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1 **Obesity III: Obesogen Assays: Limitations, Strengths, and New Directions**

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55

56 **Abstract**

57 There is increasing evidence of a role for environmental contaminants in disrupting metabolic health in
58 both humans and animals. Despite a growing need for well-understood models for evaluating adipogenic
59 and potential obesogenic contaminants, there has been a reliance on decades-old *in vitro* models that have
60 not been appropriately managed by cell line providers. There has been a quick rise in available *in vitro*
61 models in the last ten years, including commercial availability of human mesenchymal stem cell and
62 preadipocyte models; these models require more comprehensive validations but demonstrate real promise
63 in improved translation to human metabolic health. There is also progress in developing three-
64 dimensional and co-culture techniques that allow for the interrogation of a more physiologically relevant
65 state. While diverse rodent models exist for evaluating putative obesogenic and/or adipogenic chemicals
66 in a physiologically relevant context, increasing capabilities have been identified for alternative model
67 organisms such as *Drosophila*, *C. elegans*, zebrafish, and medaka in metabolic health testing. These
68 models have several appreciable advantages, most notably the size, rapid development, large brood sizes,
69 and ease of high-resolution lipid accumulation throughout the organisms. They are anticipated to expand
70 the capabilities of metabolic health research, particularly when coupled with emerging obesogen
71 evaluation techniques as described herein.

72

73 **Abbreviations**

74 United States (US); white adipose tissue (WAT); peroxisome proliferator-activated receptor gamma
75 (PPAR γ); sterol-regulatory element-binding protein-1 (SREBP-1); liver X receptor alpha (LXR α);
76 glucocorticoid receptor (GR); retinoid X receptor-alpha/beta (RXR α/β); estrogen receptor alpha (ER α);
77 human preadipocytes (HPAd); Simpson-Golabi-Behmel syndrome (SGBS); mesenchymal stem cells
78 (MSCs); Human multipotent adipose-derived stem cells (hMADS); toxicant-associated fatty liver
79 diseases (TAFLD); non-alcoholic fatty liver disease (NAFLD); dimethylsulfoxide (DMSO); primary
80 human hepatocytes (PHH); cytochrome P450 (CYP); aryl hydrocarbon receptor (AhR); constitutive
81 androstane receptor (CAR); pregnane X receptor (PXR); di(2-ethylhexyl) phthalate (DEHP);
82 dichlorodiphenyltrichloroethane (DDT); bisphenol A (BPA); polychlorinated biphenyls (PCBs);
83 tetrabromobisphenol A (TBBPA); days post-fertilization (dpf); endocrine disrupting chemicals (EDCs);
84 insulin-producing cells (IPCs); brown adipose tissue (BAT); uncoupling protein 1 (UCP1); OLTAM
85 (ODD-Luc based Thermogenic Activity Measurement); ODD (oxygen-dependent degradation); hypoxia-
86 inducible factor 1 alpha (HIF1 α); quantitative structure-activity relationship (QSAR); Toxicity Testing in
87 the 21st Century (Tox21); Organization for Economic Cooperation and Development (OECD); New
88 approach methodologies (NAMs); Adverse Outcome Pathways (AOPs); integrated approaches to testing
89 and assessment (IATAs); National Institute for Environmental Health Sciences (NIEHS); PEPPER
90 (Public-privatE Platform for the Pre-validation of Endocrine disRuptors).

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100 **Essential Points**

101 There are increasing novel capabilities to identify and assess obesogens.

102

103 There is still a reliance on using well-defined models with unclear translation to human health.

104

105 There is still a need for comprehensive validations of novel metabolic health models.

106

107 Computational models show some promise in future predictions and assessments of obesogens.

108

109

110

111 1. Introduction

112

113 Over the last several decades, the global prevalence of metabolic disorders, specifically obesity, has risen
114 at an alarming rate. Despite extensive investments in exploring interventions to address this health trend,
115 the incidence rates continue to rise. In the United States (US), 8.9% of infants and toddlers [1, 2], 18.5%
116 of 2-19 year old's [1, 2], and 42.4% of adults (20+) [3] are currently classified as obese, with an
117 additional 31.2% of the adult population classified as overweight [4]. Obesity consumes >\$200 billion of
118 the US health care expenditure annually and also drives increased risks of various comorbidities (e.g.,
119 type II diabetes, cardiovascular disease, hypertension) [5-8]. High societal costs [8, 9] have driven support
120 for research into causal factors, including exposure(s) to environmental contaminants. Previous research
121 estimated extremely high economic costs of obesity, diabetes, and associated health costs reasonably
122 attributable to environmental contaminants in the European Union [9], even when only considering five
123 chemicals for which sufficient epidemiological data were available.

124

125 As detailed in the companion review, Obesity II, "obesogens" are environmental chemicals that increase
126 the size of white adipose tissue (WAT) stores in the body as a result of exposure *in vivo* [10, 11].
127 Chemicals that can induce adipogenesis in cellular models *in vitro* but have not yet been shown to
128 increase WAT stores *in vivo* are designated as potential obesogens [12]. Considering the complexity of
129 human chemical exposures, the increasing reports of obesogens, and the rising incidence of metabolic
130 disorders, it is critical to identify and validate comprehensive models (*in silico*, *in vitro*, and *in vivo*) for
131 the identification and evaluation of obesogens. One of the major challenges in the obesity field is to
132 develop a robust set of tests that can reveal adipogenic and/or obesogenic properties of chemicals and
133 have strong predictive capacity in humans. These tests should be in line with the 3R principles (i.e.,
134 reducing the number of animals, refining experiments to minimize the number of animals used, and
135 replacing animal experiments where possible). Practically speaking, the high costs of animal experiments
136 limit the use of mammals in screening for potential obesogens. This supports an urgent need for increased

137 use of lower-order (*in silico*, *in vitro*) testing to prioritize higher-order (*in vivo*) testing. There is also an
138 urgent need for new *in vivo* models that are less time and cost-intensive to support *in vivo* testing that is
139 still required for the tens of thousands of chemicals used in commerce. While the number and diversity of
140 cellular models of adipocyte differentiation and metabolic health is increasing, these require
141 comprehensive validation to determine the strengths and weaknesses of each for their relevance to human
142 metabolic health

143
144 Despite the potential limitations of available animal models to reproduce human disease fully, they help
145 evaluate exposure pathways, generation of *in vivo* metabolites, elucidating tissue and/or disease biology,
146 and underlying molecular mechanisms involved in adverse health outcomes. The choice of the animal
147 model should consider the degree to which the outcomes being examined are relevant to humans and the
148 sensitivity of these outcomes to environmental chemicals. The relevance of the model to human health
149 depends considerably on the evolutionary conservation of biological processes impacted by candidate
150 chemical or pharmacological molecules between humans and the animal model used. It is likely that a
151 single test might not reveal all relevant properties and that a battery of tests should be developed. This set
152 of tests should address the following issues: 1) evaluate *in vivo* obesity according to its different
153 characteristics, including the type and importance of different adipose depots; 2) reveal *in vitro* and *in*
154 *silico* assays/models that reliably predict obesity; 3) identify *in vivo* biomarkers that are predictive of
155 obesity, and 4) account for delays between exposure(s) to putative obesogens and the appearance of a
156 phenotype.

157
158 Mammalian models have been relied on for metabolic health testing due to clear translation of adipose
159 physiology. However, non-mammalian model species are increasingly appropriate for the screening and
160 rapid identification of chemicals and mixtures and the exploration of disease mechanisms. Knowledge
161 acquired from non-mammalian model systems (e.g., vertebrates such as teleost fish and invertebrates such
162 as flies and worms) can provide insights into mechanisms involved in regulating lipid metabolism and

163 transport processes that have been intractable by other approaches [13]. Due to the conservation of lipid
164 metabolism processes among vertebrates, the zebrafish model has become an attractive alternative to
165 rodents, with lower costs and time investments.

166

167 **2. In vitro assays**

168 The most well-established lower-order testing protocols are the adipogenesis cell assays, although newly
169 developed cell models have allowed an increasing breadth of metabolic disruption assessment (Figure 1).
170 Several *in vitro* models were developed in various species (primarily human and murine) to identify
171 potential obesogens [14, 15]. These models generally assess three endpoints: commitment to the
172 adipocyte lineage (via multipotent MSC models), preadipocyte proliferation (proliferation of early-stage
173 adipocyte lineage cells), and differentiation into mature adipocytes (adipogenesis; generally determined
174 via quantification of intracellular triglyceride accumulation).

175

176 *2.1 Preadipocyte models*

177 Preadipocytes are already committed to the adipocyte lineage and thus can be used to examine both
178 proliferation (*via* nuclear staining) and adipogenesis (*via* triglyceride quantification). These cells are in an
179 early stage of adipocyte development and require activation of signaling pathways to promote further
180 development/maturation. Adipogenesis can be achieved by treating cells with a “differentiation cocktail”
181 that contains a variety of hormonal and/or growth factors to initiate the process. These factors are often
182 different between laboratories, but generally always include a mixture of fetal bovine serum, insulin, and
183 isobutylmethylxanthine (IBMX); some laboratories also include thyroid hormone and/or glucocorticoids,
184 though the presence of these and concentrations varies widely. Once the cocktail is removed, the relative
185 roles of various test chemicals in the role of differentiation (assessed *via* triglyceride accumulation) and
186 proliferation (of adipocyte precursor cells) can be assessed [16-19].

