

**BPAF-INDUCED ADIPOGENESIS:  
ROLE OF PERIPHERAL 5-HT SIGNALING**

BPAF-INDUCED ADIPOGENESIS IN 3T3-L1 CELLS: ROLE OF PERIPHERAL SEROTONIN  
SIGNALING

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TITLE: BPAF-Induced Adipogenesis in 3T3-L1 Cells: Role of Peripheral Serotonin Signaling

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## **ABSTRACT**

There is evidence that synthetic chemicals in our environment called, *obesogens*, may play an important role in the global obesity epidemic. Bisphenol A (BPA) is one of the most studied obesogens. Due to concerns surrounding the use of BPA, BPA structural analogues are being used as substitutes in consumer products. However, there is currently little information available regarding their effects on pathways important for the development of obesity. Interestingly, serotonin (5-HT) signaling in peripheral tissues plays an important role in regulating metabolic homeostasis. Moreover, BPA has been shown to alter 5-HT signaling in the central nervous system. Therefore, the objective of this study was to determine the effects of BPA analogues on adipogenesis and elucidate whether their obesogenic effects involve peripheral 5-HT signaling.

3T3-L1 preadipocytes were treated with 10  $\mu$ M BPA or four commonly used BPA analogues during differentiation. Lipid accumulation was assessed by Oil Red O staining. Adipogenic markers and genes important for 5-HT synthesis/metabolism were subsequently examined following treatment with BPA and the structural analogue bisphenol AF (BPAF). The roles of tryptophan hydroxylase 1 (TPH1) and monoamine oxidase (MAO), in addition to glucocorticoid receptor (GR) signaling were also evaluated.

Treatment with 10  $\mu$ M BPAF and Bisphenol S (BPS) significantly increased lipid accumulation in differentiated 3T3-L1 cells. 10  $\mu$ M BPAF significantly increased adipogenic gene markers, as well as GR gene transcripts. Moreover, BPAF induced

adipogenesis in the absence of the synthetic glucocorticoid dexamethasone. Further, although BPAF significantly increased *Tph1* mRNA levels, blocking its activity with para-chlorophenylalanine (pCPA), did not block the adipogenic effects of BPAF. However, co-treatment with the MAO inhibitor, phenelzine, significantly decreased lipid accumulation and peroxisome proliferator activated receptor gamma (*Pparg*) expression.

Therefore, these data demonstrate that BPAF may act as an obesogen and suggests that its action might be mediated, in part, by altered 5-HT signaling.

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## **LIST OF ABBREVIATIONS**

5-HT – serotonin  
AADC – aromatic amine decarboxylase  
ADIPOQ – adiponectin  
ANOVA – Analysis of Variance  
ATCC – American Type Culture Condition  
BMI – body mass index  
BPA – Bisphenol A  
BPA-G – Bisphenol A glucuronide  
BPAF – Bisphenol F  
BPB – Bisphenol B  
BPC – Bisphenol C  
BPF – Bisphenol F  
BPS – Bisphenol S  
BPZ – Bisphenol Z  
C/EBP; Cebp – CCAAT-enhancer binding protein  
CCL20 – chemokine (C-C motif) ligand 20  
cDNA – complimentary DNA  
Cfd – complement factor D; adipsin  
CHAMACOS – Center for the Health Assessment of Mothers and Children of Salinas  
CNS – central nervous system  
Ct – comparative cycle times  
DDC – dopa decarboxylase  
ddH<sub>2</sub>O – double distilled water  
Dex – dexamethasone  
DHC – dehydrocorticosterone  
DMEM – Dulbecco's Modification of Eagle's Medium  
DMSO – dimethyl sulfoxide  
DOHaD - Developmental Origins of Health and Disease  
EDC – endocrine disrupting chemical  
EFSA – European Food Safety Authority  
EPA – Environmental Protection Agency  
FABP4 – fatty acid binding protein 4  
FAS – fatty acid synthase  
FBS – fetal bovine serum  
FDA – Food and Drug Administration  
FMI – fat mass index  
GD – gestational day  
GR; Nr3c1 – glucocorticoid receptor; nuclear receptor subfamily 3 group c member 1  
HIAA – 5-hydroxyindoleacetic acid

HSL – hormone sensitive lipase  
Htr – serotonin receptor  
IBMX – 3-isobutyl-1-methylxanthine  
IFN- $\gamma$  – interferon gamma  
IL-6 – interleukin 6  
iWAT - inguinal white adipose tissue  
LPL – lipoprotein lipase  
MAO – monoamine oxidase  
MCP-1 – monocyte chemoattractant protein 1  
MIA – 5-methoxy-indolacetate  
MSC - mesenchymal stem cell  
NHANES – National Health and Nutrition Examination Survey  
NOAEL – no observed adverse effect level  
pCPA – para-chlorophenylalanine  
PEPCK; Pck1 – phosphoenolpyruvate kinase  
PGC-1 $\alpha$ ; Ppargc1a – peroxisome proliferator-activated receptor gamma coactivator 1 alpha  
Plin – perilipin  
PND – postnatal day  
PPAR $\gamma$ ; Pparg – peroxisome proliferator activated receptor gamma  
pWAT - parametrial white adipose tissue  
PZ – phenelzine  
ROSI – rosiglitazone  
RT-qPCR – Real-Time Quantitative Polymerase Chain Reaction  
S.E.M – standard error of the mean  
SERT – serotonin transporter  
SREBP1c – sterol regulatory element binding protein 1c  
SSAO/PrAO – primary amine oxidase  
TBBPA – tetrabromobisphenol A  
TCBPA – tetrachlorobisphenol A  
TNF- $\alpha$  – tumor necrosis factor alpha  
TPH – tryptophan hydroxylase  
tTDI – temporary tolerable daily intake  
WC – waist circumference  
WHO – World Health Organization

## CHAPTER 1: INTRODUCTION


### ***1.1 Adipogenesis and White Adipose Tissue Function (WAT)***

Adipogenesis is the conversion of fibroblast precursors to lipid-laden adipocytes<sup>1</sup>. New adipocytes arise from adipose stromal mesenchymal stem cells (MSCs), are committed to preadipocytes and undergo differentiation into mature adipocytes over the course of six distinct stages<sup>1,2</sup> (Figure 1). Adipose mass is expanded through adipocyte hypertrophy (increase in cell size) and hyperplasia (increase in cell number)<sup>3</sup>. The formation of adipocytes in humans begins during the 14<sup>th</sup> week of development<sup>4</sup>. It is postulated that the majority of new adipocytes are recruited early in childhood, and thus, adipocyte number remains relatively stable in adulthood, with 10% of cells renewed every year<sup>5</sup>.

Adipogenesis is a highly regulated process consisting of a cascade of transcription factors. As reviewed by Rosen et al. (2000) and Farmer (2006), CCAAT-enhancer binding proteins (C/EBP $\beta$ , C/EBP $\delta$ ) activate peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), the master regulator of adipogenesis<sup>6,7</sup>. This results in activation of C/EBP $\alpha$  resulting in a positive feedback loop between PPAR $\gamma$  and C/EBP $\alpha$ <sup>6-8</sup>. Another important regulator is sterol regulatory element binding protein 1c (SREBP1c) which has been shown to activate PPAR $\gamma$ <sup>6,9</sup> and induce expression of the SREBP1c targets of a mature adipocyte, fatty acid synthase (FAS) and lipoprotein lipase (LPL)<sup>9,10</sup>. Other markers highly

expressed in mature adipocytes include fatty acid binding protein 4 (FABP4) and the adipokines, adiponectin (ADIPOQ) and adipisin<sup>11</sup>.

**Table 1. Characteristics of the various stages of adipogenesis**



Stage of adipogenesis <sup>a</sup>	Characteristics
Mesenchymal precursor	Proliferation Ability to differentiate into multiple lineages
Committed preadipocyte	Proliferation Commitment to differentiation along adipocyte lineage Fibroblast-like morphology
Growth-arrested preadipocyte	Lack of proliferation due to contact inhibition
Mitotic clonal expansion	Re-entry into the cell cycle induced by hormonal stimulation Several rounds of cell divisions (i.e. mitotic clonal expansion) Induction of C/EBP $\beta$ and C/EBP $\delta$ expression and activity
Terminal differentiation	Cell-cycle arrest Induction of PPAR $\gamma$ and C/EBP $\alpha$ expression Transcriptional activation of adipocyte genes (lipid and carbohydrate metabolism genes, adipokines)
Mature adipocyte	High expression of adipocyte genes Transcriptionally active PPAR $\gamma$ , C/EBP $\alpha$ and C/EBP $\beta$ Signet-ring morphology: large lipid droplet occupies majority of cell volume

<sup>a</sup>These distinctions are based primarily on *in vitro* studies.

**Figure 1.** Summary of stages of adipogenesis from mesenchymal precursor to a mature adipocyte. Figure adapted from<sup>1</sup>.

