Adipogenic activity of chemicals used in plastic consumer products

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Table 1

Abstract

Bisphenols and phthalates, chemicals frequently used in plastic products, promote obesity in cell and animal models. However, these well-known metabolism disrupting chemicals (MDCs) represent only a minute fraction of all compounds found in plastics. To gain a comprehensive understanding of plastics as a source of exposure to MDCs, we characterized all chemicals present in 34 everyday products using nontarget high-resolution mass spectrometry and analyzed their joint adipogenic activities by high-content imaging. We detected 55,300 chemical features and tentatively identified 629 unique compounds, including 11 known MDCs. Importantly, chemicals that induced proliferation, growth, and triglyceride accumulation in 3T3-L1 adipocytes were found in one third of the products. Since the majority did not target peroxisome proliferator-activated receptor γ, the effects are likely to be caused by unknown MDCs. Our study demonstrates that daily-use plastics contain potent mixtures of MDCs and can, therefore, be a relevant yet underestimated environmental factor contributing to obesity.

Teaser

Plastics contain a potent mixture of chemicals promoting adipogenesis, a key process in developing obesity.

17 # Introduction

18 Obesity is a global pandemic that generates a considerable burden of disease, in particular
19 considering comorbidities such as type 2 diabetes, cardiovascular diseases, hypertension, non-
20 alcoholic fatty liver, stroke, and certain types of cancer (*1*). Worldwide, the number of obese people
21 has nearly tripled since 1975, and more than 41 million children under the age of five were
22 overweight or obese in 2016 (*2*). This is problematic since a high body mass index (BMI) is one of
23 the top risk factors for deaths (*3*), and overweight in childhood or adolescence is a good predictor
24 of adult obesity (*4*). Accordingly, a high BMI contributed to four million deaths globally in 2015 with
25 cardiovascular diseases as leading cause of death followed by diabetes, chronic kidney diseases
26 and cancer (*5*).
27 This public health problem has mainly been attributed to genetic background and changes in
28 lifestyle, such as diet, exercise, sleep deficiency, and aging. However, epidemiological evidence
29 suggests that these factors insufficiently explain the magnitude and speed of the obesity pandemic
30 (*6*). For instance, even when normalizing for caloric intake and exercise, the BMI of US adults
31 increased by 2.3 kg m^{-2} in 2006 compared to 1998 (*6*). Consequently, identifying and understanding
32 other environmental factors than lifestyle is crucial to manage obesity (*7*). Given that the endocrine
33 system controls appetite, satiety, metabolism, and weight, exposure to endocrine disrupting
34 chemicals is one such factor (*8*). In addition to prominent endocrine disruptors, such as the biocide
35 tributyltin and the pesticide dichlorodiphenyltrichloroethane (DDT), plastic chemicals such as
36 bisphenols or phthalates disrupt metabolic functions and promote obesity in cell and animal
37 experiments (*8*). This is further supported by epidemiological studies that have linked weight gain
38 in humans to bisphenol A (BPA) exposure (*9*), while contradicting outcomes have been reported
39 regarding a link to phthalate exposure (*10-12*).
40 Considering the chemical complexity of plastic consumer products, bisphenols and phthalates
41 represent only the tip of the iceberg. A final article often consists of one or more polymers, multiple
42 intentionally added substances, such as fillers or additives, as well as non-intentionally added

43 substances, for instance residues from the manufacturing (*13*). Based on regulatory inventories,
44 over 4000 substances are associated with plastic food packaging alone (*13*) and, overall, 10,547
45 chemicals are known to be used in plastics (*14*). Moreover, empirical data suggests that plastics
46 contain more chemicals than currently known. Using nontarget chemical analysis, we detected
47 hundreds to thousands of chemicals in plastic consumer products, most of these remaining
48 unknown (*15*). Importantly, the totality of plastic chemicals in a product was toxic *in vitro*, inducing
49 baseline toxicity, oxidative stress, cytotoxicity, and endocrine effects.
50 Building on these results and the fact that bisphenols and phthalates are known metabolism
51 disrupting chemicals (MDCs; *8, 16, 17*), we hypothesized that MDCs are present in plastic
52 consumer products and that metabolic disruption might represent a common but understudied
53 toxicological property of plastic chemicals. To explore this, we used the same plastic consumer
54 products we have extensively characterized previously (*15*), and investigated the extracts'
55 adipogenic activity in differentiation experiments with murine 3T3-L1 cells. Following exposure to
56 MDCs, 3T3-L1 preadipocyte differentiate into adipocytes, accumulate triglycerides until they finally
57 resemble mature white fat cells (*18*). The bioassay targets the induction of adipogenesis at the
58 cellular level and represents a well-established *in vitro* model for metabolic disruption *in vivo* (*19*).
59 We performed optimization experiments and applied high-content fluorescence microscopy
60 combined with an automated image processing to increase the sensitivity and throughput. We also
61 investigated the underlying mechanisms of the adipogenic response and tested whether the
62 extracted plastic chemicals activate the human peroxisome proliferator-activated receptor gamma
63 (PPARγ) or glucocorticoid receptor (GR). We selected PPARγ as a key regulator of adipogenesis
64 (*20*), and included GR because glucocorticoids are important regulators of lipid metabolism (*21*).
65 Accordingly, an excess of agonists for these receptors is associated with obesogenic effects in
66 animal models and humans (e.g., weight gain; *19*). Moreover, we performed nontarget, ultra-high
67 performance liquid chromatography coupled to a quadrupole of flight spectrometer (LC-QTOF-
68 MS/MS) to characterize the chemicals present in plastics and compare these with a list of
69 compounds known to induce adipogenesis.