187

188 The 3T3-L1 mouse cell line was isolated and described in the 1970s and has been utilized for decades as

189 an *in vitro* screen to examine the mechanisms regulating adipogenesis and evaluate potential adipogenic
190 chemicals [16, 17, 20]. This cell line has been used to carefully explore mechanisms promoting and
191 underlying various stages of adipogenesis [21, 22] and has been shown to appropriately select chemicals
192 for further testing (linking *in vitro* results to *in vivo* health outcomes; e.g., bisphenol A and tributyltin)
193 [23-31]. While this line has been well-characterized [21], its sourcing can be unreliable [32, 33]. For
194 example, nuclear receptor expression related to adipogenesis is markedly different between lots and
195 sources of this cell line [32]. These and other cell line integrity issues can contribute to discrepancies in
196 replication efforts between laboratories [34, 35]. We recently undertook an interlaboratory reproducibility
197 effort of 3T3-L1 responses to a positive control chemical (rosiglitazone) and three blinded test chemicals
198 [35]. While the determination of “active” *versus* “inactive” were consistent across the ten participating
199 laboratories, the potencies and efficacies of the blinded chemical responses varied by orders of
200 magnitude. The cross-over study design allowed for determinations of the sources of variation, and our
201 results demonstrated that inconsistencies of the cell line sources and differentiation protocol differences
202 promoted most of the variation. Thus the harmonization of protocols across laboratories may help support
203 consistent reporting of adipogenic results [35]. Despite these limitations, 3T3-L1 cells remain the most
204 popular model for assessing adipogenic outcomes. Specifically, numerous publications have assessed
205 bisphenols [26, 32, 36], brominated and organophosphate flame retardants [37-39], per and
206 polyfluoroalkyl substances [40, 41], and diverse other environmental contaminants [20, 24, 37] and
207 mixtures [42] using this cell model. There is an emerging interest in determinations of whether
208 environmental contaminant exposures promote the development of normal or abnormal adipocytes, and
209 some preliminary data has begun to evaluate this. For example, BPA enhanced levels of leptin,
210 interleukin-6, and interferon gamma in mature adipocytes, resulting in hypertrophic adipocytes with
211 impaired insulin signaling, increased pro-inflammatory cytokine production, and reduced glucose
212 utilization [43].

213

214 The OP9 mouse bone marrow-derived stromal cell line is another established preadipocyte model [19, 44]

215 that allows faster differentiation (2-3 *versus* 10-14 days). This cell line is considered to be a later stage
216 preadipocyte than 3T3-L1 cells because it expressed key adipogenic factors such as CCAAT/enhancer-
217 binding proteins alpha and beta, peroxisome proliferator-activated receptor gamma (PPAR γ), sterol-
218 regulatory element-binding protein-1 (SREBP-1), perilipin, and other adipocyte markers that are not
219 expressed in basal 3T3-L1 cells before adipogenic induction [19]. Therefore, OP9 cells can be induced to
220 accumulate triglycerides within two days, differentiation is not diminished by maintenance in culture at
221 high cell density, their adipogenic potential is maintained for >100 passages, and they do not require
222 contact inhibition and reversion to clonal expansion before initiating the differentiation induction [19].
223 These characteristics suggest a promising model with lower time and cost investments, though this does
224 require careful validation to understand the translation of responses to human health effects. We have
225 reported that these cells do differentially express nuclear receptors relative to 3T3-L1 cells, including
226 PPAR γ/α , liver X receptor alpha (LXR α), glucocorticoid receptor (GR), retinoid X receptor-alpha/beta
227 (RXR α/β), and estrogen receptor alpha (ER α) [32]. As a result, responsiveness to adipogenic chemicals in
228 OP9 cells is significantly different from 3T3-L1 cells, characterized by lower responsiveness *via*
229 activation of GR and greater responsiveness *via* the RXR pathway [32, 45]. While still an uncommon
230 model for assessing obesogens, OP9 cells have been used to evaluate bisphenols [32], pesticides [45], and
231 other environmental contaminants [45].

232

233 More recently, several human preadipocyte models have become available that hold promise for future
234 evaluations of adipogenicity by environmental contaminants. Since the basis for much of our
235 understanding of adipogenesis has been evaluated using the murine 3T3-L1 cells, utilizing these newer
236 human models may help elucidate any species-specific differences that may be present. Many companies
237 now supply primary human preadipocytes (HPAd) isolated from several human subcutaneous depots,
238 visceral depots, and/or adipose surrounding the heart. Moreover, suppliers also provide source-specific
239 HPAd cells, i.e., those sourced from donors with normal, overweight, or obese body mass indices and
240 those with or without diabetes (e.g., see, <https://www.zen->

241 bio.com/products/cells/subcutaneous_adipocytes.php). These discrete preadipocyte populations allow
242 more targeted questions and potentially a better molecular understanding of adipogenesis. However,
243 human preadipocyte cell models are cryopreserved at the end of primary culture. They can generally be
244 propagated at most two additional passages before losing their ability to differentiate into mature
245 adipocytes [46, 47]. As such, these models, while potentially more translationally relevant to human
246 health, are extremely costly, as numerous cryopreserved vials are needed to complete any well-designed
247 experiment (e.g., multiple biological replicates). Limitations aside, researchers have begun to utilize
248 human preadipocytes to assess adipogenic and anti-adipogenic effects of botanical and biological
249 mixtures [48-50], bisphenols [51], and flame retardants [38].

250
251 The Simpson-Golabi-Behmel syndrome (SGBS) cell line addresses some of these limitations of using
252 primary human preadipocytes. These cells were isolated from an infant with an extremely rare (250
253 reported cases) metabolic health condition characterized by excess growth; this infant demonstrated
254 expanded subcutaneous fat depots, and a sample of this tissue was obtained postmortem [52]. Profiling
255 these cells suggests that they can be maintained and retain robust differentiation capability over 50
256 passages [53], a significant advantage over normal human donor preadipocytes, and profiling has
257 suggested morphological, biochemical, and functional similarities to differentiated adipocytes from
258 healthy subjects [52, 54]. These cells also transiently express brown adipocyte markers [55-57],
259 suggesting that this cell line might be useful for assessments of adipocyte browning. Proteomic and
260 transcriptomic analyses of SGBS cells have been used to evaluate the molecular underpinnings of SGBS
261 differentiation, with >1100 proteins and >300 genes differentially expressed in differentiated cells relative
262 to undifferentiated [58]. However, some research comparing this model to existing models has suggested
263 notable differences. Metabolomics and lipidomics profiling revealed a diverse grouping of lipid classes
264 markedly changed throughout the differentiation process, suggesting a radically different metabolite
265 profile than previously observed in 3T3-L1 cells [59]. SGBS cells have been used to evaluate the
266 adipogenic effects of various bisphenols [60], though have not yet seen frequent use in this context. Other

267 human cell lines obtained from tumors or transformed can be differentiated into either white (Lisa, LS-14,
268 AML-1, Chub-S7) or brown (PAZ6) adipocytes [61], but their use in toxicology is rare [60].

269

270 *2.2 Mesenchymal stem cells (MSCs)*

271 Another option in assessing adipogenesis is the utilization of multipotent mesenchymal stromal stem cells
272 (mesenchymal stem cells, MSCs). MSCs are multipotent cells that can assess adipocyte lineage
273 commitment in addition to adipocyte differentiation [18, 62, 63]. MSCs are isolated from either bone
274 marrow or adipose tissue, and cells from both sources have been used to assess adipogenesis. The use of
275 MSC models has been reviewed previously in the context of obesogens and their potential impacts on cell
276 commitment and subsequent differentiation [64]. Recent work described a novel protocol for separately
277 evaluating adipogenic commitment and subsequent differentiation in primary MSCs [63], previously
278 described for the C3H10T_{1/2} murine stem cell model [65, 66]. This protocol allows a complete
279 characterization of potential obesogens and their role in disrupting cell commitment and differentiation.
280 While the focus has been on evaluating effects on the adipocyte lineage, a growing body of research has
281 begun to evaluate potential chemical impacts on osteogenic development using these models [67-70].
282 Some limited research has evaluated chemical impacts on development down the chondrogenic,
283 myogenic, or other cell lineages [64]. Human MSCs are readily available from diverse vendors, although
284 murine models are also routinely used [45, 70-72].