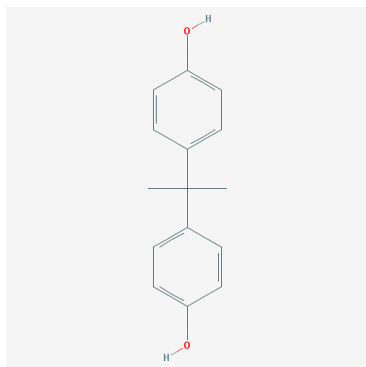
Adipocytes play a critical role in regulating global energy homeostasis<sup>12</sup>. When there is caloric excess, adipocytes expand and store energy as triglycerides protecting cells from ectopic lipid accumulation and lipotoxicity (Reviewed in: <sup>12</sup>). When there is a shortage of nutrients, free fatty acids and glycerol are subsequently released from the stored triglyceride pool<sup>12</sup>. Free fatty acids are incorporated into other tissues where they are re-esterified to triglycerides or undergo  $\beta$ -oxidation<sup>12</sup>. Free fatty acids may also remain within adipocytes to undergo  $\beta$ -oxidation<sup>12</sup>.

While it was once thought that adipose tissue was merely a storage depot, adipose tissue also acts as an endocrine organ capable of secreting factors termed adipokines<sup>13</sup>. These biologically active factors include leptin, ADIPOQ, resistin and inflammatory mediators such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1)<sup>13</sup>. There is considerable evidence demonstrating the effects of these adipokines on insulin sensitivity, lipid metabolism, energy homeostasis and inflammatory processes<sup>13</sup>. Therefore, perturbations in adipose tissue development and/or function are an essential component in the etiology of obesity.

### ***1.2 Endocrine Disrupting Chemicals and Bisphenol A***

It has been postulated that endocrine disrupting chemicals (EDCs) play a key role in mediating the obesity epidemic<sup>14</sup>. An endocrine disruptor has been defined by the World Health Organization (WHO) as: “an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse effects in intact organism, or its progeny or (sub) population”<sup>15</sup>. The “obesogen hypothesis” proposes that EDCs found in our environment and food play a key role in mediating adipose tissue development and function<sup>16</sup>. This can be through increasing the formation of new adipocytes, promoting lipid accumulation in existing fat cells or through alterations in food intake and satiety<sup>17</sup>. Exposure to potential endocrine disruptors is widespread. Phthalates, pesticides, herbicides, pharmaceuticals, flame

retardants, industrial waste and food additives have been extensively evaluated in order to determine their effects on adipogenesis and adipose tissue function (Reviewed in: <sup>14</sup>). One of the most widely studied EDCs, Bisphenol A (BPA), has also been identified as a potential obesogen<sup>18</sup>.



**Figure 2.** BPA chemical structure (<https://pubchem.ncbi.nlm.nih.gov>).

### **1.2.1 Human Exposure**

BPA is predominantly used in the manufacturing of polycarbonate plastics, and epoxy resins<sup>19</sup>. Additionally, BPA is found in thermal paper products such as cash register receipts<sup>20</sup>. Human exposure is ubiquitous; BPA has been detected in urine and serum samples from infant to adult populations around the world<sup>21–23</sup>. Biomonitoring studies have reported BPA concentrations in urine and serum in the ng/ml range (urine 1–5 ng/ml; serum 0.5–2 ng/ml)<sup>24</sup>. A comparison between two large scale studies in the U.S (National Health and Nutrition Examination Survey (NHANES) 2007–2008) and Canada (Canadian Health Measures Survey (CHMS) 2007–2009) revealed urinary levels of 2.1 ng/ml (95% CI 1.9, 2.3) and 1.2 ng/ml (95% CI 1.1, 1.2) respectively<sup>25</sup>. This corresponded



to an estimated intake of 36.9 ng/kg body weight/day (95% CI 34.1, 40.0) in the U.S and 21.4 ng/kg body weight/day (95% CI 20.0, 22.9) in Canada.

BPA exposure through dietary sources is regarded as the primary route of human exposure, although exposure can also occur via dermal, sublingual and inhalation<sup>19,21</sup>. Once ingested, BPA is rapidly metabolized in the liver and gut to BPA glucuronide (BPA-G) and sulfate metabolites<sup>26</sup>. Close to 100% of BPA taken orally is excreted in urine in its conjugated form within 24hrs<sup>26</sup>. In its conjugated form, BPA-G, has been thought to be biologically inactive, however, a recent study has demonstrated a possible active role for this metabolite<sup>27</sup>. The no observed adverse effect level (NOAEL) set by the Food and Drug Administration (FDA) for BPA is 5 mg/kg body weight/day and the European Food Safety Authority (EFSA) has set the temporary tolerable daily intake (tTDI) at 4 µg/kg body weight (EFSA 2015). Recently, Huang et al. (2017) used urinary levels of BPA from studies worldwide to estimate global daily intake of BPA<sup>29</sup>. They predicted the average global daily intake as 30.76 ng/kg body weight /day for adults, 60.08 ng/kg body weight/day for children and 42.03 ng/kg body weight/day for pregnant women. This corresponds to levels 2 and 1.4 times greater in children and pregnant women compared to exposures in non-pregnant adults, suggesting that these two groups may have increased susceptibility to the deleterious effects of BPA. Because these estimated intakes are below the tTDI and NOAEL, some controversy remains as to whether these thresholds should be lowered further.

### **1.2.2 Epidemiological Studies**

Although there remains some question about the importance of BPA exposure in overall obesity rates, cross-sectional human studies in children, adolescents and adults have shown primarily positive associations between higher urinary BPA levels and odds of obesity. Indeed, a recent meta-analysis of 12 studies reported that when comparing the highest versus the lowest urinary BPA concentrations, the pooled odds ratios (ORs) were 1.67 (95% CI 1.41, 1.98) for obesity and 1.48 (95% CI 1.25, 1.76) for increased waist circumference (WC)<sup>30</sup>. A cross sectional study of 3390 adults determined BPA was positively associated with obesity and insulin resistance in individuals in the highest quartile of urinary BPA exposure<sup>31</sup>. Similarly, a prospective study following 888 adults for 4 years found that participants with higher urinary BPA levels had an increased risk of central obesity<sup>32</sup>.

In child/adolescent populations, a recent study using data from NHANES 2003–2008 found a positive association between increasing BPA urinary concentrations and obesity after taking into account confounding factors, with an OR of 2.57 (95% CI 1.72, 3.83) for obesity in the highest urinary quartile<sup>33</sup>. Using data from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) longitudinal study, Harley et al. (2013) found higher urinary BPA levels during pregnancy were associated with decreased BMI-z score, percent body fat and obesity in females at age 9<sup>34</sup>. However, when urinary BPA levels were measured at 9 years of age in both males and females,

levels were positively associated with BMI, WC, body fat percentage and obesity<sup>34</sup>. Similarly, a recent study evaluating prenatal BPA exposure in 500 mother-child pairs from Crete, Greece evaluating BMI, WC and skinfold thickness found a negative and positive association in girls and boys respectively at age 4<sup>35</sup>. When urinary levels of BPA were measured at 4 years, a positive association was determined for all the above parameters<sup>35</sup>. Conversely, another study evaluating BPA exposure during pregnancy found no change in BMI but positive associations with percent body fat, WC and fat mass index (FMI) at age 7. When sex was taken into account, a positive association was only observed with FMI in girls<sup>36</sup>.

Taken together, based on current epidemiological studies higher levels of BPA in childhood and adolescence have most consistently been associated with an increased odds of obesity<sup>37</sup>. Importantly, this is a period of extensive adipose tissue growth and development<sup>5,38-40</sup>.

### **1.2.3 *In vitro* and *in vivo* studies**

In both *in vitro* and *in vivo* models, BPA has been shown to promote adipogenesis and alter genes essential to lipid metabolism<sup>41-44</sup>.

Appendix Table 1 provides a summary of studies evaluating the effects of BPA on lipid accumulation *in vitro*, demonstrating that BPA has adipogenic capacity at both low and

high doses. Moreover, in addition to enhanced lipid accumulation, BPA has also been shown to alter adipokine expression/secretion<sup>45–48</sup>, glucose uptake/insulin stimulated glucose utilization<sup>49–51</sup> and inflammatory markers<sup>50,51</sup> in adipocytes *in vitro*. Taken together, these studies demonstrate that BPA not only has effects on lipid accumulation, but also on key metabolic processes regulating adipose tissue function.

Studies evaluating the effects of *in vivo* BPA exposure in rodents have not shown alterations in body weight<sup>52,53</sup>; however, disruptions of other metabolic parameters, such as glucose homeostasis, insulin sensitivity, cholesterol biosynthesis as well as genes involved in *de novo* lipogenesis have been shown to be altered by BPA treatment. For example, in mice Batista et al. (2012) did not observe changes in body weight following exposure to 100 µg/kg BPA for 8 days<sup>53</sup>. However, the BPA-treated animals displayed dysregulated glucose/insulin homeostasis. Similarly, Marmugi et al. (2014) did not find any alterations in body weight when male CD1 mice were treated from 6 weeks of age for 8 months with BPA (5–5000 µg/kg/day)<sup>52</sup>. The authors did observe increased parametrial white adipose tissue (pWAT) weight, glucose intolerance, hyperglycaemia, hypercholesterolemia and increased cholesterol biosynthesis in the liver. Although exposure to BPA in adulthood has been associated with metabolic disruption, its effects on body weight may be more profound when the exposure occurs during critical windows of development (i.e., during pregnancy, early childhood, during puberty), when formation of new fat cells is rapidly occurring and adipocyte number is established<sup>5,38</sup>.