70 # Results

71 **Adipogenic activity of plastic consumer products.** To exclude cytotoxic effects masking the
72 adipogenic response, we used nuclei count data to assess cytotoxicity. Most extracts were not
73 cytotoxic up to the maximum concentration tested (3 mg plastic well^{-1}), except for PP 4, PUR 3 and
74 PUR 4. The latter two were the most cytotoxic samples with the highest noncytotoxic concentration
75 (HNC) of 0.75 mg plastic well^{-1}. The HNC for PP 4 was 1.5 mg plastic well^{-1} (Fig. S1). To assess
76 the induction of adipogenesis by the plastic extracts, we present the numbers of adipocytes and
77 mature adipocytes in the cell populations and the total lipid droplet count per image for the HNC of
78 each sample. Data were compared to both vehicle and rosiglitazone–treated controls (Fig. 1).
79 Further specifications on the endpoints of the automated image processing, dose-response
80 relationships and example images can be found in the supplementary material (Fig. S2–S17).

81 The extracts of eleven plastic consumer products induced adipogenesis with four samples having
82 an equal or stronger effect than the maximal response of cells exposed to rosiglitazone (PVC 2 and
83 4, PP 4, PUR 3). Similarly to rosiglitazone (Fig. 1A), the proliferative effects induced by plastic
84 extracts was driven by an increase in the numbers of adipocytes and mature adipocytes, while the
85 number of preadipocytes remained stable (Fig. 1C). Regarding the polymer type, the most potent
86 extracts were PUR and PVC, with seven out of eight samples inducing adipogenesis, whereas for
87 PP, PS and LDPE only specific samples induced adipogenic responses. In contrast, PET, HDPE,
88 and PLA samples were consistently inactive. The same pattern is reflected by the lipid droplet count
89 data (Fig. 1D). Here, however, some additional samples induced a slight increase in lipid droplets
90 (LDPE 1, PS 1, PP 2).

91 Given the propensity of environmental pollutants to promote unhealthy adipogenesis, we used the
92 single-cell data to explore a size shift towards hypertrophy and increased accumulation of
93 triglycerides in comparison to rosiglitazone (Fig. 2). We here present the results of one out of four
94 experiments due to the large size of the dataset (results of the other experiments can be found in
95 Fig. S18). Exposure to rosiglitazone dose-dependently increased the lipid content of adipocytes

96 with a median lipid droplet area of 137 pixels cell^{-1} in the lowest concentration to 290 pixels cell^{-1} in
97 the highest concentration (Fig. 2A). The average fluorescence intensity of the lipid droplets
98 remained stable (Fig. 2B), thus the adipocytes increased in size in response to rosiglitazone but
99 triglyceride accumulation within the droplets remained constant. Compared with the maximal
100 response to rosiglitazone, adipocytes exposed to many of the active plastic extracts had a higher
101 lipid content, resulting both from an increase in adipocyte size and from the amount of triglycerides
102 contained within the lipid droplets. The lipid droplet area per adipocyte was greater in nine out of
103 the eleven active samples with a median increase of 21.6–114% (PS 2–LDPE 4). In line with that,
104 the average fluorescence intensity was higher in ten out of the eleven active samples with a median
105 increase of 25.1–60.4% (PS 2–PVC 4). These effects are consistent over all experiments, except
106 for PVC 3 (Fig. S18).

107 **Reporter gene assays.** We observed a higher cytotoxicity of the extracts on the U2OS cells
108 compared to the 3T3-L1 cells with five samples being cytotoxic. The most cytotoxic sample was
109 PP 4 with a HNC of 0.19 mg plastic well^{-1}, followed by PS 2, PP 3 and PLA 1 as well as PVC 2 with
110 a HNC of 0.38 and 0.75 mg plastic well^{-1}, respectively (Tab. S1).

111 None of the samples activated GR (Fig. S19) and five extracts activated PPARγ (Fig. 3A). PLA1
112 was the most potent sample with a median receptor activity of 34.7%, followed by PS 2 (24.4%),
113 PVC 2 (10.3%), LDPE 2 (8.4%) and PVC 1 (7.3%). Accordingly, the PPARγ activity of plastic
114 chemicals is a poor predictor of their adipogenic activity (Fig. 3B), except for PVC 2 and PS 2 which
115 induced both PPARγ and adipogenesis at similar effect concentrations. Moreover, three out of the
116 five samples activating PPARγ did not induce adipogenesis in 3T3-L1 cells (PLA 1, LDPE 2,
117 PVC 1).

118 **Chemicals tentatively identified in plastics.** Using nontarget GC-QTOF-MS/MS, we previously
119 identified 260 unique chemicals in extracts of the same plastic products (*15*). This corresponds to
120 231 tentatively identified chemicals with 227 unique PubChem CIDs in the samples used in this
121 study (Tab. S2). In the nontarget LC-QTOF-MS/MS analysis, we detected in total 55,300 features
122 (i.e., unidentified chemicals) across all samples that were only present in samples or had a >10-

123 fold higher abundance compared to the blanks. Here, the number of features in individual samples
124 ranged from 107 (HDPE 2) to 6242 (PUR 3, Tab. 1). In total, 5500 features had spectral MS/MS
125 data we could use for compound identification, out of which we detected between 30 (PS 4) and
126 2117 features (PUR 3) per sample.

127 For tentatively identifying the plastic chemicals, we used the MS/MS data with the MassBank library
128 (14,788 compounds) and three *in silico* fragmented databases of chemicals potentially used in
129 plastics or (pre-)registered for authorization on the European market (in total 75,510 compounds;
130 *22*). These queries resulted in a successful identification of 2364 features across all samples,
131 corresponding to 629 unique chemicals (SM Excel Tab. S1). Accordingly, between 6 (PLA 2) and
132 33 % (PET 1) of the features in each sample were tentatively identified. For the 25 compounds with
133 the highest identification scores (≥ 50) and abundance in the samples, we confirmed the plausibility
134 of the identification by checking whether the compounds are known to be used in plastics (SM Excel
135 Tab. S2). We found that 14 out of 25 compounds are used in plastics, including five plasticizers
136 (e.g., acetyl tributyl citrate), four flame retardants (e.g., tris(2-butoxyethyl) phosphate, tris(3-
137 methylphenyl) phosphate) and multiple processing aids, such as the lubricant 2-nonyl-N-(2-
138 nonylphenyl) aniline, the hardener 4-methylphthalic anhydride and the slip additive (Z)-docos-13-
139 enamide. We also identified compounds that likely migrated from the packed content into the
140 packaging (two octadecanamides used in cosmetics) and one compound that was implausible (the
141 veterinary drug febarbamate).