285

286 Recent research elegantly described protocols for distinguishing assays to evaluate adipogenic lineage
287 commitment and subsequent adipocyte differentiation [63]; briefly, cells can be pre-treated with test
288 chemicals prior to the differentiation cocktail exposure. These pre-treated cells can be subsequently
289 exposed to the differentiation cocktail and evaluated at the end of the differentiation window. The extent
290 of triglyceride accumulation can be compared with standard adipogenesis plates; chemicals with effects
291 on commitment should have equivalent effects to those differentiated for the full two weeks, whereas
292 cells without effects on commitment should not accumulate more triglycerides than the vehicle control in

293 the commitment assays, regardless of effects in the standard adipogenesis assay [63].
294
295 The human MSCs lack the issues inherent in the primary human preadipocyte models; they can be
296 maintained in culture for several more passages, have less variability in sourcing, and are easier to isolate
297 and culture, increasing the utility of this model. This should lead to an increased reliance on human MSCs
298 for adipogenic *in vitro* testing. However, rigorous reproducibility assessments and comprehensive
299 validation testing are still needed to ensure accurate translation to and/or prediction of *in vivo* and human
300 health outcomes. Diverse bisphenols [72-74] and their mixtures [75], flame retardants [18], parabens [76],
301 and other environmental contaminants [63, 77-79] have been evaluated using MSC models. Research in
302 female MSCs demonstrated that RXR agonists attenuated glucose uptake; blunted adiponectin expression;
303 promoted a sustained interferon signaling, inhibiting markers of adipocyte browning; and unlike
304 activation of PPAR γ , failed to downregulate proinflammatory and profibrotic transcripts [77]. As the
305 authors described, these data implicated RXR agonists in the development of dysfunctional white adipose
306 tissue that could potentially exacerbate obesity and/or diabetes risk *in vivo*. Future research is needed to
307 evaluate these functional differences in adipocyte physiology to determine more subtle effects of
308 obesogenic contaminants. There has also been some initial research to evaluate the interplay between
309 lineage commitment, suggesting that exposures to certain chemicals can not only commit cells to the
310 adipocyte lineage but can also suppress the osteogenic lineage [45]; this interplay between different cell
311 lineages is an area of research that still requires further investigation and mechanistic assessment.
312
313 Human multipotent adipose-derived stem cells (hMADS), obtained from human infant adipose tissue,
314 have also been used to study the effects of aryl hydrocarbon receptor ligands that demonstrated an
315 inflammatory response in pre- and adipocytes, a phenomenon observed in obesity [80]. hMADS were also
316 used to screen 49 contaminants prioritized through ToxCast screening, reporting 26 active chemicals
317 across diverse chemical groups (i.e., pesticides, phenolics, phthalates, etc.) [81].
318

319 2.3 Spheroid adipocyte models

320 Spheroid cell cultures of both MSCs and preadipocytes are being developed and evaluated [82-87]. These
321 culture techniques may allow some inherent benefits over the traditional adherent monolayer cultures.
322 Spheroid culture of adipocyte models may improve differentiation efficiency relative to monolayer
323 cultures [82-86, 88], reducing time and cost investment. The fundamental goal of spheroid models is to
324 maintain greater *in vivo* or whole tissue-relevant signaling than monolayer models. Indeed, several papers
325 have demonstrated greater adipogenic and osteogenic gene expression relative to monolayer cultures and
326 a down-regulation of stemness markers [82, 83]. Other researchers have demonstrated increased plasticity
327 of spheroid constructs through multiple generations of these cells able to commit to and differentiate into
328 numerous cell lineages [89]. This plasticity might signal a greater variance in these models that requires
329 further investigation. While these models have received no apparent use for the interrogation of putative
330 obesogens, they have been demonstrated to exhibit improved relevance to the *in vivo* condition [90].
331 Specifically, researchers have demonstrated that human unilocular vascularized adipocyte spheroids have
332 unilocular morphology and large lipid droplets, and these cells develop key features of adipocyte
333 dysfunction (e.g., insulin resistance, impaired lipolysis, and disrupted adipokine secretion; [90, 91]) and
334 respond to stress (toxin or culture-related) by secreting pro-inflammatory adipokines [92]. These spheroid
335 cultures also maintain expression of markers specific to certain adipocyte types (e.g., brown) for longer
336 than is possible in 2D culture [92]. These 3D cultures also exhibit more physiologically relevant gene
337 expression (>4500 differentially expressed genes relative to 2D culture) and lipid profiles of >1000 lipid
338 species resemble the *in vivo* condition [93]. As such, these models may allow for a clearer understanding
339 of adipose physiology than was possible with monolayer cultures and hence requires further evaluation
340 and comprehensive validation and testing; this should also include evaluation of known adipogenic and/or
341 obesogenic contaminants to compare responses with existing models.

342

343 2.4 Liver cell assays

344 Obesogens are also known to target liver (either directly or indirectly) and promote metabolic diseases
345 such as toxicant-associated fatty liver diseases (TAFLD) or non-alcoholic fatty liver disease (NAFLD);
346 thus, there is a need to have accurate *in vitro* hepatocyte models for testing chemicals. Liver cell assays
347 are frequently used as surrogate models to predict *in vivo* hepatotoxicity related to chemicals and decipher
348 the determinants of NAFLD development and progression. The use of various hepatocyte models for
349 evaluating NAFLD and other metabolic disorders has been covered recently in detail [94-97]. These
350 models have been used to evaluate diverse environmental contaminants, including bisphenols [98, 99],
351 phthalates [99-101], pesticides [102], other environmental contaminants [99, 101], and therapeutics [103]
352 for effects on NAFLD and other metabolic dysfunction.

353
354 Among many liver cell lines, HepG2 cells a human hepatoma cell line commonly used for drug
355 metabolism and hepatotoxicity studies. HepG2 cells express certain differentiated hepatic functions like
356 lipoprotein metabolism, triglyceride metabolism, bile acid synthesis, glycogen synthesis, or insulin
357 signaling, making them a useful tool for some studies targeting hepatotoxicity and drug metabolism
358 [104]. HepG2 cells exposed to a low concentration of BPA alter lipid metabolism, mitochondrial function
359 and promote lipid accumulation leading later one to steatosis [105]. Co-incubation of HepG2 with fatty
360 acids palmitic acid and oleic acid, induced lipid accumulation in a dose-dependent manner which will
361 contribute to steatosis [106].

362
363 Comparatively, human THLE-2 and murine AML12 cell lines are derived from healthy liver cells and
364 express characteristics of normal adult liver epithelial cells [107]. Insulin receptor expression was low in
365 THLE-2 cells relative to AML12 and HepG2 cells, suggesting disparities in their application to insulin
366 receptor signaling. Gluconeogenesis and hepatokine expression was impaired in both THLE-2 and
367 AML12 cells; while expression of Angiopoietin Like 4 (ANGPTL4) was regulated by PPAR δ activation
368 similarly across THLE-2, AML12, and HepG2 cells, only HepG2 cells reflected the *in vivo* environment

369 with regulation by cAMP [107]. These models have been utilized to evaluate fatty acid induced lipid
370 droplet accumulation and the presence and causes of heterogeneity in the lipid droplet content [108],
371
372 The most prevalent human liver cell line is HepaRG. HepaRG cells can differentiate into hepatocyte-like
373 and biliary-like phenotypes after dimethylsulfoxide (DMSO) (1.75 - 2%) exposure, and possess the ability
374 to stably express several liver-specific genes such as albumin, aldolase B, CYP2E1 and CYP3A4 [109].
375 Changes in metabolites related to energy metabolism, oxidative stress, and insulin resistance have also
376 been observed in differentiated HepaRG cells supplemented with an oleate/palmitate mixture [110].
377 These are consistent with alterations observed in the liver tissues of human patients and animal models of
378 NAFLD [111, 112]. Altogether, these data further support the suitability of the fatty acid-supplemented
379 HepaRG model to study the impact of obesogens on steatosis progression towards steatohepatitis in the
380 context of the “two-hit” model [113]. In line with these data, an oleate/stearate mixture is sufficient to
381 decrease the expression of CYP1A1, 1A2, 1B1 and decrease their activity after steatosis induction [114].
382 These results corroborate data obtained from NAFLD rodent models, especially regarding CYP1A1 and
383 1A2 [115-117].
384
385 In addition, several 3D liver culture models have also been developed to create a cell environment closer
386 to *in vivo* conditions. In 3D cell cultures, cell growth and interaction with surrounding conditions exhibit
387 higher differentiation and benefit from more extended culture than 2D cultures [118]. When cultured as
388 3D spheroids, HepaRG cells express genes involved in lipoprotein metabolism, energetic lipid synthesis,
389 gluconeogenesis, glycolysis, and bile acid metabolism, liver-specific functions, and xenobiotic
390 metabolism enzymes [119, 120].
391
392 Primary human hepatocytes (PHH) are increasingly used to predict drug metabolism and liver enzyme
393 induction in humans. However, PHH have inherent limitations: scarce and unpredictable availability,
394 limited growth activity and lifespan, and early and variable phenotypic alterations in 2D culture.