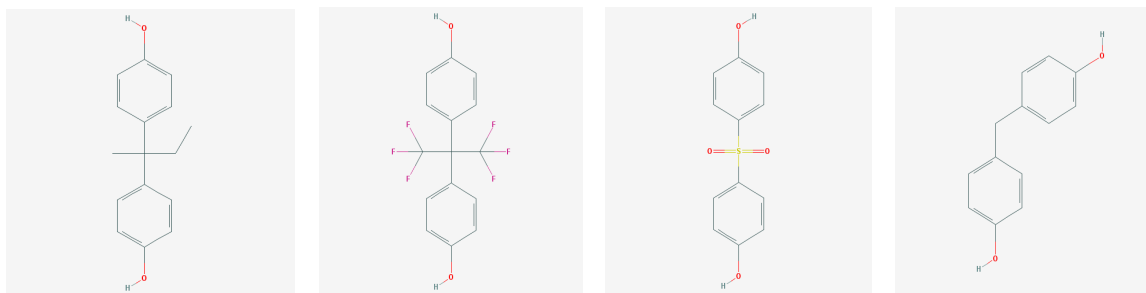
In support of the Developmental Origins of Health and Disease (DOHaD) hypothesis, that perturbations during critical periods of development may lead to adverse metabolic outcomes later in life (Reviewed in: <sup>54,55</sup>), BPA has been extensively evaluated for its effects on obesity risk when exposures occur during early life. Studies of BPA exposure during pregnancy and lactation have been shown to increase body weight and adiposity by adulthood with clear sex specific effects<sup>42,43,56–60</sup>. However, the literature is inconsistent with some studies reporting decreased body weight only in females<sup>56,61,62</sup>, while others have found no effect of BPA exposure *in utero* through lactation<sup>63–65</sup>. Further, a study evaluating BPA exposure during adolescence found increased body weight and adiposity in both males and females<sup>66</sup>.

Two recent studies, using environmentally relevant doses below the NOEL, determined dose and sex specific differences in BPA exposed offspring. Lejonklou et al. (2017) exposed rats *in utero* to 0.5 µg/kg body weight /day or 50 µg/kg body weight/day BPA<sup>63</sup>. While no differences in weight gain were observed, females had significantly elevated plasma triglyceride levels (50 µg/kg body weight/day) and significantly increased adipocyte density with exposure at the lower dose. Males, however, only displayed significant increases in plasma triglyceride levels at the lower dose<sup>63</sup>. Rubin et al. (2017) treated mice perinatally, or perinatally in addition to peripubertally, to determine whether a specific exposure window altered body weight and body composition in CD-1 mice<sup>60</sup>. They chose doses ranging from 0–250 µg BPA/kg body weight/day, with no dose

exceeding the NOEL of 5 mg/kg body weight/day. While perinatally exposed males had an increase in weight gain, when combined with peripubertal exposure, the differences in weight with control animals declined. However, females had increased body weight, elevated triglyceride levels, dysregulated glucose homeostasis and evidence of insulin resistance with the addition of peripubertal exposure. These studies highlight that many factors can lead to variations among findings including type of species, animal strain, sex, dose, mode of administration, diet and window of exposure<sup>60,63</sup>. Moreover, although data surrounding BPA exposure *in vivo* is not consistent, mounting evidence from human, animal and *in vitro* studies suggest BPA can affect pathways important for the development of obesity.

### ***1.3 Are Bisphenol A Analogues Safe Alternatives?***

With the rising concern of human exposure to BPA, the use of BPA structural analogues as replacements has become widespread<sup>67,68</sup>. This is alarming as very limited toxicological data on these analogues is available. Structural analogues of BPA include Bisphenol S (BPS), Bisphenol F (BPF), Bisphenol B (BPB) and Bisphenol AF (BPAF) among others<sup>67</sup>.



**Figure 3.** Chemical structures of BPA structural analogues from left to right: BPB, BPAF, BPS, BPF (<https://pubchem.ncbi.nlm.nih.gov>).

While these BPA-replacement compounds should ideally exhibit less harmful effects than BPA, a growing number of studies have demonstrated that these structural analogues also possess deleterious effects, comparable to or greater than BPA itself<sup>68–72</sup>. Of the structural analogues, BPS has been the most extensively studied. BPS is often used as an alternative in “BPA-free” thermal paper<sup>73</sup> as well as in baby bottles<sup>74</sup>. BPS has also been found to leach from food cans<sup>75</sup>. Héliès-Toussaint et al. (2014) showed that at low doses (i.e., fM range) both BPA and BPS increased triglyceride levels in 3T3-L1 cells. Additionally, lipolysis was decreased significantly in both BPA and BPS treatments, with a greater reduction observed with BPS at lower concentrations<sup>76</sup>. Additionally, BPS was shown to act through a PPAR $\gamma$  mediated mechanism in 3T3-L1 cells, significantly increasing lipid accumulation (10–50  $\mu$ M)<sup>77</sup>. Of concern, BPS was shown to act as a stronger inducer of adipogenesis than BPA. Further, another study has also reported the obesogenic effects of BPS *in vivo*<sup>78</sup>. In an earlier study, 80  $\mu$ M of BPB,

BPE, BPF and BPS was able to increase triglyceride levels in 3T3-L1 cells, to levels similar to that seen with BPA<sup>41</sup>. Recently, the adipogenic effects of BPAF were evaluated in American Type Culture Condition (ATCC) 3T3-L1 cells at doses ranging from 1 nM to 10 µM. BPAF treatment resulted in significant lipid accumulation<sup>79</sup>, an effect which may be due to its potential interaction with the PPARγ ligand binding domain<sup>80</sup>.

With the use of BPA declining and the use of its structural analogues on the rise, it is not surprising that this parallels the increase in detection in the environment and in human exposure levels. BPAF and BPF have been detected in high levels in surface water in Taihu Lake, China<sup>81</sup>. Recently, BPAF, Bisphenol C (BPC) and Bisphenol Z (BPZ) were detected in the highest quantities in aquatic organisms, displaying higher bioaccumulative ability than BPA<sup>82</sup>. Another study measured urine samples of American adults during 2000-2014 at eight time points to detect concentrations of BPA and three analogues, BPS, BPF and BPAF<sup>83</sup>. BPA was detected most frequently but displayed the greatest decline, (99–74%) whereas BPS showed the greatest rise in detection (19–74%). BPF was the highest detected analogue (42–88%), however BPAF was only detected in <3% of samples. Appendix Table 2 shows urinary concentrations of BPA and BPA structural analogues across human studies.

Recently, data from NHANES 2013–2014 was used to see if BPA, BPF and BPS were positively associated with obesity in adults<sup>84</sup>. While BPA was shown to be positively



associated with both general and abdominal obesity, no associations were observed for BPS and BPF. However, for BPS, the authors highlighted that a significant association was found with BMI, but only in the third urinary quartile<sup>84</sup>. Moreover, the metabolic toxicity of these compounds is largely unknown despite the fact that they are structurally similar to BPA. Thus, they may target many of the same pathways that are important for energy homeostasis including serotonin signaling, which is disrupted by BPA in the brain.

#### ***1.4 Role of Peripheral Serotonin on Adipogenesis and White Adipose Tissue Function***

Serotonin (5-hydroxytryptamine, 5-HT), a monoamine formed from the amino acid tryptophan, has been shown to play an important role in both central and peripheral nervous system functions<sup>85,86</sup>. Tryptophan hydroxylase (TPH; *Tph*), the rate limiting enzyme in the synthesis of 5-HT, has two isoforms<sup>87</sup>. TPH1 is responsible for the production of 5-HT in peripheral tissues whereas TPH2 is predominantly found in the central nervous system (CNS)<sup>87</sup>. As 5-HT is unable to cross the blood brain barrier, it is important to note that two distinct functional pools of 5-HT exist<sup>87</sup>. Serotonin is produced in two enzymatic steps<sup>85</sup>. Firstly, L-tryptophan becomes 5-hydroxytryptophan through the activity of TPH1. Secondly, aromatic amine decarboxylase (AADC), also known as dopa decarboxylase (DDC; *Ddc*), acts to convert 5-hydroxytryptophan to 5-HT.

