developed adipocytes indicates the possibility of a new way of metabolic disruption by chemicals in white adipose tissue of adults. Further research is needed to fully understand the mechanisms leading to the changed extracellular lipid profiles and their ultimate consequences. The study sheds light on the complexity of metabolism disruption by environmental chemicals and shows the importance of including lipidomic endpoints in the assessment of MDCs.

Author contribution statement

PB – Validation, Formal analysis, Investigation, Data curation,

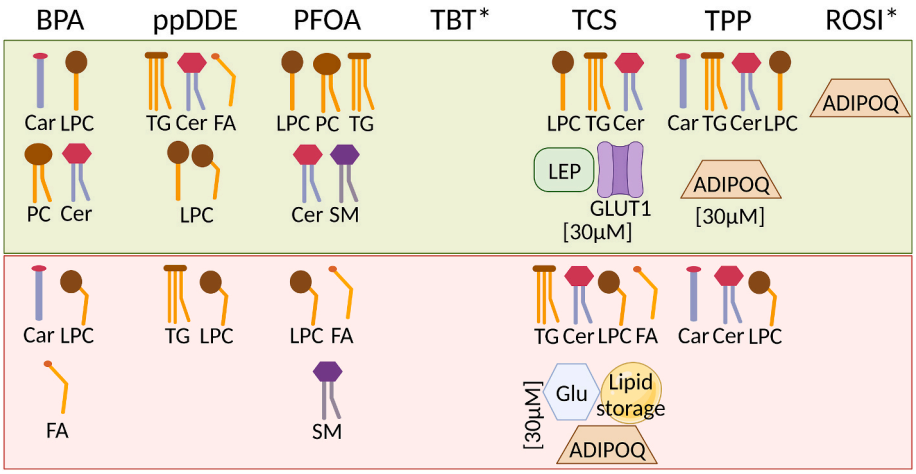

Fig. 7. Summary of the effects of MDCs on mature human white adipocytes in 72 h exposure. Upper (green) panel – features that were increased, lower (red) panel – features that were decreased upon exposure. Shown lipids represent the classes of discriminatory lipids found by multivariate analysis of the extracellular lipidome (cell medium). The figure was made using BioRender.

Writing – original draft, Visualization; SAPD – Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization; ARRT – Methodology, Formal analysis, Investigation; KN – Methodology, Formal analysis, Investigation; XL – Formal analysis, Investigation; VN – Investigation; JLG – Conceptualization, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition; VMK – Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Funding

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

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.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Prof Matej Oresic for sharing his valued scientific advice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140852.

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