142 When cross-referencing the chemicals tentatively identified in plastics and a list of known MDCs
143 inducing adipogenesis in 3T3-L1 cells (Tab. S3), we found eleven compounds known to induce
144 adipogenesis *in vitro*. The MDCs present in our samples include four phthalates and six
145 organophosphates (Tab. 1). Benzyl butyl phthalate (BBP), di-butyl phthalate (DBP) and di(2-
146 ethylhexyl) phthalate (DEHP) were present in PVC 4. Di-iso-nonyl phthalate (DINP) was detected
147 in PVC 3 and 4. Diphenyl phosphate (DPP), 2-ethylhexyl diphenyl phosphate (EHDP) and triphenyl
148 phosphate (TPP) were detected in multiple samples. When using raw abundance as a proxy for
149 concentration, high levels of TPP, DPP and EHDP (the MDCs present in at least three samples)

150 were detected in two to three active PVC samples (Tab. S4). In contrast, the other active samples
151 contain very low levels of these chemicals (PS 2, PP 4, PUR 2, PUR 3). Interestingly, we did not
152 detect organotin compounds or bisphenols (SM Excel Table S1) despite these are known MDCs
153 and thought of as being common in PVC and other plastics (*16, 17*).

154 # Discussion

155 **Adipogenic activity of plastic consumer products.** We hypothesized that plastic products
156 contain MDCs based on the fact that a small set of compounds used in plastics is known to induce
157 adipogenesis *in vitro* and *in vivo* (*8, 16, 17*). However, the current focus on few, individual plastic
158 chemicals neglects the chemical complexity of plastic materials given that we know that thousands
159 of compounds are either used in plastics or non-intentionally present (*13, 14*). Thus, we decided to
160 characterize the adipogenic activity of all compounds extractable from plastic consumer products.

161 Eleven out of 34 products contain chemicals that induce adipogenesis and are, thus, MDCs *in vitro*
162 (Fig. 1). The chemicals extracted from some plastics are not only quite potent but also trigger effects
163 that are similar to or higher than those induced by the reference compound rosiglitazone (PVC 2
164 and 4, PP 4). Supramaximal efficacies have previously been reported for single compounds, such
165 as dibutyl phthalate and tert-butyl phenyl diphenyl phosphate (*23*) but only at concentrations
166 ≥10 μM, illustrating the potency of the extracted mixtures.

167 Products with multiple applications, including two FCMs (PS 2, PP 3) and nine non-FCMs, contain
168 adipogenic chemicals. While chemicals migrating from packaging into food represent an obvious
169 source of human exposure (*24*), compounds released from non-FCMs can also contribute via
170 dermal uptake (e.g., PUR 4 shower slippers) or inhalation. For instance, dust contains chemical
171 mixtures that induce adipogenesis (*23*). Here, we show that plastic flooring (e.g., PVC 4) contains
172 MDCs that may contribute to human exposure if they partition into dust. Given the potency of the
173 extracted mixtures and considering our close and constant contact with plastics, it appears

174 plausible that plastic chemicals contribute to an obesogenic environment and, thus, the obesity
175 pandemic.
176 The chemicals present in PVC and PUR products most consistently induce potent adipogenic
177 responses, while compounds extracted from PET, HDPE, and PLA products were inactive. Apart
178 from the PLA samples, this is in line with our previous findings for other toxicity endpoints (*15*). This
179 suggests that PVC and PUR are more likely to contain MDCs compared to other polymers.
180 However, the chemicals extracted from some PP, PS, and LDPE products also induce
181 adipogenesis (Fig. 1). This supports the idea that caution is needed when trying to generalize the
182 occurrence of toxic chemicals based on polymer type (*15*).
183 Unhealthy or dysfunctional adipocytes are part of the obesity phenotype. They are larger in size,
184 have an impaired glucose uptake and insulin signaling, an elevated inflammatory response and
185 decreased respiration (*25*). While we did not investigate the latter characteristics, adipocytes
186 exposed to plastic chemicals were larger and contained more triglycerides compared to those
187 treated with rosiglitazone (Fig. 2). Since rosiglitazone promotes the development of healthy white
188 adipocytes (*26, 27*), these results suggest that exposure to plastic chemicals could shift adipocytes
189 towards an unhealthy phenotype. Similar trends have been reported for a range of MDCs, including
190 BPA (*28*), organotin compounds (*29, 30*) and DEHP (*31*), which we detected in PVC 4 (Tab. 1).
191 Hence, it will be interesting to investigate whether plastic chemicals also trigger the other hallmarks
192 of unhealthy, dysfunctional adipocytes.

193 **Plastic chemicals and adipogenesis**. Using nontarget high resolution mass spectrometry, we
194 show that plastic products contain hundreds to thousands of extractable chemicals; few of those
195 identifiable using spectral libraries and *in silico* tools. This is in line with our previous research (*15,*
196 *22*) and points towards the presence of unknown chemicals in plastics (e.g., non-intentionally
197 added substances). Accordingly, the relatively low identification performance in our study is a result
198 of the limited coverage of chemical databases. These limitations notwithstanding, we tentatively
199 identified a range of known plastic chemicals providing confidence in the accuracy of the
200 identifications.

201 Plastic products contain known MDCs, including four phthalates (only in PVC 3 and 4) and six
202 organophosphates (Tab. 1). Biomonitoring data suggests that humans are commonly exposed to
203 some of these compounds (*32-34*). As an example, the phthalates DBP and DEHP as well as the
204 flame retardants TPP and TBEP we found in plastics were recently detected in matched maternal
205 and cord blood samples (*35*). Accordingly, plastic products can be one source of exposure to these
206 MDCs.
207 Known MDCs may explain the adipogenic response to chemicals extracted from some but not all
208 plastic samples. Most active samples contain at least one MDC with TPP, DPP and EHDP being
209 present in multiple samples. Interestingly, the floor covering (PVC 4) contained ten known MDCs.
210 While the active PVC samples contain high levels of TPP, DPP and EHDP, the abundance of these
211 chemicals was very low in the other active samples (Tab. S4). This suggests that other than the
212 known MDCs contribute to the adipogenesis induced by plastic chemicals.