In the CNS, 5-HT acts as a neurotransmitter controlling physiological processes including feeding, behavior and energy expenditure<sup>88,89</sup>. In peripheral tissues, 5-HT has been

shown to regulate metabolic homeostasis in adipocytes, liver, pancreas, muscle and macrophages (Reviewed in: <sup>85</sup>). Most of the 5-HT found in the periphery arises from the enterochromaffin cells located in the gut<sup>85</sup>. Serotonin produced by the gut is either stored in platelets or circulates in the blood<sup>85</sup>. Serotonin signaling in the periphery is mediated through binding to one of fourteen receptors found on target tissues which have been separated into seven distinct classes (Htr1 to Htr7)<sup>85</sup>. Serotonin receptors are G-protein coupled receptors, with the exception of Htr3, the only ligand-gated ion channel<sup>85,90</sup>.

Serotonin has been shown to promote adipogenesis and affect adipose tissue function. In rodents on a high-fat diet, an increase in 5-HT levels been shown to be associated with obesity<sup>91,92</sup> whereas *Tph1* deficient mice have lower adiposity and gain less weight while on a high fat diet<sup>93,94</sup>. In differentiating primary rat adipocytes, *Tph1* and mRNA levels of 5-HT receptors, *Htr2a*, *Htr2b* and *Htr2c*, were significantly upregulated when compared to undifferentiated control cells<sup>95</sup>. Moreover, primary rat adipocytes were able to independently produce and secrete 5-HT<sup>95</sup>. Kinoshita et al. (2010) reported that TPH1 is essential for adipogenesis in 3T3-L1 cells<sup>96</sup> and 5-HT has been shown to be produced over the course of differentiation<sup>94</sup>. In these cells, the 5-HT receptor subtypes, Htr1a, Htr1b, Htr1d, Htr1f, Htr2a, Htr2c, Htr5a, Htr5b, Htr6, Htr7 have been identified<sup>96</sup>. Additionally, antagonists for both Htr2c and Htr2a decreased adipogenesis, suggesting a role for these receptors during the differentiation process<sup>96</sup>. Another group showed

inhibition of the Htr2a receptor resulted in significantly reduced expression of lipogenic genes<sup>94</sup>. Therefore, Htr2a receptor activation in WAT may promote lipogenesis<sup>94</sup>. Conversely, stimulation of Htr2b seems to suppress lipogenesis<sup>97</sup> while stimulating lipolysis through its effects on hormone sensitive lipase (HSL) in WAT<sup>98</sup>. Stimulation of Htr2a has also been shown to block adiponectin expression in adipocytes<sup>99</sup>, which could lead to elevated gluconeogenesis in the liver and impaired insulin sensitivity (Reviewed in: <sup>100</sup>). Taken together, these studies suggest a functional role for 5-HT signaling in regulating key processes of adipogenesis and lipid metabolism in WAT.

Following 5-HT uptake by 5-HT transporters (SERT), 5-HT is metabolized to 5-hydroxyindoleacetic acid (HIAA) and 5-methoxy-indolacetate (MIA) by monoamine oxidase (MAO; *Mao*)<sup>101</sup>. While adipocytes have both MAOA and MAOB, MAOA is expressed in higher amounts in human adipocytes<sup>102</sup> and has been shown to be present in 3T3-L1 cells during differentiation<sup>96</sup>. Moreover, the metabolites of 5-HT, HIAA and MIA, have been implicated in the adipogenic process. MIA is believed to act as a PPAR $\gamma$  agonist stimulating adipogenesis in 3T3-L1 cells<sup>101</sup>. Additionally, MIA and HIAA have been shown to directly bind to the activation function 2 pocket of PPAR $\gamma$ <sup>101</sup>. To further support this hypothesis, Grès et al. (2013) showed that oxidation of 5-HT in adipocytes increases lipid accumulation and stimulates the expression of downstream PPAR $\gamma$  genes<sup>103</sup>. Pharmacologically inhibiting MAO with an irreversible non-selective MAO inhibitor, phenelzine, significantly decreases triglyceride content in 3T3-L1 cells<sup>104</sup>. Therefore, the

role of 5-HT in promoting adipogenesis may be two-fold. Serotonin can promote adipogenesis by acting through 5-HT receptors such as Htr2a or Htr2c or in a receptor independent pathway through 5-HT catabolism.

### **1.5 BPA and Serotonin Signaling**

There is evidence linking BPA exposure and 5-HT synthesis and metabolism in the CNS. Previous reports in rodents have shown BPA significantly increases 5-HT levels<sup>105-107</sup>. A study evaluating perinatal exposure to BPA in mice reported increased mRNA levels of *Tph2* and *Htr1a* in the hippocampus, *Htr2a* in the midbrain and decreased *Htr2c* expression in the striatum<sup>108</sup>. Additionally, the effects of BPA, BPF and BPS were evaluated on the 5-HT system in the prefrontal cortex of female rats<sup>109</sup>. BPA increased 5-HT receptors *Htr3a* and *Htr7* while both BPF and BPS increased expression of *Htr4*<sup>109</sup>. *Tph1* was decreased in all three treatment groups<sup>109</sup>. Further support for the association between BPA and analogues and serotonergic signaling comes from the U.S Environmental Protection Agency's (EPA) ToxCast database (<http://www.epa.gov/ncct/toxcast/>). BPA, BPAF and BPB have all been predicted active against 5-HT receptors (Htr1a, Htr2a, Htr2c, Htr3a, Htr4, Htr5a, Htr6, Htr7). This coupled with the effects of BPA on 5-HT signaling in the brain demonstrates a plausible mechanism for the action of BPA and its structural analogues in peripheral tissues.

### **1.6 BPA and Glucocorticoid Signaling**

Glucocorticoid signaling plays an important role during adipogenesis<sup>8,110,111</sup> and thus any alterations in signaling may impact the adipogenic process. In adipogenesis, GR activation leads to the induction of C/EBP $\delta$ , a transcription factor critical during early adipogenesis<sup>112</sup>; both C/EBP $\delta$  and C/EBP $\beta$  have been shown to activate C/EBP $\alpha$  in 3T3-L1 cells<sup>113</sup>. GR knockdown in 3T3-L1 cells, significantly impairs lipid accumulation<sup>8</sup>. Additionally, upon hormonal induction, both GR and C/EBP $\beta$  co-localize resulting in histone H3 acetylation which in turn promotes expression of adipogenic markers such as PPAR $\gamma$ <sup>8</sup>. Therefore, as PPAR $\gamma$  and C/EBP $\alpha$  are key regulators in adipogenesis, it is not surprising that glucocorticoids are used to stimulate 3T3-L1 differentiation<sup>111</sup>.

A number of EDC's, including BPA, have been evaluated for their ability to modulate glucocorticoid signaling<sup>114,115</sup>. Through a luciferase reporter assay in 3T3-L1 cells, BPA was reported to significantly activate GR activity<sup>115</sup>. While BPA alone was not able to induce adipogenesis in the absence of the synthetic glucocorticoid, dexamethasone, under submaximal conditions with the addition of dehydrocorticosterone (DHC), significant increases in adipocyte differentiation were observed<sup>115</sup>. Additionally, others have investigated the potential link between BPA and GR signaling as a potential mechanism of BPA-induced adipogenesis<sup>44,116</sup>. Interestingly, in contrast to the above study<sup>115</sup>, BPA was shown to promote adipogenesis in the absence of dexamethasone (0.01–10 nM), however the authors found no transcriptional activation of the GR using luciferase gene assays<sup>116</sup>. In primary human preadipocytes, the GR antagonist, RU-486

did not block BPA-induced adipogenesis suggesting that the actions of BPA may not be mediated solely via GR signaling<sup>44</sup>. Nonetheless, recently the binding affinities of several bisphenol compounds with GR were determined using a competitive binding assay. Of the eleven compounds compared, BPA and BPS displayed intermediate binding affinity with GR, while BPAF had the least potent binding affinity<sup>117</sup>. Interestingly, a relationship between glucocorticoid and 5-HT signaling has been suggested in a previous study. Moreover, treatment with a glucocorticoid in rats has been linked to significantly increased mRNA and protein expression of *Tph1* and *Ddc*, in addition to significantly higher 5-HT levels in intra-abdominal adipose tissue<sup>118</sup>. Taken together, these reports suggest the potential role for BPA in modulating GR signaling during adipogenesis and highlights a possible mode of action for other bisphenol compounds.

## CHAPTER 2: HYPOTHESIS AND OBJECTIVES

### *2.1 Rationale and Hypothesis*

#### **Rationale:**

Concerns have been raised about the impact of human exposure to environmental chemicals. The “obesogen hypothesis” postulates that EDCs found in our environment and food play a key role in mediating adipose tissue development and function<sup>16</sup>. Although a number of EDCs have been shown to cause dysregulated lipid metabolism<sup>14</sup>, the mechanistic pathways have yet to be fully elucidated. BPA, one of the highest volume chemicals produced globally, is used for the production of polycarbonate plastic and epoxy resins<sup>19</sup>. Based on human, animal and cell culture studies, BPA has been identified as a potential obesogenic compound<sup>37,41,42</sup>. With the increasing health risks associated with BPA exposure, BPA analogues have commonly been used as substitutes in consumer products. More alarming, the associated risks of BPA analogues on metabolic diseases are poorly defined. There is now substantial evidence that 5-HT signaling in peripheral tissues plays an important role in regulating metabolic homeostasis, including effects on adipose tissue deposition and lipid homeostasis<sup>85</sup>. Interestingly, BPA has been shown to alter 5-HT signaling in the CNS<sup>106,108</sup>. Therefore, it is plausible that BPA and its structural analogues may disrupt peripheral serotonergic networks leading to the increased fat deposition and altered lipid homeostasis observed

in obesity.

