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Short title: Overall perspective on obesogen assays

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Abstract

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

Abbreviations

United States (US); white adipose tissue (WAT); peroxisome proliferator-activated receptor gamma (PPARγ); sterol-regulatory element-binding protein-1 (SREBP-1); liver X receptor alpha (LXRα); glucocorticoid receptor (GR); retinoid X receptor-alpha/beta (RXRα/β); estrogen receptor alpha (ERα); human preadipocytes (HPAd); Simpson-Golabi-Behmel syndrome (SGBS); mesenchymal stem cells (MSCs); Human multipotent adipose-derived stem cells (hMADS); toxicant-associated fatty liver diseases (TAFLD); non-alcoholic fatty liver disease (NAFLD); dimethylsulfoxide (DMSO); primary human hepatocytes (PHH); cytochrome P450 (CYP); aryl hydrocarbon receptor (AhR); constitutive androstane receptor (CAR); pregnane X receptor (PXR); di(2-ethylhexyl) phthalate (DEHP); dichlorodiphenyltrichloroethane (DDT); bisphenol A (BPA); polychlorinated biphenyls (PCBs); tetrabromobisphenol A (TBBPA); days post-fertilization (dpf); endocrine disrupting chemicals (EDCs); insulin-producing cells (IPCs); brown adipose tissue (BAT); uncoupling protein 1 (UCP1); OLTAM (*O*DD-*L*uc based *T*hermogenic *A*ctivity *M*easurement); ODD (oxygen-dependent degradation); hypoxia-inducible factor 1 alpha (HIF1α); quantitative structure-activity relationship (QSAR); Toxicity Testing in the 21st Century (Tox21); Organization for Economic Cooperation and Development (OECD); New approach methodologies (NAMs); Adverse Outcome Pathways (AOPs); integrated approaches to testing and assessment (IATAs); National Institute for Environmental Health Sciences (NIEHS); PEPPER (Public-privatE Platform for the Pre-validation of Endocrine disRuptors.

Essential Points

There are increasing novel capabilities to identify and assess obesogens.

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

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

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

1. Introduction

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

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

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

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

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

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

2. In vitro assays

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

2.1 Preadipocyte models

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

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 impared 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]

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

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

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

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

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

2.2 Mesenchymal stem cells (MSCs)

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

Recent research elegantly described protocols for distinguishing assays to evaluate adipogenic lineage commitment and subsequent adipocyte differentiation [63]; briefly, cells can be pre-treated with test chemicals prior to the differentiation cocktail exposure. These pre-treated cells can be subsequently exposed to the differentiation cocktail and evaluated at the end of the differentiation window. The extent of triglyceride accumulation can be compared with standard adipogenesis plates; chemicals with effects on commitment should have equivalent effects to those differentiated for the full two weeks, whereas 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 PPARγ, 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

2.3 Spheroid adipocyte models

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

2.4 Liver cell assays

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

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

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

with regulation by cAMP [107]. These models have been utilized to evaluate fatty acid induced lipid droplet accumulation and the presence and causes of heterogeneity in the lipid droplet content [108],

The most prevalent human liver cell line is HepaRG. HepaRG cells can differentiate into hepatocyte-like and biliary-like phenotypes after dimethylsulfoxide (DMSO) (1.75 - 2%) exposure, and possess the ability to stably express several liver-specific genes such as albumin, aldolase B, CYP2E1 and CYP3A4 [109]. Changes in metabolites related to energy metabolism, oxidative stress, and insulin resistance have also been observed in differentiated HepaRG cells supplemented with an oleate/palmitate mixture [110]. These are consistent with alterations observed in the liver tissues of human patients and animal models of NAFLD [111, 112]. Altogether, these data further support the suitability of the fatty acid-supplemented HepaRG model to study the impact of obesogens on steatosis progression towards steatohepatitis in the context of the “two-hit” model [113]. In line with these data, an oleate/stearate mixture is sufficient to decrease the expression of CYP1A1, 1A2, 1B1 and decrease their activity after steatosis induction [114]. These results corroborate data obtained from NAFLD rodent models, especially regarding CYP1A1 and 1A2 [115-117].

In addition, several 3D liver culture models have also been developed to create a cell environment closer to *in vivo* conditions. In 3D cell cultures, cell growth and interaction with surrounding conditions exhibit higher differentiation and benefit from more extended culture than 2D cultures [118]. When cultured as 3D spheroids, HepaRG cells express genes involved in lipoprotein metabolism, energetic lipid synthesis, gluconeogenesis, glycolysis, and bile acid metabolism, liver-specific functions, and xenobiotic metabolism enzymes [119, 120].

Primary human hepatocytes (PHH) are increasingly used to predict drug metabolism and liver enzyme induction in humans. However, PHH have inherent limitations: scarce and unpredictable availability, limited growth activity and lifespan, and early and variable phenotypic alterations in 2D culture.