213 **Underlying mechanisms**. PPARγ is a key regulator of adipogenesis (*20*), and many MDCs that
214 induce adipogenesis also activate PPARγ (*17*). Despite the common idea that PPARγ activation is
215 a main mechanism via which anthropogenic chemicals trigger adipogenesis, most of the adipogenic
216 plastic samples in fact do not activate this receptor (Fig. 3). Only in two cases (PVC 2, PS 2) does
217 a high PPARγ activity correspond to a strong induction of lipid droplet formation. Moreover, three
218 samples (PLA 1, LDPE 2, PVC 1) activated PPARγ but were inactive in the adipogenesis assay.
219 Thus, the adipogenic effects of the plastic extracts is not necessarily dependent on direct activation
220 of PPARγ and other mechanisms must be involved.

221 GR is another important nuclear receptor that participates in adipogenesis, and various MDCs
222 activate GR (*36*). In particular, glucocorticoids are essential in inducing adipocyte differentiation
223 (Fig. S20). However, none of the plastic extracts activated GR rendering this an unlikely mechanism
224 of action in this case.
225 Elucidating the mechanism by which plastic chemicals induce adipogenesis is complex since we
226 are dealing with two black boxes, namely the complex chemical mixtures present in plastics and
227 the multitude of potential mechanism of actions involved in adipogenic responses in 3T3-L1 cells

228 (*19*). In addition to PPARγ and GR, (ant)agonists of multiple other nuclear receptors, such as the
229 retinoid x receptor α, estrogen receptor, androgen receptor, liver x receptor, thyroid receptor β,
230 have been demonstrated or are discussed to contribute to an adipogenic response (*37*). In the light
231 of the diversity of compounds we detected in plastics, it appears probable that these act via multiple
232 mechanisms that are in most cases PPARγ and GR independent. Although more work needs to be
233 done to elucidate the underlying mechanisms, our results underline the importance of using
234 integrative methods, such as the adipogenesis assay to identify MDCs triggering cellular responses
235 rather than assessing (anta)agonism at selected nuclear receptors.

236 **Limitations and future directions.** To the best of our knowledge, this is the first study investigating
237 the adipogenic activity of chemicals extractable from plastic consumer products. Considering the
238 diversity of plastic products and their chemical composition, the sample set is certainly not
239 representative of all plastic chemicals, humans will be exposed to. While it is challenging to
240 comprehensively characterize the human exposure to plastic chemicals from all types of products,
241 given their ubiquity and diversity, a way forward is to prioritize polymer types that are likely to
242 contain MDCs, such as PVC and PUR.

243 Given that our aim was to investigate whether MDCs are present in plastic products, we used
244 methanol to extract the samples. This simulates a worst-case scenario. Thus, even though we
245 demonstrated that potent (mixtures of) MDCs are present in consumer products, it remains to be
246 investigated whether these will migrate under more realistic conditions into air, water or food, or
247 can be taken up dermally. Using the same samples as in the present study, we recently
248 demonstrated that a significant number of chemicals and *in vitro* toxicity, such as antiandrogenic
249 compounds, migrate into water (*22*). However, it remains unknown if this is also the case for the
250 present MDCs.

251 Moreover, we analyzed plastic packaging that contained foodstuff or personal care products
252 because we aimed at investigating final products. Since chemical migration is not a one-way street,
253 we cannot exclude that compounds from the contents migrated into the packaging. The detection
254 of compounds used in cosmetics in its packaging underlines this limitation. Such compounds may

255 contribute to the observed adipogenic activity or PPARγ activation and future research should cover
256 unused final packaging.
257 The nontarget chemical analysis resulted in the tentative identification of several MDCs. However,
258 many compounds remain unidentified and there is some likelihood of false-positive identifications.
259 The challenge of a rather low identification success is well-known for environmental pollutants (*38*)
260 and can be addressed by building more comprehensive spectral databases. Recent efforts to build
261 specific databases for plastic chemicals are promising (*13, 14*) but must be complemented with
262 spectral information and non-intentionally added substances. Moreover, we show that known
263 MDCs only partially – if at all – contribute to the adipogenesis induced by plastic extracts. This
264 points towards the presence of unidentified MDCs in plastics. To identify the compounds that are
265 indeed causative for the observed responses, future research should apply effect-directed analysis.
266 Moreover, our results indicate that plastic chemicals may promote a development towards
267 unhealthy adipocytes. However, more evidence is needed to further support this hypothesis. For
268 instance, one needs to extend the adipogenesis assay to cover later stages of adipocyte
269 development and investigate biomarkers of inflammation and metabolic function (e.g., glucose
270 uptake, insulin sensitivity).
271 Taken together, we demonstrated that plastic consumer products contain potent (mixtures of)
272 MDCs inducing adipogenesis *in vitro* via mechanisms that are mostly not mediated via PPARγ and
273 GR. Accordingly, plastic chemicals may contribute to an obesogenic environment considering our
274 constant contact with a broad range of plastic products. Given that the plastic products containing
275 MDCs also contained compounds triggering other toxicological endpoints (*15*), a shift towards
276 chemically less complex plastics represents a way forward to a non-toxic environment.

277 # Materials and Methods

278 A list of used chemicals is provided in the supplementary material (Tab. S5).

279 **Sample selection and plastic extraction.** We used the same 34 plastic samples (Tab. 1) as in
280 Zimmermann *et al.* (*15*). The samples cover petroleum-based polymers with the highest market
281 share (polypropylene (PP) > low density polyethylene (LDPE) > high density polyethylene (HDPE)
282 > polyvinyl chloride (PVC) > polyurethane (PUR) > polyethylene terephthalate (PET) > polystyrene
283 (PS); *39*), and polylactic acid (PLA) as a bio-based alternative. The samples include 21 products
284 with and 13 products without food contact. Further specifications on the sample selection, collection
285 and polymer identification are described in Zimmermann *et al.* (*15*). We extracted 3 g sample with
286 including three procedure blanks (PB 1-3) with methanol and concentrated the extracts to a final
287 volume of 200 µL using dimethyl sulfoxide as a keeper. To contextualize the bioassay results, we
288 use "plastic equivalents" in such that "1 mg plastic" corresponds to the chemicals extracted from 1
289 mg of plastic. Accordingly, 1 µl extract corresponds to 15 mg plastic. See supplementary text for
290 details.