**Hypothesis:** Structural analogues of BPA (BPAF, BPS, BPF and BPB) will potentiate adipogenesis and cause dysregulated lipid homeostasis. I postulate these metabolic perturbations will be due to a disruption in peripheral 5-HT signaling.

## ***2.2 Objectives***

The specific aims of my M.Sc. project are:

- 1) To examine the effects of BPA analogues on adipogenesis and lipid homeostasis in 3T3-L1 cells.
- 2) To determine whether glucocorticoid signaling plays a role in BPAF-induced adipogenesis.
- 3) To determine the role of 5-HT signaling in mediating the effects of BPA and its structural analogues on adipose tissue development and function.



## CHAPTER 3: MATERIALS AND METHODS

### ***3.1 Cell culture, murine adipocyte differentiation and treatments***

The 3T3-L1 cell line, fibroblastic cells which are programmed to the adipocyte lineage, has been widely used to investigate adipocyte differentiation *in vitro*<sup>111,119</sup>. A differentiation cocktail consisting of insulin, 3-isobutyl-1-methylxanthine (IBMX), fetal bovine serum (FBS) and glucocorticoids is frequently used to stimulate adipogenesis<sup>110,111,120</sup>. This model system has been essential in evaluating preadipocyte-adipocyte differentiation *in vitro*.

3T3-L1 preadipocytes (ATCC® CL-173™) were grown in Dulbecco's Modification of Eagle's Medium (DMEM) (Corning, 10-014-CV, Manassas, VA, USA) with 1 g/L glucose supplemented with 10% FBS (Gibco, 12483-020, Grand Island, NY, USA), 1% Penicillin Streptomycin (P/S) (Gibco, 15140-122) and 1% L-Glutamine (L-Glut) (Gibco, 25030-081) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were grown in 100mm x 20mm polystyrene tissue-culture treated plates (Corning, 353003, Corning, NY, USA) and passaged once 60-70% confluence was reached. For experiments, cells were plated in 6-well plates (Corning, 353046) or maintained in 100mm x 20mm plates to grow to confluence. At 1 day post-confluence, designated as day 0, cells were differentiated into adipocytes in DMEM (Corning, 10-013-CV) with 4.5 g/L glucose containing 10% FBS using an induction cocktail consisting of 2 µg/ml insulin (Sigma-Aldrich, I0516, St. Louis, MO,

USA), 0.5 mM of IBMX (Sigma-Aldrich, I5879), and 0.25  $\mu$ M dexamethasone (Dex) (Sigma-Aldrich, D4902), unless otherwise indicated (51). After the initial 72 hours, the differentiation media was replaced with maintenance media containing DMEM with 4.5 g/L and 2  $\mu$ g/ml insulin. Media was replaced every 48hrs until day 9 (Figure 4).

Cells were treated with BPA, BPS, BPB, BPF and BPAF (Toronto Research Chemicals Inc., Toronto, ON, Canada) at a final concentration of 10  $\mu$ M by adding indicated bisphenol compounds dissolved in dimethyl sulfoxide (DMSO) starting at day 0. For dose response experiments, cells were treated with BPA and BPAF at varying concentrations (0.01–10  $\mu$ M). Cells were treated with DMSO at or below 0.02% as a vehicle control. For antagonist experiments, cells were co-treated with 50  $\mu$ M para-chlorophenylalanine (4-Chloro-DL-phenylalanine; pCPA) (Sigma-Aldrich, C6506) or 100  $\mu$ M Phenezine sulfate salt (Sigma-Aldrich, P6777) dissolved in double distilled water (ddH<sub>2</sub>O) as indicated from day 0 to day 9. For Aim 1, 2  $\mu$ M rosiglitazone (ROSI) (Sigma-Aldrich, R2408) was also added to the induction cocktail for 72hrs.

### ***3.2 Lipid Staining and Quantification***

At day 9, cells were stained with Oil Red O (Sigma-Aldrich, 00625) solution. Briefly, cells were fixed with 10% formaldehyde for 60 min at room temperature. Cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Corning, 21-031-CV) followed with 60% isopropanol for 5 min at room temperature. Filtered Oil Red O solution (300 mg of

Oil Red O added to 100 ml 99% isopropanol) was added to each well (1ml/well) for 20 min. The wells were then washed with ddH<sub>2</sub>O until clear and immediately followed with 60% isopropanol. After 10 min, 100 µl of each extracted sample was transferred to a 96-well plate in triplicate. The plates were read at 510 nm in a plate reader (Synergy™ H4 Hybrid Microplate Reader, BioTek Instruments, Winooski, VT, USA) to measure absorbance.

### ***3.3 RNA Extraction and Quantification***

At day 9, cells from differentiated adipocytes were harvested using TRIzol® Reagent (Ambion, 15596018, Carlsban CA, USA) and stored at -80°C until further use. Cells were lysed with 20G needle and RNA was extracted by adding 0.2 mL chlorophorm/1 ml trizol. Following centrifugation, the upper aqueous phase was removed and 0.5 ml isopropanol/1 mL trizol was added. After centrifuging, the pellet was washed with 75% ethanol 2–3 times. The pellet was then air dried and re-suspended in 30 µL nuclease free water (Qiagen, 129115, Hilden, Germany). RNA concentration in ng/µl was determined by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific USA) and stored at -80°C until further use.

### ***3.4 Complimentary DNA (cDNA) Synthesis***

A total of 4 µg of cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814, Foster City, CA, USA) as per manufacturer's

instructions. 40  $\mu$ L of cDNA was made using the following conditions using the iCycler thermocycler (BioRad Laboratories, Hercules, CA, USA): 25°C for 10 min; 37°C for 120 min; 85°C for 5 min. cDNA was stored at -20°C until further use.

### ***3.5 Primer Design***

Primers were designed using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD, USA). If transcript variances were present, Clustal Omega (EMBL-EBI, Hinxton, Cambridgeshire, UK) was used to find overlapping variant regions. PCR product size was set to 50–150 base pairs. The following melting temperature conditions were followed: Minimum: 58°C; Optimal: 60°C; Maximum: 60°C; Max difference: 2°C. Each primer sequence was run through OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA, USA) to check for hairpin, self-dimer and hetero-dimerization. Sequences selected had a  $\Delta G$  (kcal/mol) > than -9 for all parameters. Primer sequences were synthesized by MOBIX Lab (Sanger Sequencing and Oligo Synthesis Facility, Hamilton, ON, Canada) and validated by examining melting point curves.

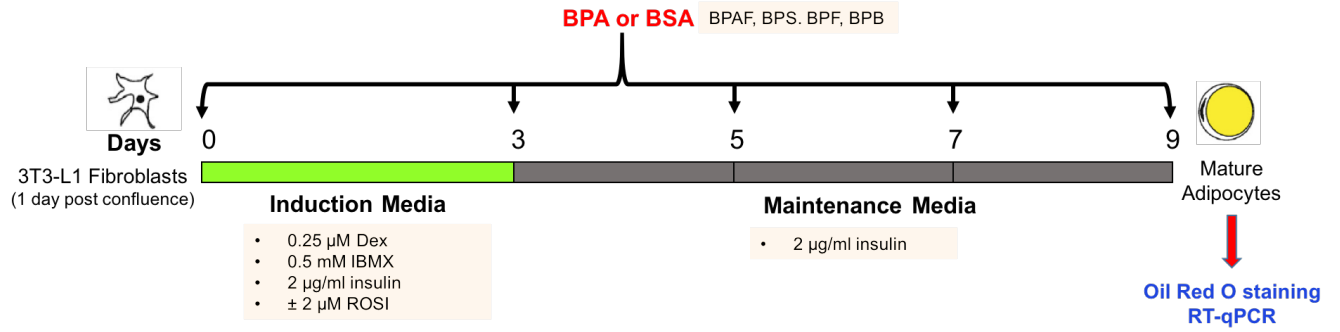
### ***3.6 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)***

RT-qPCR was performed using the Quantabio Perfecta® SYBR® Green FastMix® (95072-05K, Beverly, MA, USA) to determine relative fold change of each gene transcript. The plates (LightCycler 480 384-Multiwell Plates, 0472974900, Roche, Mannheim, Germany)

were analyzed by Light Cycler 480 II Machine (Roche) under the following conditions: polymerase activation (95°C for 10 min); 50 cycles of denaturing (95°C for 10 sec); annealing (60°C for 10 sec); elongation (72°C for 15 sec). Samples were ran in triplicate and the expression of each gene was normalized to the expression of  $\beta$ -actin. The relative fold changes were determined using the comparative cycle times (Ct) method, using the formula  $2^{\Delta\Delta CT}$ . Appendix Table 3 shows the list of primer sequences used for RT-qPCR.