395 Moreover, liver-specific functions, particularly cytochrome P450 (CYP) activities and their
396 responsiveness to prototypical inducers, are not maintained with increasing time of culture. Liver-specific
397 functions also usually decrease with time in culture and are differently altered [121, 122]. Cultivated in a
398 3D collagen matrix, they proliferate, form hollow spheroids, and undergo robust hepatic differentiation.
399 They can be maintained in this state for at least 28 days without decreasing survival rate and cellular
400 polarity and require fewer cells to generate spheroids than 2D cultures [123]. PHH 3D-spheroid models
401 co-cultured with liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, increase human
402 hepatocyte functionality (increased mRNA expression of APOB, CYP3A4, and albumin). Essential
403 factors such as spheroid size, time in culture, and culture media composition affect basal levels of
404 xenobiotic metabolism and liver enzyme inducibility *via* activators of hepatic receptors such as the aryl
405 hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR)
406 [124]. Various co-culture techniques have also been developed for liver cell assays to recreate more tissue
407 or disease-relevant environments for the evaluation of disease biology and toxicology [125].

408
409 Similarly, primary murine hepatocytes (PMHs) are readily isolated through rapid protocols and thus have
410 improved availability relative to PHH [126]. PMHs have been well-described as a model to assess fat
411 deposition, inflammatory responses, and mechanistic interrogation of fatty acid induced lipid
412 accumulation by diverse contaminants [127-129].

413 414 *2.5 Muscle cell assays*

415 While skeletal muscle is the main tissue responsible for utilization of glucose and is the main site of the
416 development of insulin resistance, the impact of toxicants on skeletal muscle has not been extensively
417 studied. Detecting effects *in vitro* can be difficult due to the specific cell culture requirements and
418 stimulation of skeletal muscle fibers required to mimic physiological function. Since skeletal muscle
419 plays a critical role in developing metabolic diseases, any chronic disturbances in muscle cells may
420 contribute to insulin resistance and subsequent obesity.

421
422 The most widely used *in vitro* myocyte model is the murine myoblast cell line, C2C12. These cells can be
423 differentiated into myotubes (immature muscle cells) over several days. BPA and estradiol have been
424 demonstrated to suppress myogenic differentiation by inhibiting Akt signaling in C2C12 cells [130],
425 potentially disrupting ER signaling. Tolyfluanid alters insulin signaling, mitochondrial function, and
426 protein synthesis in C2C12 cells in a manner dependent on fatty acid levels [131]. The rat myoblast cell
427 line, L6, has a longer differentiation time relative to C2C12 cells, as well as appreciable differences in
428 mitochondrial respiration and glucose utilization [132]. In L6 rat myotubes, di(2-ethylhexyl) phthalate
429 (DEHP) exposure was shown to affect insulin receptor expression, GLUT4 expression, as well as glucose
430 uptake and oxidation, indicating that it may negatively influence insulin signaling [133]. The pesticides
431 dichlorodiphenyltrichloroethane (DDT) and lindane impair insulin signaling in L6 myotubes, promoting
432 insulin resistance-like conditions [134].

433
434 Human and rodent primary myoblasts are also used. However, they are unsuitable for extended cultures
435 and more extensive screening studies due to relatively low numbers of cells obtained at a relatively high
436 cost. Some polychlorinated biphenyls (PCBs) have been shown to inhibit myogenic differentiation of
437 primary murine myoblasts and L6 cells [135]. In primary murine myoblasts differentiated to myotubes,
438 low micromolar concentrations of BPA and tetrabromobisphenol A (TBBPA) were shown to affect
439 calcium signaling and resting potential. In a similar study, using rabbit skeletal muscle microsomes, BPA
440 and TBBPA were shown to differently affect the function of proteins involved in calcium signaling [136].

441
442 Notably, there are distinct differences between mature muscle tissue and myotubes derived from myoblast
443 cell lines or primary myoblasts [132]. Myotubes have lower energy demand, lower oxidative
444 phosphorylation, higher glycolysis, and lower insulin responsiveness [137]. There is a considerable
445 knowledge gap regarding the effects of environmental chemicals in more complex and physiologically
446 relevant skeletal muscle systems, which require additional validations.

447

448 3. *In vivo* assays

449 While *in vitro* mechanistic studies are a critical component in environmental chemical research, these
450 studies cannot replace the need for *in vivo* integrative models, particularly for adverse health outcomes
451 that develop later in life following developmental exposures. Research examining the environmental
452 health consequences of exposure to environmental chemicals using animal models has demonstrated that
453 some adverse health effects of chemical exposures reported in humans are also apparent across other
454 vertebrates [138]. These findings are essential for understanding the impact of environmental chemicals,
455 including obesogens, across all vertebrates [139]. These tests are critical because the classification of
456 obesogens into different classes according to the strength of evidence is highly dependent on the tests
457 used.

458

459 Beyond the classical rodent *in vivo* models used to investigate human obesity, new models have emerged
460 based on alternative model organisms, *e.g.*, bony fishes, worms, and flies [140] (Figure 2). These model
461 organisms, including *Danio rerio* (zebrafish), *Caenorhabditis elegans* (*C. elegans*; roundworm), and
462 *Drosophila melanogaster* (fruit flies), offer several advantages to accurate discernment of the metabolic
463 processes involved in metabolic diseases such as obesity [141]. These organisms share small size, large
464 numbers of progeny, relatively rapid development, and sequenced genomes. They are well suited to
465 moderate throughput screening of chemicals to study metabolic diseases [142-146]. Moreover, most
466 genes and gene families implicated in metabolic diseases are conserved among flies, worms, zebrafish
467 and humans [144]. Below we present a short overview of the utility of each model and some summarized
468 obesogenic chemical evaluation using these emerging models (Table 1).

469

470 3.1 *Danio rerio* (Zebrafish)

471 Zebrafish, a small tropical freshwater fish native to South Asia (*e.g.*, India and Bangladesh), has found
472 wide use in almost all areas of biological research [147, 148]. Zebrafish is one of the most widely used

473 models to study metabolic dysfunction. They have indeed all the critical organs that regulate energy
474 homeostasis and metabolism, including adipose tissue, digestive organs, i.e., pancreas and liver, and
475 skeletal muscles, all physiologically and anatomically like humans [141, 149, 150]. The rapid
476 development of zebrafish promotes metabolically functional organs only a few days post-fertilization
477 (dpf; e.g., pancreas and liver develop around three dpf). Organogenesis and biological processes can be
478 easily monitored due to the extra-uterine development and the semitransparency of the embryo and larva
479 stages that persist until a relatively late stage of development [151].

480

481 Zebrafish store excess neutral triglycerides in lipid droplets within white adipocytes similar to mammals
482 [152] and have well-described anatomically, physiologically, and molecularly distinct adipose depots
483 throughout their bodies [153-155]. This contrasts with *Drosophila* and *C. elegans*, where fat is stored in
484 non-specialized cells (within the fat body or within the intestine, respectively) that carry out several other
485 functions besides lipid storage [156]. Regulations of body weight, appetite, lipid, and sugar homeostasis
486 share similar mechanisms between humans and zebrafish and are similarly affected by endocrine
487 disrupting chemicals (EDCs) [145, 157, 158]. The development of WAT starts in the pancreatic and
488 abdominal adipose depots, then in various cranial and ocular depots, and finally expands throughout the
489 fish. The appearance correlates with the size rather than the age of the fish [159-161]. The first adipocytes
490 develop from 8-12 dpf or at a minimal larval size of approximately 5 mm [160].

491

492 Zebrafish obesity models enable the evaluation of diet, chemical or genetic, phenotypic modifiers through
493 several different techniques [162-165]. Measurement of total body triglycerides may be used as an
494 indicator for evaluating adiposity and/or obesity progression [161]. Adipocytes can also be visualized and
495 quantified by lipid staining with the Oil Red O neutral dye or with various fluorescent lipophilic dyes
496 (e.g., Nile Red, Lipid Green) in live fish, adult zebrafish sections, or fixed zebrafish larvae. Since
497 zebrafish larvae are transparent, live-imaging and fluorescent staining allow ready detection and
498 quantification of intracellular lipid droplets and adipose tissue, including its regional body distribution

499 [166, 167]. These methodological advantages have been exploited for developing a bioassay to evaluate
500 the obesogenic properties of chemicals in zebrafish larvae [161]. Zebrafish models can also help assess
501 specific windows of sensitivity during life as well as transgenerational effects of obesogens [168-170] and
502 can be used to study the interaction between the diet composition and metabolic health effects promoted
503 by subsequent chemical exposures [114, 152, 159, 160, 171]. Interesting recent research demonstrated
504 that long-term dietary vitamin D deficiency promoted stunted growth and increased central adiposity via
505 both adipocyte hypertrophy and hyperplasia in both visceral and subcutaneous depots [172]. Through
506 lipidomics analysis, these fish were demonstrated to have increased bioactive lipids that seemed to be
507 mediated through disrupted endocannabinoid signaling [173].