291 **Bioassays**. We performed differentiation assays with murine 3T3-L1 adipocytes (Zenbio Inc., SP-
292 L1-F, lot 3T3L1062104) to examine the induction of adipogenesis as well as CALUX reporter gene
293 assays (BioDetection Systems B.V., Amsterdam, The Netherlands) to investigate the agonistic
294 activity at the human peroxisome proliferator-activated receptor γ (*40*) and glucocorticoid receptor
295 (GR, *41*). All experiments were conducted with negative controls, vehicle controls, positive controls,
296 and PB 1–3. Samples, controls, and blanks were diluted 1000-fold (adipogenesis assay) or 500-
297 fold (reporter gene assays) with medium, resulting in a maximum final solvent concentration of
298 0.1% or 0.2% (v/v), respectively. Each sample was analyzed in serial dilutions of 1:2 with four
299 replicates per concentration in at least three independent experiments per assay. Moreover, the
300 respective reference compound was included on every microtiter plate to control for potential
301 variations between plates, and the sample arrangement was randomized to exclude position

302 effects. As negative and vehicle controls did not differ significantly, the results from both were
303 pooled. Further, there was no contamination during sample extraction and analysis since none of
304 the controls and blanks induced activity (Fig. S21 and S22). See supplementary text for details on
305 the cell culture conditions.

306 **Adipogenesis assay**. We performed the differentiation assay with 3T3-L1 cells in accordance with
307 a previously described method (*42*). In brief, an experiment consists of three days pre-
308 differentiation (one day seeding, two days allowing cells to enter the resting state) followed by an
309 8-d differentiation window (two days differentiation, six days maintenance). Subconfluent cells of
310 passage 10 were trypsinized and counted with a flow cytometer (NovoCyte, Acea Biosciences).
311 15,000 cells well^{-1} were seeded in 200 µL preadipocyte medium (PAM: DMEM-high supplemented
312 with 10% bovine calf serum and 1% penicillin/streptomycin) into 96-well black, clear bottom tissue
313 culture plates (655090, Greiner Bio-One) and incubated at 37 ℃ and 5% CO_2. After 24 h, we
314 checked that the cells reached confluency, replaced the medium with 200 µL fresh PAM well^{-1}, and
315 cultured the cells for another 48 h to initiate growth arrest. We included on every plate a pre-
316 adipocyte control (undifferentiated cells) which was kept in PAM, while the rest of the cells were
317 differentiate as described below.

318 **Optimization experiments.** Given that a systematic analysis of dexamethasone (DEX) effects on
319 triglyceride accumulation and differentiation efficiency in 3T3-L1 cells was lacking, we conducted
320 optimization experiments to identify a suitable DEX concentration to initiate adipocyte differentiation
321 that results in the lowest baseline as well as the highest sensitivity and dynamic range when co-
322 exposed to the reference compound rosiglitazone. Moreover, we compared two methods to
323 quantify triglycerides based on Nile Red Staining. We determined the total NileRed fluorescence
324 well^{-1} and compared it to an automated imaging and analysis platform to determine whether the
325 latter improves the sensitivity and dynamic range for the screening of adipogenic activity.

326 Based on the results (Fig. S20) and in comparison with previous studies, we found that a rather
327 low optimal DEX concentration (6.25 nM) was sufficient to initiate adipocyte differentiation without

328 increasing the assay's baseline. Compared to the fluorescence readout well^{-1}, an automated
329 imaging approach is more sensitive to assess proliferation, enhances the dynamic range of the
330 assay (Fig. S20) and provides more information because it enables single-cell analysis for a more
331 in-depth characterization of the adipocyte population (pre-adipocytes, adipocytes and mature
332 adipocytes). Accordingly, we analyzed the effects of the plastic extracts using 6.25 nM DEX during
333 the differentiation window and the automated imaging approach. See supplementary text for
334 details.

335 **Dosing of samples**. To initiate differentiation, we replaced the PAM medium with 200 µL
336 differentiation medium well^{-1} (DM: DMEM-high supplemented with 10% FBS, 1%
337 penicillin/streptomycin, 20 mM HEPES, 1 µg mL^{-1} insulin, 0.5 mM 3-isobutyl-1-methylxanthine
338 (IBMX), and 6.25 nM DEX) containing 5 concentrations of samples serially diluted 1:2 (0.19–3 mg
339 plastic well^{-1} equivalent to 0.94–15 mg plastic mL^{-1}) or rosiglitazone (1.17–300 nM). Following the
340 48-h differentiation, the medium was replaced with 200 µL adipocyte maintenance medium well^{-1}
341 (DM without IBMX and DEX) containing the respective controls, samples, or rosiglitazone and
342 changed the medium every other day during the 6d maintenance period.

343 **Fixation and staining**. After 11 d, cells were fixed with 2% paraformaldehyde and co-stained with
344 NileRed and NucBlue. Imaging was carried out on the Cytation 5 Cell Imaging Multimode reader
345 (BioTek). Three images per field (Brightfield, NucBlue and NileRed) and nine fields per well were
346 captured. See supplementary text for details.

347 **Image analysis**. Images were analyzed in the open-source software CellProfiler (*43*). A description
348 of the image analysis protocol and the CellProfiler pipelines are available in the supplementary
349 material. We quantified proliferation (nuclei count), number of lipid droplets (lipid droplet count),
350 total area occupied by lipid droplets (total area), and the total intensity of the NileRed staining within
351 the lipid droplets (total intensity) per image. We further quantified the total area occupied by lipid
352 (lipid droplet area), and the total intensity of NileRed staining in the lipid droplet area per cell.