### **3.7 Statistical Analysis**

Statistical Analysis was conducted by SigmaPlot v11 (Systat Software Inc., San Jose, CA, USA) and graphs were created using GraphPad Prism 7.0c, (GraphPad Software Inc., San Diego, CA). Images were taken using the Moticam X2 camera (National Optical & Scientific Instruments Inc, Schertz, TX, USA). Data were assessed for outliers using the Grubb's test (GraphPad QuickCalcs, GraphPad Software Inc.). Comparisons among two groups were analyzed using Student's T-tests. Comparisons among multiple groups were analyzed using One-Way Analysis of Variance (ANOVA) followed by the appropriate multiple comparisons test. When normality or equal variance failed, the Mann-Whitney Rank Sum Test or Kruskal-Wallis One-Way ANOVA on Ranks were used to determine significance. The level of significance was set at  $p=0.05$ . All data are presented as mean  $\pm$  standard error of the mean (S.E.M).



**Figure 4.** Model of adipogenesis *in vitro*.

## CHAPTER 4: RESULTS

### **4.1 BPAF and BPS significantly induce lipid accumulation in 3T3-L1 cells.**

To identify adipogenic BPA structural analogues, the effects of BPA, BPAF, BPS, BPB and BPF on lipid accumulation were evaluated by Oil Red O staining. Both BPAF and BPS significantly increased lipid accumulation in differentiated 3T3-L1 cells ( $P < 0.05$ ) (Figure 5).

### **4.2 BPAF significantly increases mRNA expression levels of adipogenic gene markers.**

mRNA levels of key markers of adipogenesis (PPAR $\gamma$ ; *Pparg*, C/EBP $\alpha$ ; *Cebpa*) and of mature adipocytes (FABP4; *Fabp4*, ADIPOQ; *Adipoq*) were evaluated by RT-qPCR. Treatment of cells with 10  $\mu$ M BPAF significantly increased mRNA levels of *Pparg*, *Cebpa*, *Fabp4* and *Adipoq* ( $P < 0.05$ ) (Figure 6). BPA at an equimolar dose (i.e., 10  $\mu$ M) did not have any effect on the expression of *Pparg*, *Cebpa* or *Adipoq*, but resulted in decreased ( $P < 0.05$ ) *Fabp4* mRNA levels (Figure 6).

### **4.3 Dose-dependent effects of BPAF-induced adipogenesis.**

Because the effects of BPAF on adipogenic gene markers had not previously been evaluated, I determined the effects of this compound over a range of concentrations (0.01–10  $\mu$ M) on the expression of key markers of adipogenesis (PPAR $\gamma$ , C/EBP $\alpha$ ) and of mature adipocytes (FABP4, ADIPOQ). BPAF significantly increased mRNA expression of

the key adipogenic marker *Pparg* at doses ranging from 0.1 to 10  $\mu\text{M}$  ( $P < 0.05$ ). However, markers of mature adipocytes (*Fabp4*, *Adipoq*) were only significantly increased at the 10  $\mu\text{M}$  dose ( $P < 0.05$ ) (Figure 7). No changes in gene expression with BPA were observed at any dose tested.

***4.4 24hr treatment with 10  $\mu\text{M}$  BPA or BPAF does not increase lipid accumulation in mature adipocytes.***

To determine whether BPA or BPAF increases lipid accumulation in mature adipocytes, fully differentiated 3T3-L1 cells at day 9 were treated with control (DMSO), 10  $\mu\text{M}$  BPA or 10  $\mu\text{M}$  BPAF for 24hrs. Lipid accumulation was not significantly different among treatment groups ( $P < 0.05$ ) (Figure 8).

***4.5 10  $\mu\text{M}$  BPAF significantly increases mRNA expression levels of *Nr3c1* (GR).***

Treatment with 10  $\mu\text{M}$  BPAF during differentiation significantly increased nuclear receptor subfamily 3 group c member 1 (*Nr3c1*; GR) gene transcripts in 3T3-L1 cells ( $P < 0.05$ ) (Figure 9a).

***4.6 *Nr3c1* (GR) and *Tph1* gene transcripts are positively correlated with 10  $\mu\text{M}$  BPAF treatment ( $R^2=0.829$ ,  $P < 0.0001$ ) (Figure 9b).***

***4.7 BPAF can induce adipogenesis in the absence of the synthetic glucocorticoid***



***dexamethasone.***

To determine whether glucocorticoid signaling plays a role in BPAF-induced adipogenesis, dexamethasone was omitted from the induction cocktail. Treatment with 10  $\mu$ M BPAF in the absence of dexamethasone from Day 0 to Day 3 significantly increased lipid accumulation compared to (-) Dex (DMSO control) treated group ( $P < 0.05$ ) (Figure 9d), but this did not reach the same level as cells which received dexamethasone in the induction media. Treatment with BPAF from Day 0 to Day 9 elicited an increase in lipid accumulation, however this was not significantly different from the (-) Dex (DMSO control) treated group (Figure 9e).

***4.8 BPAF increases 5-HT synthetic enzyme gene transcripts.***

To determine whether BPA and its structural analogues affect 5-HT synthesis, the expression levels of *Tph1* and *Ddc* were evaluated by RT-qPCR in differentiated 3T3-L1 cells. Steady-state mRNA expression of the 5-HT synthetic enzyme *Tph1* was significantly increased by 10  $\mu$ M BPAF ( $P < 0.05$ ) (Figure 6a). *Ddc* mRNA levels were also increased, however this did not reach significance ( $p = 0.103$ ; Figure 10b).

***4.9 TPH1 inhibition does not inhibit BPAF-induced adipogenesis.***

To determine whether BPAF-induced adipogenesis is mediated through increased 5-HT synthesis, cells were co-treated with pCPA, a TPH inhibitor, during differentiation. Co-treatment with 10  $\mu$ M BPA or BPAF  $\pm$  50  $\mu$ M pCPA did not impair differentiation in 3T3-

L1 cells; pCPA administration did not reduce BPAF-induced lipid accumulation (Figure 11b) or the BPAF-induced increase in mRNA expression of adipogenic gene markers (*Adipoq* and *Fabp4*) ( $P < 0.05$ ) (Figure 12). Interestingly, pCPA + 10  $\mu$ M BPA resulted in a significant increase in *Pparg* expression; an effect not seen with BPA alone (Figure 8e) and it did not block the BPA-induced increase of *Adipoq* (Figure 12h).

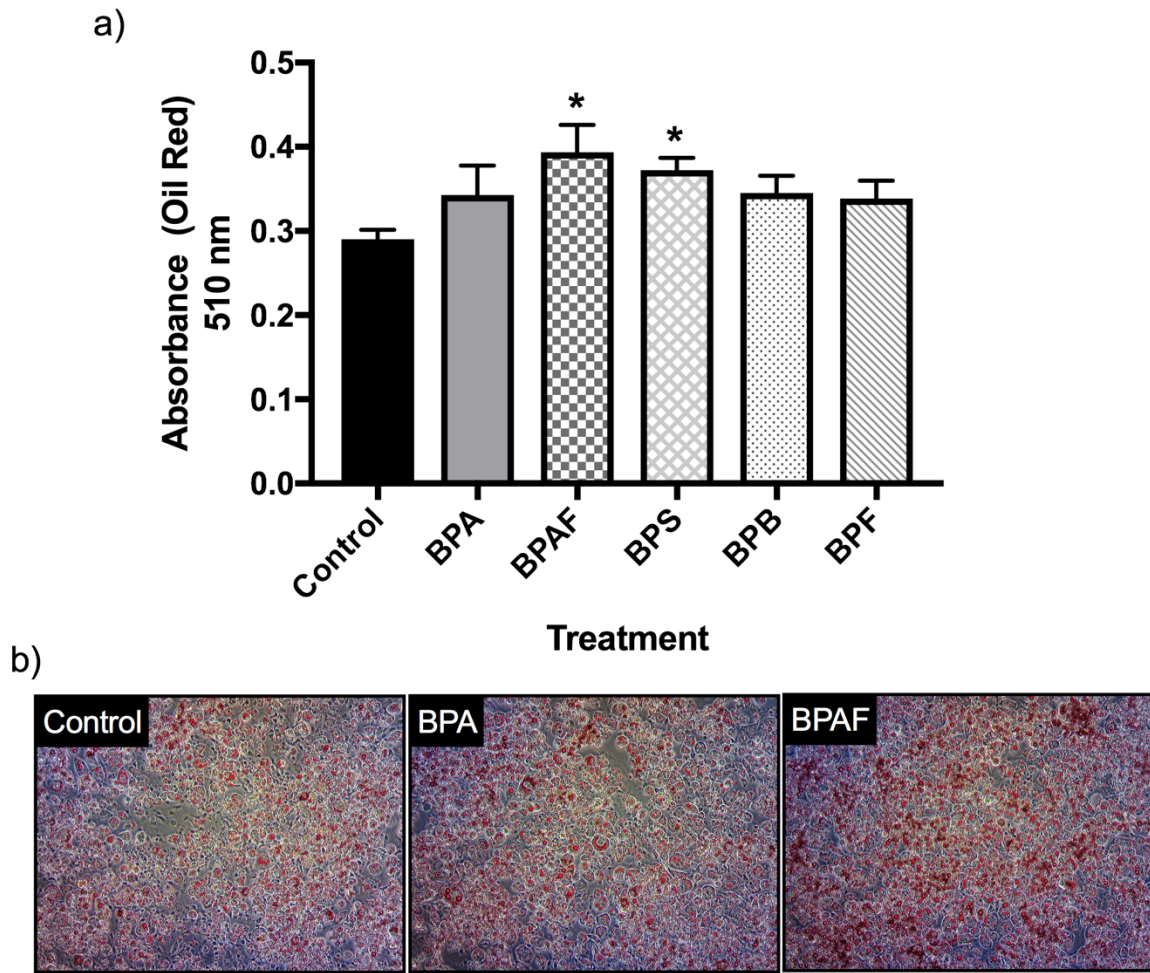
#### **4.10 BPAF significantly increases *Maoa* gene expression.**