508
509 Zebrafish have been widely applied to obesogenic chemical testing, with expanding capacity for
510 modulation of diverse metabolic disrupting effects [27, 169, 174-177]. Among other obesogenic chemical
511 evaluations, developmental exposure of bisphenol S in combination with overfeeding promoted increased
512 triacylglycerol and visceral adiposity via disrupted lipid metabolism [175], while BPA exposures both
513 transiently and persistently disrupted food intake, increased body weights, and disrupted gene expression
514 related to glucose and lipid metabolism [165]. Halogenated BPA analogs also promoted lipid
515 accumulation in zebrafish larvae in a manner correlated with their activity as zebrafish PPAR γ agonists
516 [27]. Developmental exposures to nonylphenol and nonylphenol polyethoxylates increased body weights
517 and adiposity (in both viscera and subcutaneous adipose depots) and disrupted energy expenditure [79].
518 Tributyltin exposure has been described to increase body weights, hepatic triglycerides, and
519 hepatosomatic index, along with disrupting genes related to adipogenesis, lipogenesis, and diverse other
520 metabolism and growth-related pathways [178] as well as increasing adiposity [161]. Developmental
521 cadmium exposures have also been demonstrated to increase lipid accumulation, though this effect was
522 transient (observed at one and two months post fertilization but was no longer observed by 3.5 months
523 [177]). Perfluorooctane sulfonate (PFOS) exposures have also been described to increase adiposity and

524 disrupt pancreatic islet morphology and area in developmentally exposed zebrafish, along with increasing
525 fatty acid concentrations and disrupting PPAR gene expression [169].

526

527 *3.2 Oryzias latipes (Medaka)*

528 The Japanese rice fish, also known as the medaka, are a valuable model for environmental chemical and
529 epigenetic transgenerational research [179]. Similar to zebrafish, this model can be used for estimating
530 adipose tissue volumes and the effects of nutritional factors (dietary soy sauce oil) or various
531 environmental chemicals such as per/polyfluoroalkyl substances and tributyltin chloride [180-182].
532 However, they lack the thorough characterization of adipose depots and the transparent bodies that
533 zebrafish benefit from. They have also been utilized for determining transgenerational effects on
534 metabolic health outcomes such as lipid metabolism [183]. Research using medaka has also evaluated
535 chemical exposures and effects on bone formation [184], suggesting a potential strength for this model in
536 the evaluation of differential MSC lineage commitment.

537

538 Medka have not yet been widely used in obesogenic chemical evaluations, but some preliminary research
539 suggests utility in this model for diverse obesogenic endpoints. Specifically, exposure of medaka to both
540 tributyltin and perfluorooctane sulfonate (PFOS) individually promoted adipose accumulation in larvae,
541 with mixtures of these two obesogens resulting in enhanced effects (even below the individual no-effect
542 concentrations) [181]. In related research, tributyltin exposures disrupted signaling pathways related to
543 PPAR signaling, hormonal metabolism, and genes related to obesity in humans via mRNA-Seq analysis
544 in exposed zebrafish [185]. Similarly, BPA exposure was reported to disrupt genes related to lipid
545 metabolism (cholesterol and lipid synthesis, regulation, and transport, etc.) in a sex-specific manner [186].

546

547 *3.3 C. elegans (Roundworm)*

548 The roundworm is a small nematode living in temperate soil environments that has been used as a model
549 organism since the 1960s in everything from developmental biology to neurodegenerative disease and

550 aging. Although *C. elegans* is generally considered genetically and physiologically distant from humans,
551 several studies have shown that the main regulatory pathways of energy homeostasis are shared between
552 mammals and nematodes [144, 187, 188]. These advantages make *C. elegans* a suitable *in vivo* model to
553 identify compounds that modulate fat storage and promote obesity [141, 189]. Both simple fluorescence
554 (Nile red or Sudan-black probes) and biochemical (triglyceride assays) techniques can be used to quantify
555 lipid amount and fat storage in these worms [188]. In addition, genetic approaches using mutant or
556 transgenic animals can help evaluate molecular mechanisms underlying metabolic health effects [187,
557 188]. Moreover, *C. elegans* can be readily used to measure food intake and energy expenditure [188,
558 190]; several diets, food-derived or nutraceutical compounds, and fat-increasing compounds have been
559 described to modulate fat accumulation [189-191]. Limitations of this model include lower conservation
560 of biological pathways with humans and a lack of particular organs and circulatory systems [192]. *C.*
561 *elegans* also lack PPAR γ , though they do express orthologs of both PPAR α and δ , and have no
562 identifiable homolog for leptin [193, 194]. Perhaps unsurprisingly, they thus have no cells specifically
563 designed for lipid storage (i.e. adipocytes), though they do still store lipids, primarily in intestinal and
564 epidermal skin-like cells, which are comprised of diverse saturated, monounsaturated, and polyunsaturated
565 fatty acids [193]. This model has also been used to assess transgenerational effects, with research
566 demonstrating that starvation of the parental generation promoted disrupted metabolism in the F3
567 generation, whereas BPA exposures resulted in transgenerational modulation of epigenetic germline
568 silencing through up to five subsequent (non-exposed) generations (reviewed in [195]).
569
570 Despite these limitations, this model has been utilized widely in better understanding the genetics of fat
571 accumulation, storage, and obesity [194, 196], and has been applied to obesogenic chemical evaluation
572 successfully. Specifically, methylmercury exposure promotes triglyceride accumulation, lipid storage, and
573 alter feeding behaviors [197], erythromycin promotes increased fat content and triacylglycerol levels as
574 well as promoting overeating, presumably mediated through stimulation of serotonin, dopamine, and
575 acetylcholine and/or disruption of lipogenesis and lipolysis [198]. Recent research demonstrated a non-

576 monotonic increase in overall fat deposition and triglyceride content following bisphenol S exposures,
577 along with modulation of fat synthesis and fatty acid oxidation gene expression [199].

578

579 *3.4 Drosophila melanogaster (Fruit fly)*

580 The fruit fly is one of the most used model organisms throughout biological research. The small size,
581 short generation time, low cost, ease of breeding, and a large panel of genetic tools have spurred use in
582 genetic and developmental biology research [192, 200]. Many studies have demonstrated the usefulness
583 of this model in nutrition and obesity studies based on the manipulation of diet composition and genes
584 involved in nutrient sensing and regulation of energy balance [201]. Although this model is anatomically
585 different from mammals, many organ systems perform similar functions relative to mammals. For
586 example, the fruit fly fat body covers metabolic functions of liver and adipose tissue (e.g., fat and
587 carbohydrate storage). Instead of a fully differentiated pancreas, there are neurosecretory insulin-
588 producing cells (IPCs), which allow carbohydrate and lipid homeostasis *via* the production and secretion
589 of an insulin-like peptide [146, 201]. Few studies have utilized this model to evaluate potential obesogens
590 and/or obesity biology, though its suitability for evaluating endocrine impact(s) on development and
591 fertility is well accepted [202]. The efficiency of this model in assessing obesogenic properties of EDCs is
592 highlighted by several studies demonstrating alterations of lipid homeostasis with chemical exposure
593 (e.g., DEHP) and subsequent increase in lipid/adipose accumulation and/or transgenerational effects [203-
594 205].

595

596 *3.5 Rodents*

597 A critical issue in selecting an animal model is whether the outcomes examined are relevant to human
598 anatomy, physiology, molecular mechanisms and show homology with humans, which has historically
599 driven a reliance on rodent models (e.g., rats and mice). The use of rodents in metabolic health research is
600 well-described and assessed by several previous reviews [206-208]. Here we will address other
601 considerations for *in vivo* model organism research revealed through comprehensive evaluations in rodent

602 models. Many of these factors have yet to be evaluated or considered for the emerging models described
603 above but will need to be assessed as they are increasingly used.

604
605 Dozens of publications have clearly delineated the use of the rodent model in metabolic health research.
606 A number of studies (reviewed in [207, 208]) have explicitly described the use of hypercaloric and/or
607 high fat diets to promote metabolic disorders and the clear translation of this preclinical model to human
608 metabolic syndrome. However, other approaches, such as creating a crowded uterus in pregnant mice due
609 to prior hemiovariectomy, have also been used to generate metabolically abnormal intrauterine growth
610 restricted (IUGR) and macrosomic offspring in the same litter [209].

611
612 There are diverse genetic models of obesity, including *db/db* mice (leptin receptor mutation that promotes
613 higher body weights, triglycerides, and cholesterol, hyperinsulinemia, and impaired glucose tolerance),
614 *ob/ob* mice (leptin gene mutation resulting in inactive leptin protein and promoting obesity,
615 hyperinsulinaemia and hyperglycaemia), *fa/fa* diabetic fatty rats (different leptin receptor mutation
616 promoting hyperinsulinaemia, hypertriglyceridaemia, and increased serum inflammatory markers), and
617 Otsuka Long-Evans Tokushima fatty rats (Pancreatic acini cells insensitive to cholecystokinin, which
618 controls food intake, promoting obesity, hypertriglyceridaemia, impaired glucose tolerance), that have
619 been described in detail previously [206]. Rodents can be robust models for body weight, adiposity,
620 development of specific adipose depots, measurement of diverse lipid classes, glucose and insulin
621 signaling, inflammatory markers, blood pressure, controlled measurement of food and water intake and
622 metabolic activity, as well as NASH and NAFLD, among other metabolic outcomes [206].