353 Analysis of the single-cell data was applied to quantify the numbers of adipocytes and mature
354 adipocytes per image. An adipocyte was defined as a cell containing at least one lipid droplet and
355 a mature adipocyte was defined as a cell containing a total lipid content equivalent to ≥ 8 average
356 sized lipid droplets (1000 pixels).

357 Single-cell measurements of lipid droplet area, and NileRed staining were analyzed further to
358 explore how triglyceride accumulation was distributed within an adipocyte population. To control
359 for potential cross-plate differences in staining intensity within an experiment, the average
360 fluorescence intensity per adipocyte was normalized to the mean average fluorescence intensity
361 for an internal plate control (cells treated with 300 nM rosiglitazone). For an independent
362 experiment, single-cell data were grouped based on the treatment. The median lipid droplet area
363 and normalized average fluorescence intensities per cell were calculated for each experiment. An
364 example of the images and visualized output of the image analysis is shown in Fig. S2.

365 **Reporter gene assays**. We performed the CALUX reporter gene assays in 384-well plates and
366 used imaging to count nuclei per well to normalize the reporter gene response and assess
367 cytotoxicity. Rosiglitazone was the reference compound for PPARγ and DEX for GR (Fig. S23).
368 See supplementary text for the detailed protocol.

369 **Analysis of bioassay data**. We used GraphPad Prism 9 (GraphPad Software, San Diego, CA) for
370 non-linear regressions and statistical analysis, and interpolated plastic equivalents inducing 10 or
371 20% effect (effect concentration, EC_{10} or EC_{20}) from the respective dose-response curves. The limit
372 of detection (LOD) of each endpoint and experiment was calculated as three times the standard
373 deviation (SD) of pooled controls. See supplementary text for details.

374 **Nontarget chemical analysis**. We analyzed all samples, except PLA 3, using ultra-high
375 performance liquid chromatography coupled to a quadrupole time of flight spectrometer (LC-QTOF-
376 MS/MS) with an Acquity UPLC Waters liquid chromatography system coupled to a SYNAPT G2-S
377 mass spectrometer (both Waters Norge, Oslo, Norway). The analytical method has been described

378 in Zimmermann *et al.* (*22, 44*) and a brief description as well as information about the data analysis
379 and compound identification can be found in the supplementary text.

380 **Comparison with chemicals known to induce adipogenesis**. We built a list of 120 know
381 adipogenic chemicals (Tab. S3) by searching Web of Science (Core Collection) for studies
382 investigating chemicals in the adipogenesis assay and complemented the search with chemicals
383 reviewed by Amato *et al.* (*16*). We cross-referenced the list with the tentatively identified
384 compounds in the plastic samples to determine whether some of these compounds are MDCs
385 (Tab. 1). See supplementary text for details.

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Author contributions

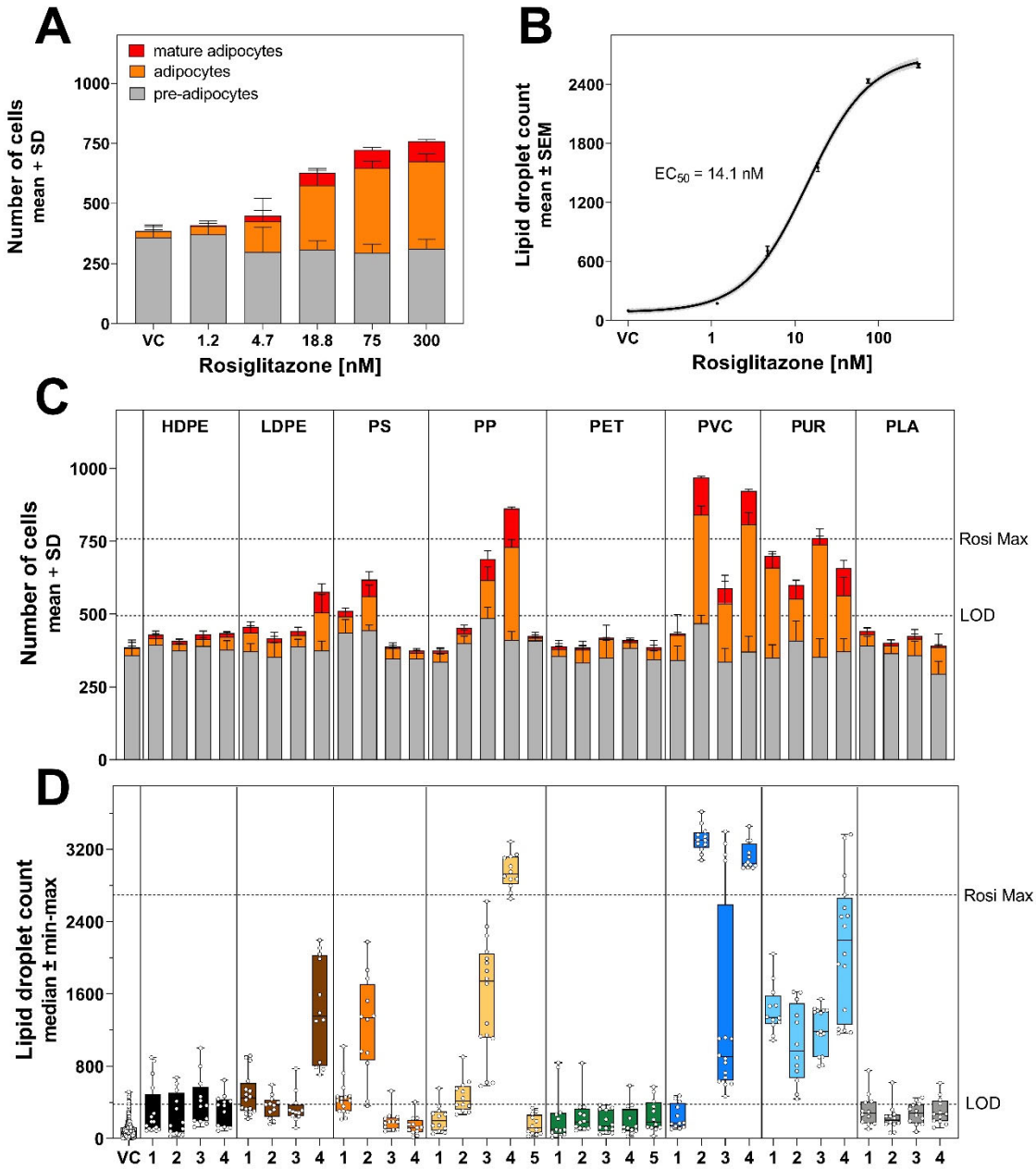
J.V., F.A. and M.W. conceived the study, J.V. and Å.V. performed the experiments, L.Z. performed the chemical analysis, J.V., F.A., and M.W. analyzed the data and wrote the manuscript, and all authors provided comments on the manuscript.