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

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

2.5 Muscle cell assays

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

3. *In vivo* assays

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

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

3.1 Danio rerio (Zebrafish)

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

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

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

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

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

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

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

3.2 Oryzias latipes (Medaka)

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

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

3.3 C. elegans (Roundworm)

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

aging. Although *C. elegans* is generally considered genetically and physiologically distant from humans, several studies have shown that the main regulatory pathways of energy homeostasis are shared between mammals and nematodes [144, 187, 188]. These advantages make *C. elegans* a suitable *in vivo* model to identify compounds that modulate fat storage and promote obesity [141, 189]. Both simple fluorescence (Nile red or Sudan-black probes) and biochemical (triglyceride assays) techniques can be used to quantify lipid amount and fat storage in these worms [188]. In addition, genetic approaches using mutant or transgenic animals can help evaluate molecular mechanisms underlying metabolic health effects [187, 188]. Moreover, *C. elegans* can be readily used to measure food intake and energy expenditure [188, 190]; several diets, food-derived or nutraceutical compounds, and fat-increasing compounds have been described to modulate fat accumulation [189-191]. Limitations of this model include lower conservation of biological pathways with humans and a lack of particular organs and circulatory systems [192]. *C. elegans* also lack PPARγ, though they do express orthologs of both PPARα and δ, and have no identifiable homolog for leptin [193, 194]. Perhaps unsurprisingly, they thus have no cells specifically designed for lipid storage (i.e. adipocytes), though they do still store lipids, primarily in intestinal and epidermal skin-like cells, which are comprised of diverse saturated, monounsatured, and polyunsatured fatty acids [193]. This model has also been used to assess transgenerational effects, with research demonstrating that starvation of the parental generaton promoted disrupted metabolism in the F3 generation, whereas BPA exposures resulted in transgenerational modulation of epigenetic germline silencing through up to five subsequent (non-exposed) generations (reviewed in [195]).

Despite these limitations, this model has been utilized widely in better understanding the genetics of fat accumulation, storage, and obesity [194, 196], and has been applied to obesogenic chemical evaluation successfully. Specifically, methylmercury exposure promotes triglyceride accumulation, lipid storage, and alter feeding behaviors [197], erythromycin promotes increased fat content and triacylglycerol levels as well as promoting overeating, presumably mediated through stimulation of serotonin, dopamine, and acetylcholine and/or disruption of lipogenesis and lipolysis [198]. Recent research demonstrated a non-

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

3.4 Drosophila melanogaster (Fruit fly)

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

3.5 Rodents

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

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

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

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

3.6 Use of inbred vs. outbred models

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

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

3.7 Animal feed as a source of variability

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

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

This observation by Thigpen demonstrated that there can be significant batch-to-batch variability of phytoestrogen levels in laboratory animal feed with presumably the same nutrient profile; a constant level of soy protein in different batches of a feed can have markedly different levels of phytoestrogens, which vary in soy based on many environmental factors [216]. It has been assumed for some time that the only issue of concern with soy-based feeds was variability in the soy phytoestrogens genistein and daidzein, but findings described below suggest other components of soy-based feeds (e.g., contaminated fish meal, source of lipid) may also lead to significant differences in phenotype in mice. Second, the study revealed that specific batches of feed could promote replication failure relative to most prior studies reporting that DES (a known human carcinogen) disrupted development in mice, just as it did in humans [218]. Developmental exposure to DES also promoted obesity during later adulthood in mice maintained on a soy-based (NIH31) open formula feed [219]. This demonstrates that a core issue should be whether the feed used is resulting in an inability to see effects in response to treatments that others are reporting. Not surprising is that industry-funded research on BPA, which claimed to be a replication of findings from multiple laboratories [220], in fact, had used 5002 feed [221, 222]. This led to a failure to demonstrate a BPA-induced effect in both CF-1 mice and Crl:CD Sprague-Dawley (CD-SD) rats. This research also failed to demonstrate effects of DES with this food (included as positive control) [221], suggesting an inappropriate model to detect BPA-induced effects [223].

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

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

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

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

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

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

3.8 The role of positive controls in animal model selection

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

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

environmental chemical being examined.

3.9 Animal housing

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

3.10 Assays for detecting thermogenic brown fat activity

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

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

The OLTAM (*O*DD-*L*uc based *T*hermogenic *A*ctivity *M*easurement) system was developed to assay the activity of UCP1 independent thermogenesis in beige and BAT. In this *in vivo* model, a transgenic mouse that expressed the ODD (oxygen-dependent degradation) domain of hypoxia-inducible factor 1 alpha (HIF1α), tagged with luciferase, was used to measure hypoxia. Hypoxia has been shown to take place during nonshivering thermogenesis in beige and brown fat and is an indicator of thermogenesis [244]. An *in vitro* system was developed using the stromal vascular fraction of isolated brown adipocytes from these mice to measure cell-based thermogenic activity [244]. These cells could be used to evaluate the action of chemicals on the function of thermogenic beige and brown adipocytes.