623
624 *3.6 Use of inbred vs. outbred models*

625 Genetic diversity of model organisms (inbred *versus* outbred) can be an essential design consideration for
626 chemical contaminant studies. Researchers may select an inbred rodent strain without background genetic
627 variation to study the epigenetic basis of phenotypic diversity (e.g., inheritance of an epigenetic trait)

628 [210]. In contrast, a researcher may choose an outbred rodent (e.g., CD-1) for the genetically diverse
629 background to assess toxicant-induced effects more rigorously. However, there are concerns that
630 laboratory outbred rodent strains differ substantially between vendors and relative to bona fide outbred
631 animals. Inbred rodents do not represent the spectrum of sensitivity required to model genetically diverse
632 human populations accurately. For example, males at puberty have considerable heterogeneity in rodent
633 responsiveness to estrogens [211]. The C57BL/6J inbred strain is exquisitely sensitive to estradiol after
634 puberty relative to other mouse strains/stocks and exhibits hyper-estrogenization during fetal life, which
635 becomes apparent in behavioral assays [212]. Interestingly, C57 mice are insensitive to xenoestrogens
636 administered *via* the dam compared to the outbred, hyper-fertile CD-1 mouse, which exhibits high
637 sensitivity fetal-neonatal response to xenoestrogens [213]. Given this, the choice of strain used can have
638 demonstrable impacts on endpoint measurements.

639

640 *3.7 Animal feed as a source of variability*

641 Animal feed can be a substantial source of variability in toxins, phytoestrogens, sources of fats, and other
642 components. Open formula feeds provide the proportion of nutrients, which is intended to reduce, but not
643 eliminate, batch-to-batch variability. Closed formula (constant nutrition) feeds just provide information
644 about the amount of protein, fat and fiber, but the sources may vary due to price and availability [214,
645 215]. Thus, the choice of feed used in animal studies, impacted by price, can be a critical source of
646 variability in outcomes of health-related research and can also be the basis for studies that do not replicate
647 prior results [216]. For example, publications by Thigpen and colleagues reported that a batch of constant
648 nutrition rodent feed (Purina® 5002) containing elevated levels of phytoestrogens (focusing on the soy
649 isoflavones genistein and daidzein) interfered with the ability to see estrogenic effects of a positive
650 control chemical, the potent estrogenic drug diethylstilbestrol (DES). However, DES effects were
651 observed with another batch of 5002 feed that had much lower phytoestrogen levels. The rat strain used
652 also mattered, with Sprague-Dawley rats showing no effect of use of soy feed, while the CD-1 mouse (the

653 model used by the National Toxicology Program), is, as discussed below, very sensitive to components of
654 feed [217].

655
656 This observation by Thigpen demonstrated that there can be significant batch-to-batch variability of
657 phytoestrogen levels in laboratory animal feed with presumably the same nutrient profile; a constant level
658 of soy protein in different batches of a feed can have markedly different levels of phytoestrogens, which
659 vary in soy based on many environmental factors [216]. It has been assumed for some time that the only
660 issue of concern with soy-based feeds was variability in the soy phytoestrogens genistein and daidzein,
661 but findings described below suggest other components of soy-based feeds (e.g., contaminated fish meal,
662 source of lipid) may also lead to significant differences in phenotype in mice. Second, the study revealed
663 that specific batches of feed could promote replication failure relative to most prior studies reporting that
664 DES (a known human carcinogen) disrupted development in mice, just as it did in humans [218].

665 Developmental exposure to DES also promoted obesity during later adulthood in mice maintained on a
666 soy-based (NIH31) open formula feed [219]. This demonstrates that a core issue should be whether the
667 feed used is resulting in an inability to see effects in response to treatments that others are reporting. Not
668 surprising is that industry-funded research on BPA, which claimed to be a replication of findings from
669 multiple laboratories [220], in fact, had used 5002 feed [221, 222]. This led to a failure to demonstrate a
670 BPA-induced effect in both CF-1 mice and Crl:CD Sprague-Dawley (CD-SD) rats. This research also
671 failed to demonstrate effects of DES with this food (included as positive control) [221], suggesting an
672 inappropriate model to detect BPA-induced effects [223].

673
674 In other studies, the expected developmental effects of DES were again shown not to occur in CD-1 mice
675 fed 5002 feed, but were found if the mice were fed the constant nutrition, soy-based Purina[®] 5008/5001
676 breeder and maintenance feeds, respectively. Specifically, relative to Purina[®] 5008 fed to pregnant CD-1
677 mice, the 5002 feed significantly estrogenized and elevated fetal serum estradiol in fetuses. Critically, the
678 5008 feed had >50% higher total estrogenic activity (detected in a human breast cancer cell bioassay) as

679 well as higher amounts of genistein and daidzein relative to the 5002 feed, substantiating that 5002 feed
680 interfered with finding DES effects, but this was not mediated by elevated genistein and daidzein or total
681 estrogenic activity as initially proposed [224].

682
683 In addition to problems related to the use of soy-based 5002 feed, feeding casein-based low phytoestrogen
684 5K96 feed to pregnant CD-1 mice also elevated endogenous serum estradiol in fetuses compared to CD-1
685 mice fed Purina® 5008; 5K96 casein feed thus also promoted estrogenization of mouse fetuses, similar to
686 effects in mice exposed as fetuses to xenoestrogens such as DES or BPA [225]. Relevant to this review,
687 the 5K96 feed resulted in morbid obesity in adult CD-1 male mice (all internal organs were encased in
688 fat) compared to Purina 5008/5001 or Harlan Teklad 8604, another soy-based constant nutrition feed
689 [225, 226].

690
691 Another example of feed-based impact on a supposed “non-replication” experiment was when prior
692 metabolic effects of BPA and DES were not found is a study in which the control CD-1 mice were
693 morbidly obese and did not show the previously reported effects of fetal exposure to BPA or DES [227]
694 while maintained on the casein-based AIN93G feed [228]. The fetal mice whose mothers were fed casein-
695 based 5K96 or soy-based 5002 feeds potentially had elevated aromatase (estrogen synthetase) activity,
696 thus elevating fetal estradiol levels, compared to other soy-based feeds. Various flavonoids and lignans
697 have been reported to inhibit aromatase activity in a human preadipocyte cell culture assay [229],
698 although the components of the different feeds that caused these effects remain unknown.

699
700 There have been many articles published about the issue of non-replication in laboratory research, mostly
701 attempting to sensationalize the problem [230], but clearly, there are issues, such as variability in feed,
702 that are a major contributing factor in non-replication in laboratory animal research. The above findings
703 demonstrate the critical importance of, whenever possible, including a positive control in toxicological or
704 pharmacological studies that will provide information about the sensitivity and validity of the assays and

705 results [223]. The vast diversity of animal feed components, including the casein or soy backbone and
706 multiple sources of protein and lipids, can markedly impact research findings related to metabolic health.

707

708 *3.8 The role of positive controls in animal model selection*

709 A National Toxicology Program (NTP) panel addressed animal models for EDCs or drug research. It
710 stated: “Because of clear species and strain differences in sensitivity, animal model selection should be
711 based on responsiveness to active endocrine agents of concern (i.e., responsive to positive controls), not
712 on convenience and familiarity.” The rat strain (CRL: CD(SD)) is used by many investigators to examine
713 gestational exposure to estrogenic chemicals and drugs, although this rat strain required over a 15-fold
714 higher dose of ethinylestradiol to show a response relative to women [231]. It is well known that selecting
715 for very high fecundity (CD-SD rats average 14-15 pups per litter), results in low sensitivity to estrogenic
716 drugs and chemicals [232].

717

718 It is also possible that the characteristics selected for in the generation of the CD-SD rat strain, with large
719 litter size and accelerated postnatal growth, may make them resistant to contaminant exposures, reducing
720 their future sensitivity and usefulness as a model; this strain is generally used in all FDA and in many
721 commercial laboratory toxicology studies. Some strains have undergone selection for large litter sizes for
722 over 100 generations in commercial laboratories, with the largest 5-10% of litters selected every
723 generation for >100 generations, regardless of whether they were exposed to pesticides (in feed or used in
724 the colony), xenoestrogens in their cage materials, or diseases in the colony, etc. The result is laboratory
725 animal strains that are precocious, excellent breeders and produce large litters. However, the laboratory
726 animal suppliers selected large litter animals not sensitive to environmental chemicals [211, 232]. Thus,
727 before proceeding with experiments using environmental chemicals such as potential obesogens, it is
728 critical to examine the sensitivity of the animal model to appropriate positive controls (e.g., DES for
729 estrogenic testing) for the endpoint examined to ensure that each experimental design is sensitive to the

730 environmental chemical being examined.