Competing interest

LZ became an employee the Food Packaging Forum (FPF) after this study was concluded. MW is an unremunerated member of the Scientific Advisory Board of the FPF and received travel support for attending annual SAB meetings. FPF is a Swiss foundation that enhances the scientific principles and recent scientific findings that are relevant to the topic of food contact chemicals and their health impacts on humans and the environment.

Data and materials availability

The raw mass spectral data can be accessed under DOI 10.5281/zenodo.4781257 (published after publication), bioassay data (SM Excel Tab. S3–10) and the CellProfiler pipelines can be found in the supplementary material.



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687 **Fig. 1. Effect of rosiglitazone on (A) the adipocyte population and (B) the lipid droplet count**
688 **(pooled data from four experiments). Effect of plastic extracts on (C) the adipocyte**
689 **population and (D) the lipid droplet count in the highest noncytotoxic concentration.** The
690 highest noncytotoxic concentration (HNC) was 3 mg plastics well^{-1} except for PP 4 (1.5 mg plastic
691 well^{-1}) as well as PUR 2 and PUR 3 (0.75 mg plastic well^{-1}). VC = vehicle control, LOD = limit of the
692 detection, Rosi Max = maximal response of rosiglitazone.

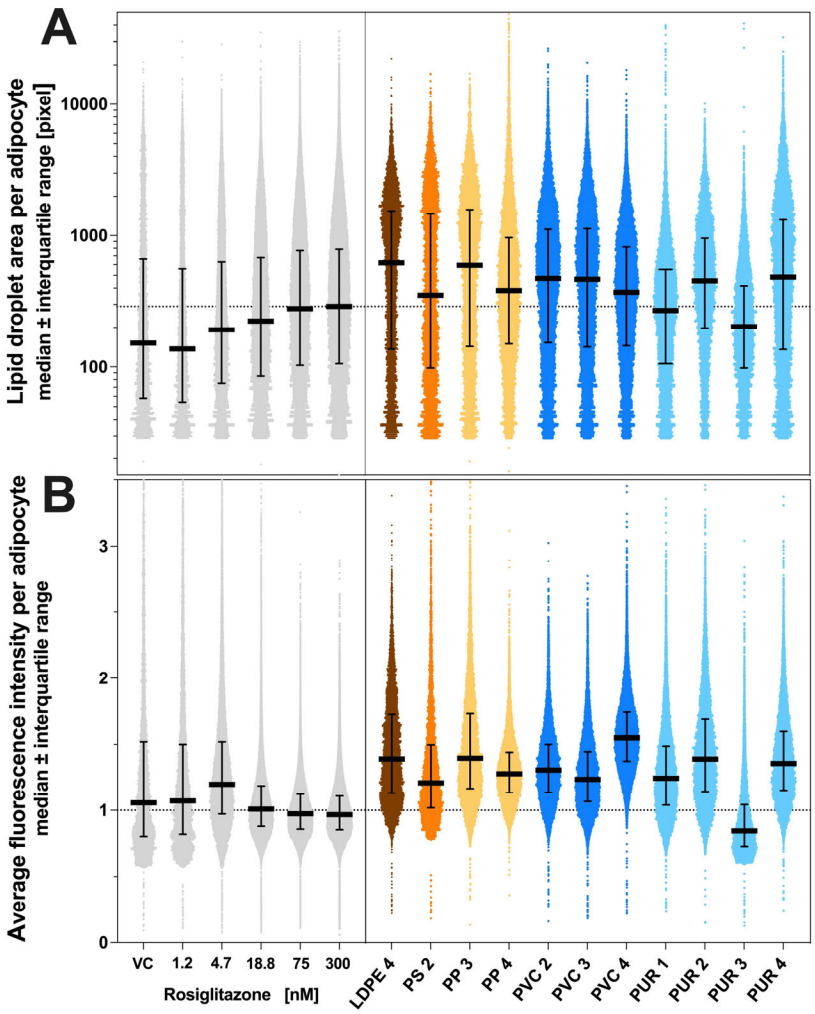

Fig. 2. (A) Size distribution of adipocyte population and (B) accumulation of triglyceride per adipocyte in cells exposed to rosiglitazone (left) or the highest noncytotoxic concentration of the eleven active plastic extracts (right). Single-cell data from one experiment. Intensity data is normalized on the mean of the highest rosiglitazone concentration (300 nM). VC = vehicle control.