To determine whether BPAF affects 5-HT catabolism, the expression levels of MAOA were evaluated by RT-qPCR in differentiated 3T3-L1 cells. mRNA expression of *Maoa* was significantly increased by 10  $\mu$ M BPAF ( $P < 0.05$ ) (Figure 13a).

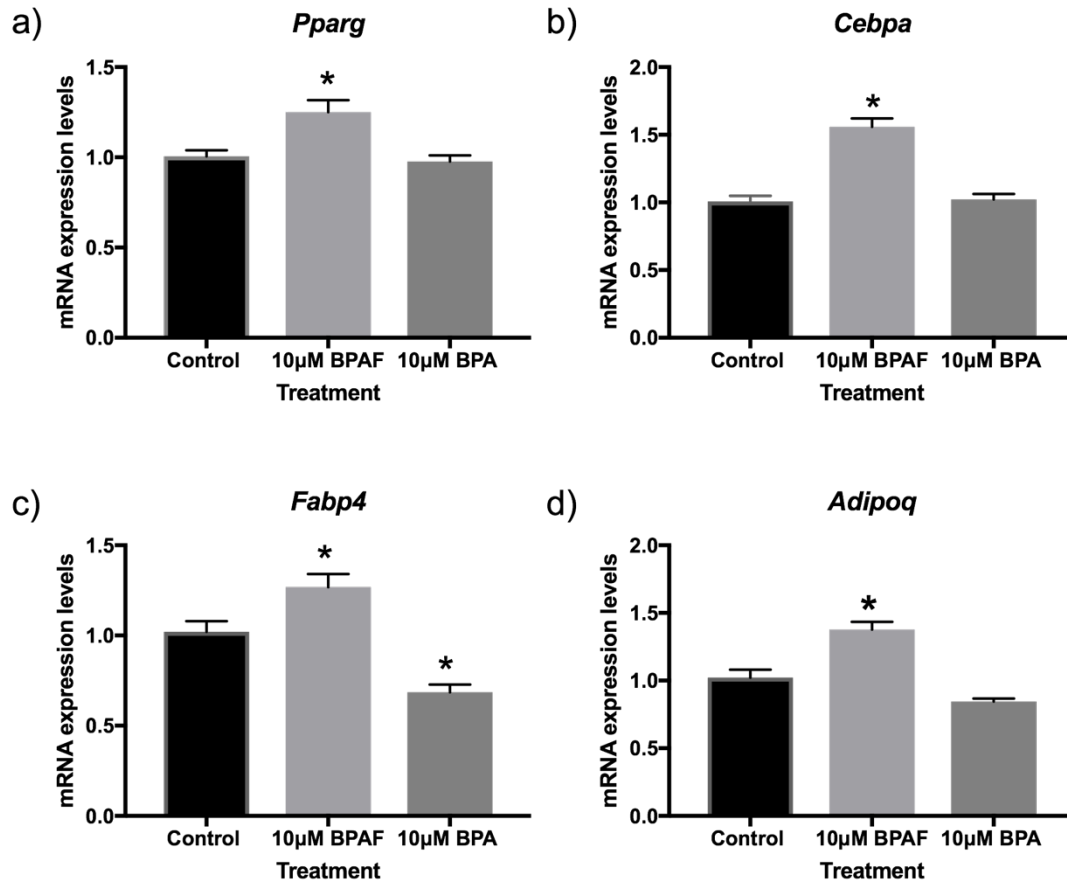
#### **4.11 *Maoa* and *Pparg* gene transcripts are positively correlated with 10 $\mu$ M BPAF treatment ( $R^2=0.5312$ , $P=0.0021$ ) (Figure 13b).**

#### **4.12 MAO inhibition blocks BPAF-induced adipogenesis.**

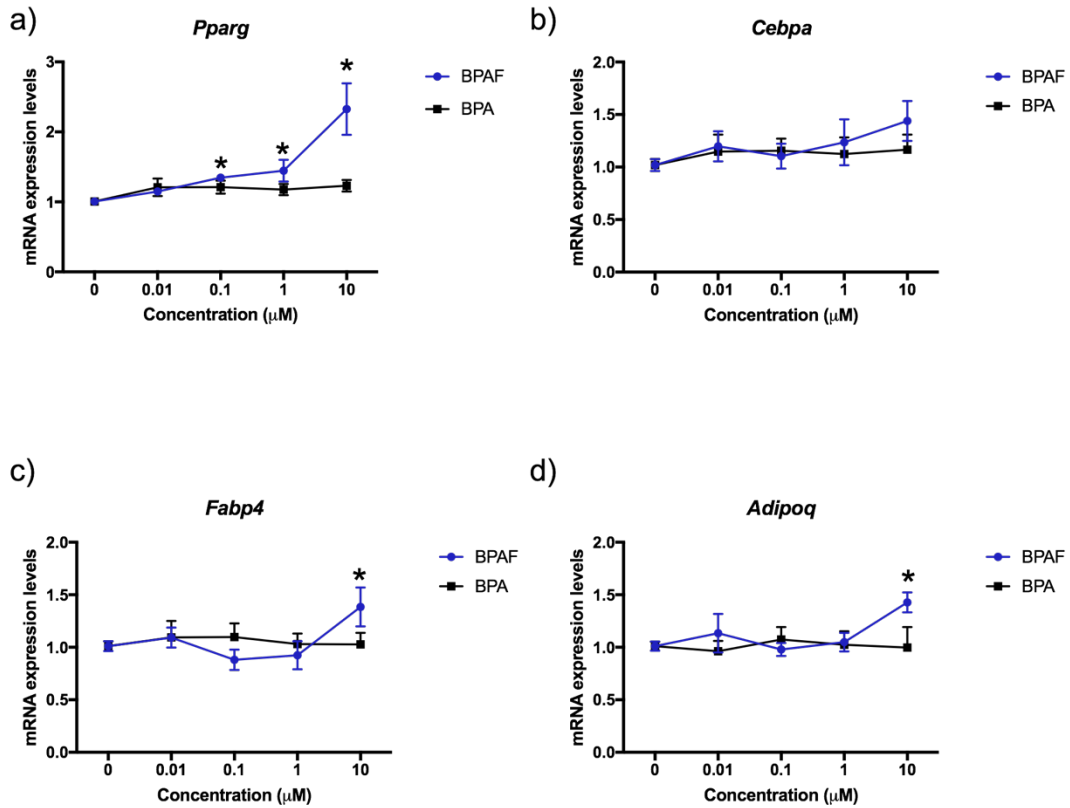
Co-treatment with 100  $\mu$ M phenelzine (PZ), a non-selective irreversible MAO inhibitor, in this study was able to block the adipogenic effects of BPAF, significantly decreasing lipid accumulation (Figure 14b). Moreover, mRNA expression levels of *Pparg* were significantly decreased in the BPAF + PZ treatment group (Figure 14d). Lipid accumulation was also significantly decreased in the 10  $\mu$ M BPA + PZ treatment group (Figure 14c).