731

732 *3.9 Animal housing*

733 The caging used in an experiment is an additional key factor. This was clearly described in studies of
734 BPA, the monomer used to make polycarbonate cages and bottles. Due to harsh washing of the cages,
735 BPA was found to leach from the polycarbonate cages; this was further shown to expose both control and
736 intervention animals to this xenoestrogen, negatively influencing the experimental determinations of
737 successful meiosis in mouse oocytes [233-235]. It is also worth noting that the vast majority of aquatic
738 housing systems use polycarbonate; there is likely to be leaching of BPA from these and potential
739 recirculation of the chemical throughout the system. While some alternatives do exist [e.g., polysulfone
740 (PS) or glass], they are often cost-prohibitive. Polycarbonate (PC) consists of BPA molecules linked by
741 ester bonds that are subject to hydrolysis under elevated temperature or either high or low pH. PS is a co-
742 polymer of BPA and bisphenol S (BPS) that is linked by ether bonds and is stable under temperature and
743 pH conditions that hydrolyze BPA bonds in polycarbonate, though PS cages are more expensive. It is
744 essential to ascertain the potential impacts of the housing materials (for rodents, also water bottles) on
745 testing estrogenic or other metabolism disrupting chemicals.

746

747 *3.10 Assays for detecting thermogenic brown fat activity*

748 Beige and brown thermogenic fat produces heat during non-shivering thermogenesis to regulate body
749 temperature by burning calories (i.e., glucose and lipids) [236]. These tissues help regulate glucose and
750 lipid levels, making them high-priority targets for future therapeutics in the treatment and prevention of
751 obesity and other metabolically related diseases [237]. The functionality of beige and brown fat and the
752 discovery that these tissues exist in adults have made the development of reliable assays a critical step to
753 better quantify and harness their therapeutic potential as well as to identify chemicals that promote or
754 inhibit function.

755

756 The energy expenditure in beige and brown adipose tissue (BAT) is made possible through the activity of
757 uncoupling protein 1 (UCP1) in brown and beige fat, which uncouples mitochondrial respiration from
758 ATP production, leading to the generation of heat [237]. Reporter systems that focus on UCP1 levels
759 have been developed to measure the activity of thermogenic fat and have been used as a screening tool to
760 identify novel small molecules that can induce thermogenesis within these tissues. Specifically, the
761 ThermoMouse model measures thermogenesis via luciferase activity linked to levels of UCP1 expression
762 in BAT following environmental stimuli (e.g., decreased temperatures) [238], which has also been
763 adapted as an *in vitro* assay to screen small molecules for luciferase activity [238]. This assay has
764 supported screening of potential drug targets that promote UCP1, and which could provide a foundation
765 for future BAT-mediated drug therapies that could induce thermogenesis and energy expenditure [239-
766 243].

767
768 The OLTAM (ODD-Luc based Thermogenic Activity Measurement) system was developed to assay the
769 activity of UCP1 independent thermogenesis in beige and BAT. In this *in vivo* model, a transgenic mouse
770 that expressed the ODD (oxygen-dependent degradation) domain of hypoxia-inducible factor 1 alpha
771 (HIF1 α), tagged with luciferase, was used to measure hypoxia. Hypoxia has been shown to take place
772 during nonshivering thermogenesis in beige and brown fat and is an indicator of thermogenesis [244]. An
773 *in vitro* system was developed using the stromal vascular fraction of isolated brown adipocytes from these
774 mice to measure cell-based thermogenic activity [244]. These cells could be used to evaluate the action of
775 chemicals on the function of thermogenic beige and brown adipocytes.

776
777 Measuring changes in heat generated within BAT offers another tool to assay thermogenic activity.
778 Noninvasive imaging techniques lack sensitivity and specificity due to the distance between the
779 instrument and the tissue, and invasive techniques lack sensitivity due to their inability to directly and
780 safely insert into BAT and their inability to detect more minute temperature fluctuations [245]. Xenon-
781 enhanced computed tomography enabled accurate measurement of BAT within mice due to the lipophilic

782 preference of xenon gas [246], which has been further enhanced through later research [245].
783 ERthermAC, a small molecule fluorescent dye that responds to changes in intracellular heat, is another
784 tool that has been found to assay chemically stimulated thermogenesis in both rodent and human brown
785 adipocytes [247], and has provided evidence comparable to existing indirect methods of measurement.
786
787 Lastly, UCP1-expressing brown adipose cells isolated from supraclavicular depots in humans have
788 revealed that the molecular makeup of these cells more closely resembled mouse beige adipocytes than
789 brown adipocytes [248]. In addition, humans who initially possessed no BAT, were found to create new
790 BAT within the supraclavicular region. This suggests that human BAT is derived from the browning of
791 beige fat. One could develop assays based on these cells to identify chemicals that promote or inhibit the
792 production of these thermogenic adipocytes.

793

794 4. *In silico* tests

795 Computational strategies offer promising tools for developing animal-free models for human risk
796 assessment of obesogens. Traditional computational methods using structural information of chemicals
797 (quantitative structure-activity relationship (QSAR), Read Across) have already been outlined as a
798 general strategy for non-animal testing approaches, for example, by the US National Research Council
799 (Tox21, Toxicity Testing in the 21st Century) [249] and the Organization for Economic Cooperation and
800 Development (OECD) guidelines. New approach methodologies (NAMs), including *in silico* methods, are
801 increasingly important in toxicant risk assessment [250].

802

803 With the recent advance in omics and high throughput screening, the amount of information on
804 gene/protein activity in response to obesogenic chemicals has expanded substantially, thereby enabling
805 the development of innovative approaches such as integrative systems biology/toxicology models.
806 Systems toxicology uses advanced bioinformatics and statistical tools to integrate heterogeneous data
807 types (functional genomic profile of obesogens, protein-protein interactions, protein-tissue associations,

808 disease annotations, etc.) to mimic the complexity of the biological organization, to identify
809 uncharacterized putative associations between an obesogen and its biological targets, and therefore to
810 prioritize further experimental testing, thereby associating these chemicals with the disease [251, 252].

811
812 Adverse Outcome Pathways (AOPs) are structured frameworks representing relationships between
813 molecular initiating events, key events, and adverse outcomes. The OECD proposed AOPs to enable
814 robust mechanistic evidence for chemical safety and risk assessment [253]. However, for chemical risk
815 assessments, a pragmatic approach has been proposed for applying AOP criteria in evaluating the safety
816 of a chemical [254], since a comprehensive understanding of the initiating events and molecular pathways
817 linking chemicals to adverse outcomes is unrealistic; for a chemical such as BPA with over 10,000
818 publications and clearly understood to result in adverse effects [255], understanding all of the AOPs is
819 still a work in progress. AOPs describe and connect data from various sources, i.e., databases and the
820 scientific literature. Key information used to build AOPs can also be gathered using computational
821 approaches based on artificial intelligence, such as frequent itemset mining and text mining [256]. AOP-
822 helpFinder is a recent hybrid tool that combines text mining and graph theory, helping identify the
823 existing linkages between variables (e.g., an obesogen and a biological event) by automatically screening
824 the available scientific abstracts [257]. Using this tool, it was possible to link exposure to bisphenol S
825 with obesity [258]. Integrative systems toxicology modeling and text mining can also link obesogens to
826 AOPs, as proposed recently for bisphenol F [259].

827

828 **5. The Future of Screening for Obesogens**

829 A single approach or assay will not yield all the information needed to identify and classify obesogens.
830 Data from epidemiological studies should be integrated with experimental data from animal models to
831 support the evidence for the obesogenic potential of an identified chemical. It is advisable to adopt a
832 tiered approach to identify and characterize EDCs, which can ultimately inform their classification as
833 obesogens, which has been proposed previously [260]. For example, if robust biomarkers such as

834 epigenetic modifications (e.g., DNA methylation), growth factors, or metabolites are identified through *in*
835 *vivo* experimental studies, they can be matched with findings from human studies. *In vitro* methods that
836 assess these changes will support prioritized screening for putative obesogens, which can then be
837 classified accordingly. Structured frameworks, such as the integrated approaches to testing and
838 assessment (IATAs), allow categorization of different tests that support the linkage of a chemical with an
839 adverse outcome and with the different events leading to that outcome. IATAs are expected to be used for
840 large scale obesogen testing and appear to be more time- and cost-effective than current approaches [261].
841 Additional *in vitro* tests are needed, including assays that will develop and characterize brown and beige
842 adipocytes to be used to define further the sites and actions of potential and actual obesogens.

843
844 Approaches like this have been previously attempted using the ToxCast dataset. The National Institute for
845 Environmental Health Sciences (NIEHS) hosted a workshop in 2011 to develop models for predicting
846 obesogenic and/or diabetogenic outcomes using ToxCast and Tox21 data [262]. Expert panels developed
847 (among others) a model to predict chemicals likely to promote adipocyte differentiation. An early
848 application of this model reported poor performance in predicting both active and inactive adipogenic
849 chemicals and suggested that better validation of primary high throughput screening assays was required
850 before using ToxCast data for this purpose [62]. Later analysis updated the predictive model and reported
851 more promising effects [81]. Computational modeling cannot substitute for experimental (*in vitro* and *in*
852 *vivo* studies) but can help prioritize obesogens, assess human health risks and trigger new epidemiological
853 and experimental studies. To be useful for screening purposes, computational models need to be grounded
854 in real-world data and continually refined such that predicted activities match the results of *in vitro* and *in*
855 *vivo* screening assays.