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

preference of xenon gas [246], which has been further enhanced through later research [245]. ERthermAC, a small molecule fluorescent dye that responds to changes in intracellular heat, is another tool that has been found to assay chemically stimulated thermogenesis in both rodent and human brown adipocytes [247], and has provided evidence comparable to existing indirect methods of measurement.

Lastly, UCP1-expressing brown adipose cells isolated from supraclavicular depots in humans have revealed that the molecular makeup of these cells more closely resembled mouse beige adipocytes than brown adipocytes [248]. In addition, humans who initially possessed no BAT, were found to create new BAT within the supraclavicular region. This suggests that human BAT is derived from the browning of beige fat. One could develop assays based on these cells to identify chemicals that promote or inhibit the production of these thermogenic adipocytes.

4. *In silico* tests

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

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

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

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

5. The Future of Screening for Obesogens

A single approach or assay will not yield all the information needed to identify and classify obesogens. Data from epidemiological studies should be integrated with experimental data from animal models to support the evidence for the obesogenic potential of an identified chemical. It is advisable to adopt a tiered approach to identify and characterize EDCs, which can ultimately inform their classification as 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. I*n 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)

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

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

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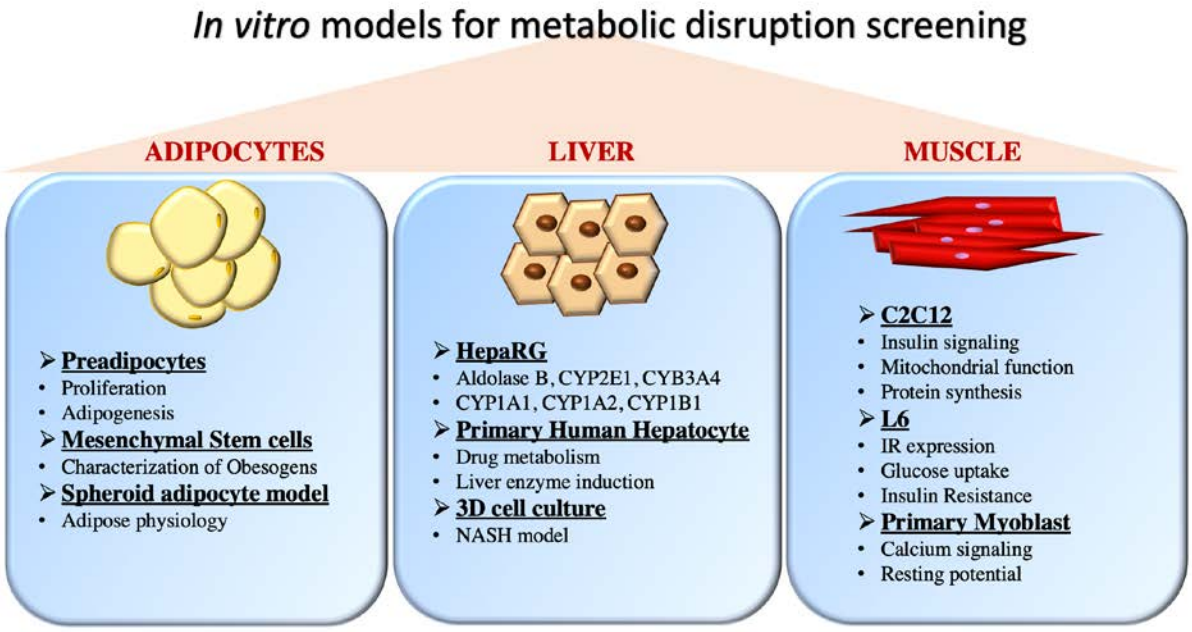
1743 **Figure Legends**



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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 	• 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	• Moderate flexibility • Moderate translational value
Medaka 	• 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	• Moderate flexibility • Moderate translational value • Less characterization of adipose relative to zebrafish
C. elegans 	• 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	• Lower conservation of biological pathways with mammals • Lack of specific organs and circulatory systems
D. melanogastor 	• Small size, short generation time, inexpensive and easy breeding • Several discrete organs perform the same as humans • Less regulations governing invertebrate animal use	• Anatomically different from mammals • Lower conservation of many relevant biological pathways with mammals
Rodents 	• 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	• 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

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

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

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]

Summary table of obesogenic activity testing in the zebrafish, medaka, roundworm, and fruit fly models. Representative obesogenic chemical testing (non-exhaustive) is included to detail the diversity of contaminants examined.