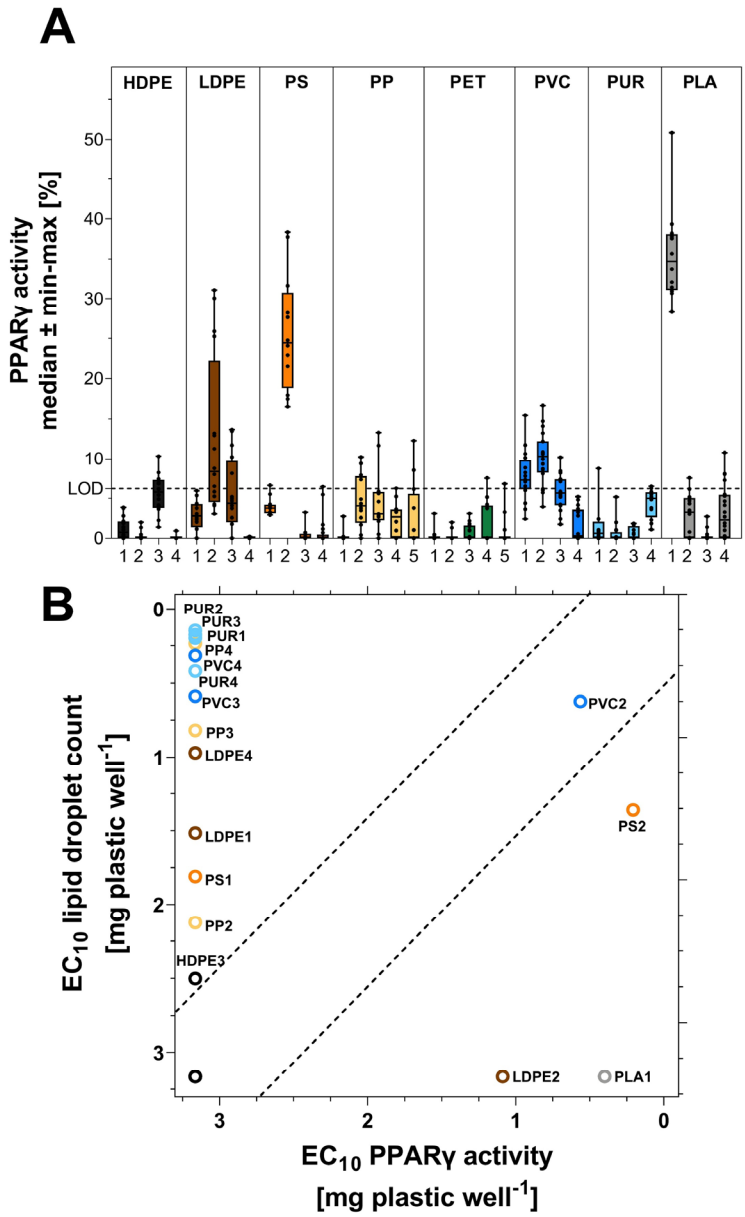

Fig. 3. (A) PPARγ activity induced by plastic extracts at the highest noncytotoxic concentration and (B) correlation of the EC_{10} of the PPARγ activity and lipid droplet count. The highest noncytotoxic concentration (HCN) was 1.5 mg plastic well^{-1} except for PP 4 (0.19 mg plastic well^{-1}), PS 2 and PP 3 (0.38 mg plastic well^{-1}) as well as PLA 1 and PVC 2 (0.75 mg plastic well^{-1}).

704 **Table 1. Plastic products analyzed in this study, results of the nontarget chemical analysis**
705 **and the tentatively identified metabolic disrupting chemicals (MDCs).**

Sample	Plastic product	LC-QTOF-MS/MS (number of features)				Tentatively identified MDCs
		in sample	with MS2	ID score ≥40	% of MS2	
HDPE 1	refillable drinking bottle[a]	779	203	38	18.7	TPP
HDPE 2	yogurt drinking bottle[a]	107	34	7	20.6	
HDPE 3	bin liner	614	153	30	19.6	TPP
HDPE 4	shower gel bottle	164	50	16	32.0	EHDP
LDPE 1	lemon juice bottle[a]	241	66	20	30.3	EHDP
LDPE 2	plastic wrap[a]	1833	543	98	18.0	TPP
LDPE 3	freezer bag[a]	1603	416	62	14.9	TPP
LDPE 4	hair conditioner bottle	1702	544	89	16.4	allethrin, TPP
PS 1	yogurt cup[a]	447	96	12	12.5	TPP
PS 2	fruit tray[a]	1122	293	44	15.0	DPP, TPP
PS 3	vegetable tray[a]	308	63	11	17.5	
PS 4	plastic cup[a]	119	30	7	23.3	
PP 1	refillable drinking bottle[a]	1365	396	87	22.0	TPP
PP 2	yogurt cup[a]	1870	549	93	16.9	TPP
PP 3	gummy candy packaging[a]	3159	910	117	12.9	TPP
PP 4	handkerchief packaging	1798	519	85	16.4	TPP
PP 5	shampoo bottle	268	101	29	28.7	
PET 1	soft drink bottle[a]	148	55	18	32.7	
PET 2	yogurt cup[a]	179	51	12	23.5	
PET 3	oven bag[a]	647	159	30	18.9	
PET 4	vegetable tray[a]	695	182	20	11.0	
PET 5	shampoo bottle	375	89	11	12.4	
PVC 1	plastic wrap[a]	3655	1374	118	8.6	
PVC 2	place mat	2426	819	145	17.7	DPP, TPP
PVC 3	pond liner	1270	450	91	20.2	DINP, TPP
PVC 4	floor covering	2361	868	145	16.7	BBP, BPDP, DBP, DEHP, DINP, DPP, EHDP, TBEP, TOCP, TPP
PUR 1	scouring pad	5619	1773	216	12.2	EHDP, TPP
PUR 2	kids bath sponge	4521	1182	151	12.8	
PUR 3	acoustic foam	6242	2117	224	10.6	EHDP, TPP
PUR 4	shower slippers	1035	300	78	26.0	EHDP, TPP
PLA 1	yogurt cup[a]	2421	772	52	6.7	TPP
PLA 2	vegetable tray[a]	1983	672	40	6.0	
PLA 3	coffee cup lid[a]	N/A	N/A	N/A	N/A	
PLA 4	coffee cup lid[a]	2575	857	73	8.5	

706 [a] = food contact material, BBP = benzyl butyl phthalate, BPDP = tert-butylphenyl diphenyl
707 phosphate, DBP = dibutyl phthalate, DEHP = bis(2-ethylhexyl) phthalate, DINP = di-iso-nonyl
708 phthalate, DPP = diphenyl phosphate, EHDP = 2-ethylhexyl diphenyl phosphate, N/A = not
709 analyzed, TBEP = tris(2 butoxyethyl) phosphate, TOCP = tri-o-cresyl phosphate, TPP = triphenyl
710 phosphate