856
857 Indeed, there is consensus regarding the need for standardized testing methods to identify new chemicals
858 that trigger metabolic dysfunction. In this context, initiatives like the French PEPPER (Public-privatE
859 Platform for the Pre-validation of Endocrine disRuptors characterization methods, <https://ed-pepper.eu>)

860 platform may facilitate development of pre-validated methods and assays in toxicology for identification
861 of novel EDCs [263]. In Europe, a collaborative group of eight projects, named EURION [264], was
862 established in 2019. EURION aimed to develop integrative tests to identify new EDCs. Among
863 EURION's projects, three projects focus on obesity and metabolic disorders (OBERON [265], GOLIATH
864 [266], and EDCMET [267]), which are expected to deliver standardized batteries of tests for the
865 identification of novel obesogens.

866
867 As the field of obesity and adiposity research develops, more research will likely utilize some of the
868 alternative models described above. While historically less utilized than rodents, these models have some
869 advantages that are likely to see increased use in the coming years. Among these are the relatively lower
870 cost and rapid development of assays and models that may allow for superior chemical mixture
871 assessments than using rodent models. *In vitro* models have also continued to expand, with an anticipated
872 shift to greater use of normal human cell models, three-dimensional culture techniques, and co-cultures
873 techniques that may recreate the physiology present in the tissue microenvironment more accurately.
874 Recent advances in high content analysis provide promising grounds for increased throughput of
875 adipogenesis models, which would enable the screening of larger number of chemicals and their mixtures
876 with increased sensitivity and the possibility to differentiate the changes in adipocyte number as well as
877 size [42, 75]. Predictive models are still early in development but have shown some promise in predicting
878 likely active adipogenic and/or obesogenic chemicals. Predictive models based on key concepts for
879 obesogens (such as those recently described for EDCs and hepatotoxicants [268, 269]) are likely to
880 support determinations of obesogens and their causal mechanisms of action. They should be prioritized on
881 an international level, such as the OECD.

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883

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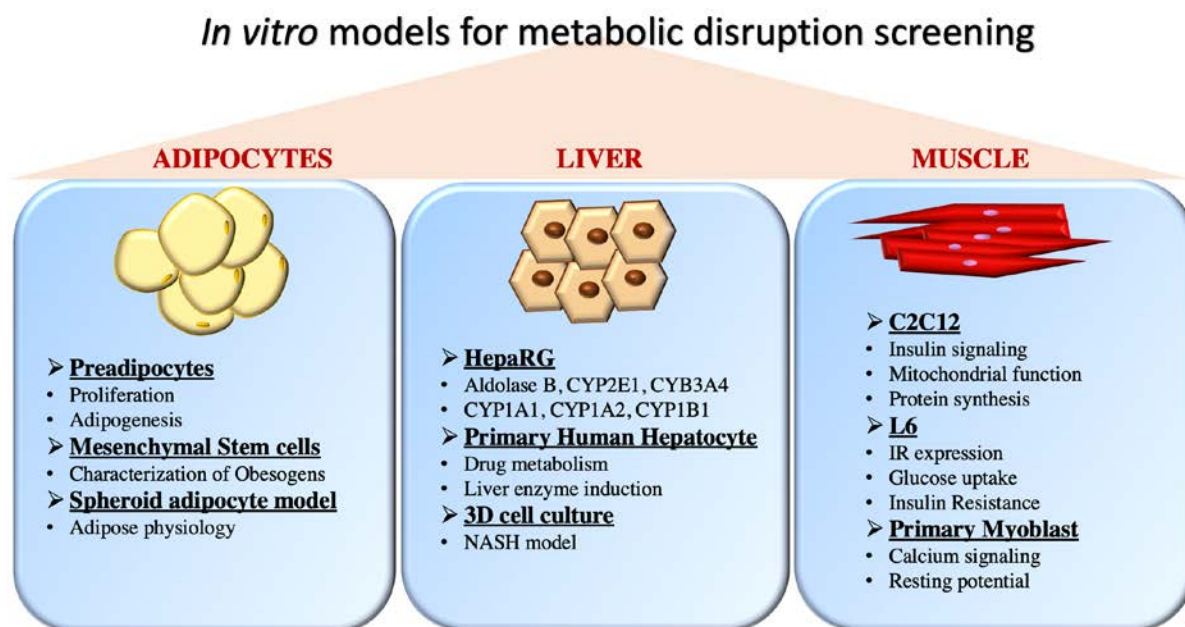
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1745 **Figure 1:** *In vitro* models used for testing the effect of metabolic disrupting chemicals on various

1746 pathways. Common uses of the various cell models are described.

In vivo models for metabolic disruption screening

Models	Advantages	Disadvantages
Zebrafish 	<ul style="list-style-type: none"> • Rapid development, ease of breeding, transparency • Metabolic organs/tissues are physiologically and anatomically similar to humans • High-resolution fluorescent imaging of total body adipose • Ease of molecular manipulation, wealth of transgenic models 	<ul style="list-style-type: none"> • Moderate flexibility • Moderate translational value
Medaka 	<ul style="list-style-type: none"> • Genetic sex determination like humans • Rapid development, ease of breeding, transparency • Metabolic organs/tissues are physiologically and anatomically similar to humans • Ease of molecular manipulation, small genome size, high diversity 	<ul style="list-style-type: none"> • Moderate flexibility • Moderate translational value • Less characterization of adipose relative to zebrafish
<i>C. elegans</i> 	<ul style="list-style-type: none"> • Compounds that modulate fat storage and obesity can be identified • Food intake and energy expenditure can be measured easily • Less regulations governing invertebrate animal use 	<ul style="list-style-type: none"> • Lower conservation of biological pathways with mammals • Lack of specific organs and circulatory systems
<i>D. melanogaster</i> 	<ul style="list-style-type: none"> • Small size, short generation time, inexpensive and easy breeding • Several discrete organs perform the same as humans • Less regulations governing invertebrate animal use 	<ul style="list-style-type: none"> • Anatomically different from mammals • Lower conservation of many relevant biological pathways with mammals
Rodents 	<ul style="list-style-type: none"> • Well described model with clear translation to human outcomes • Periconception, pregnancy, parental and offspring, short- and long-term, multi- and trans-generational outcomes can be assessed • Diverse housing materials readily available • Well-characterized & customizable feed options readily available • Inbred and outbred models available to dissect role of genes, environment, and their interactions 	<ul style="list-style-type: none"> • Time consuming and expensive compared to above alternatives, but less so with larger animal models (e.g. porcine, bovine, ovine, and non-human primates). • Ethical issues; regulatory push to reduce use of mammalian vertebrate animal models

1747

1748 **Figure 2:** Advantages and disadvantages of *in vivo* models for metabolic disrupting chemical evaluation.
 1749 Common or emerging model organisms used in metabolic health research are discussed and various
 1750 characteristics are described.

1751

1752 **Table 1: Obesogenic chemical testing in emerging *in vivo* models (zebrafish, medaka, roundworm,**
 1753 **fruit fly)**

1754

Species	Mode of action	Representative References
Danio rerio	Obesity phenotype Increased weight, adiposity, and/or lipid accumulation	Cadmium: [177], [270] DDT mixture: [271] Nonylphenol and polyethoxylates: [79] Bisphenols: [272], [27], [165] Phthalates: [176], [273], [174] PFOS: [169]
	NAFLD phenotype Steatosis, fatty liver changes	Cadmium: [270] Benzo(a)pyrene: [274], [275] Bisphenols: [276], [277], [278], [279], [280] Phthalates: [281], [282]
	Metabolism changes Metabolomics, lipids, fatty acids, diabetic phenotype, etc.	Bisphenols: [283], [278], [165] Phthalates: [176], [284], [174] PFOS: [169]
Oryzias latipes	Obesity phenotype Increased weight, adiposity, and/or lipid accumulation	TBT: [182] TBT/PFOS: [181]
	NAFLD phenotype Steatosis, fatty liver changes	
	Metabolism changes Metabolomics, lipids, fatty acids, diabetic phenotype, etc.	TBT: [182] Bisphenols: [186]
C. elegans	Obesity phenotype Increased weight, adiposity, and/or lipid accumulation	Bisphenols: [199], [285] Erythromycin: [198] PFOA: [286]
	NAFLD phenotype Steatosis, fatty liver	
	Metabolism changes Metabolomics, lipids, fatty acids, diabetic phenotype, etc.	Bisphenols: [199] Erythromycin: [198] Methylmercury: [197] PFOA: [286]
Drosophila melanogaster	Obesity phenotype Increased weight, adiposity, and/or lipid accumulation	DEHP: [287]
	NAFLD phenotype Steatosis, fatty liver changes	
	Metabolism changes Metabolomics, lipids, fatty acids, diabetic phenotype, etc.	PFOA: [288] PFOS: [289]

1755

1756 Summary table of obesogenic activity testing in the zebrafish, medaka, roundworm, and fruit fly models.

1757 Representative obesogenic chemical testing (non-exhaustive) is included to detail the diversity of

1758 contaminants examined.

1759