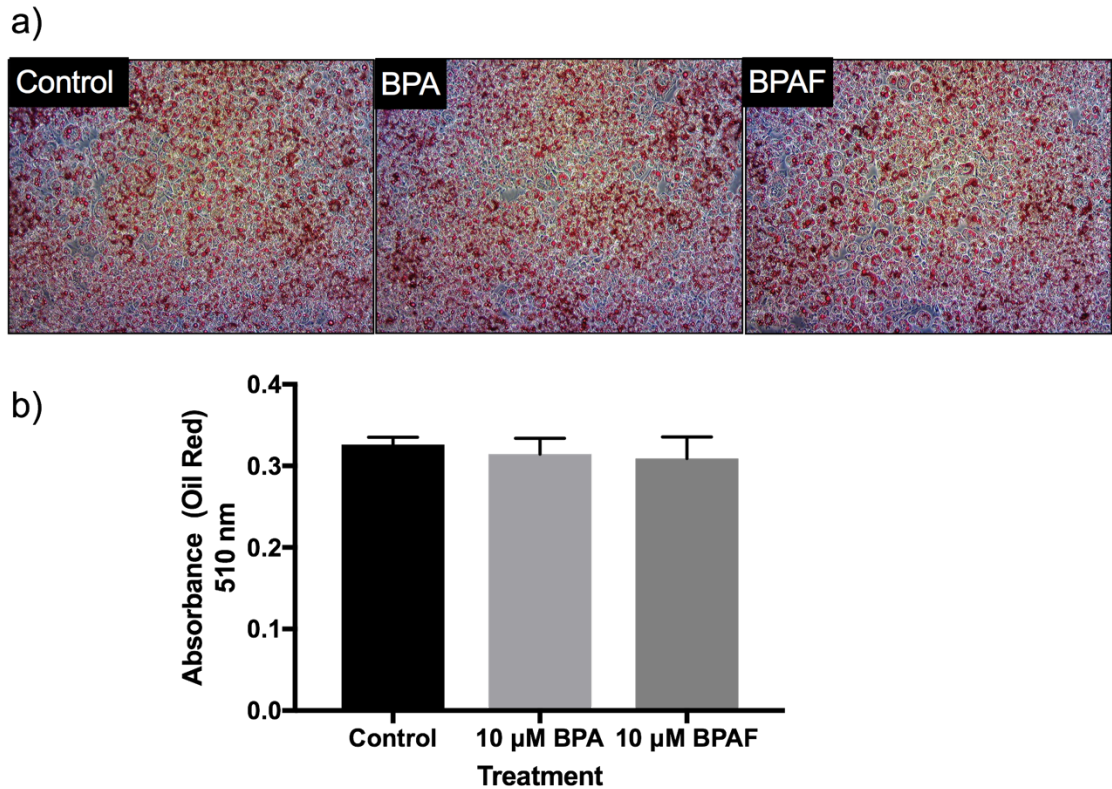
**Figure 5. BPA structural analogues induce lipid accumulation in 3T3-L1 cells.** a) Murine 3T3-L1 preadipocytes were treated with control (DMSO) or 10  $\mu$ M BPA, BPAF, BPS, BPB and BPF for 9 days. Lipid accumulation was determined using Oil Red O staining. b) Representative images of control, 10  $\mu$ M BPA and 10  $\mu$ M BPAF stained with Oil Red O (10x). Data represent mean  $\pm$  S.E.M. \* $P$ <0.05 relative to control (n=3–20) calculated by One-Way ANOVA followed by Holm-Sidak.



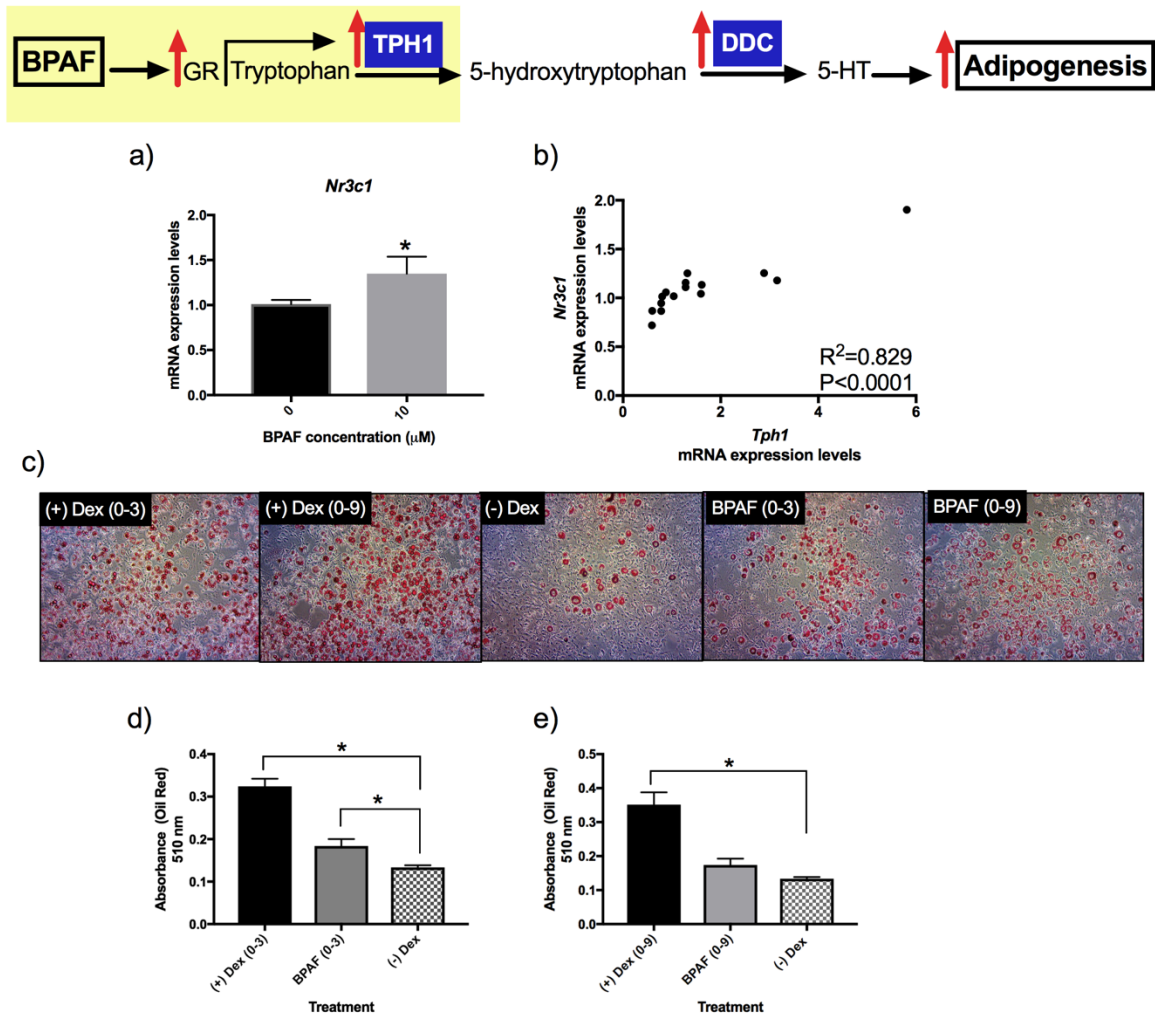
**Figure 6. mRNA expression levels of adipogenic gene markers in 3T3-L1 cells treated with 10 μM BPAF and BPA.** mRNA levels were determined by RT-qPCR in murine 3T3-L1 preadipocytes treated with control (DMSO) or 10 μM BPAF and BPA for 9 days. Data are normalized to β-actin. Data represent mean ± SEM. \*P<0.05 (n=4–15) relative to control calculated by One Way ANOVA followed by Holm-Sidak.



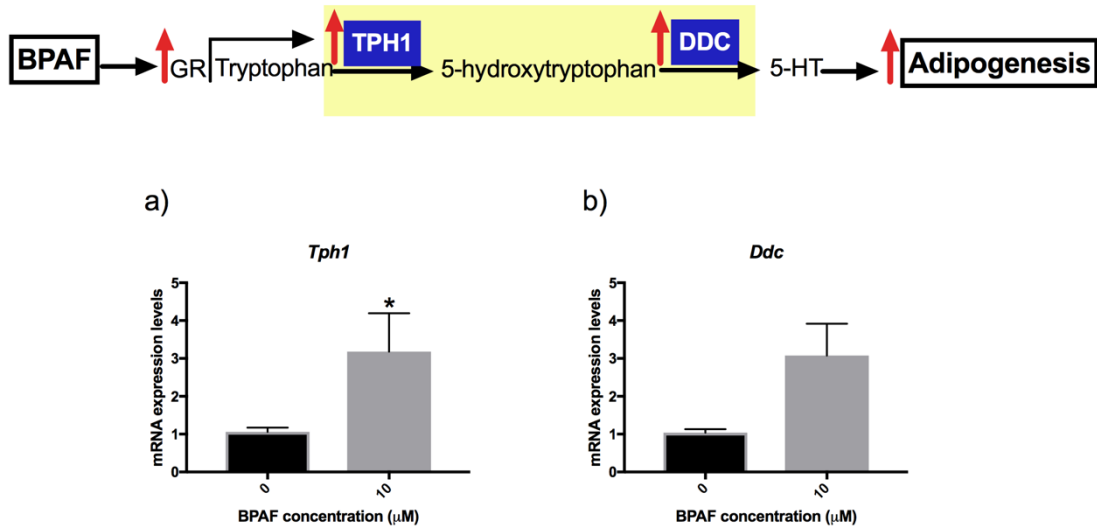
**Figure 7. Dose-dependent effects of BPAF and BPA on levels of adipogenic gene markers in 3T3-L1 cells by RT- qPCR.** Cells were treated with control (DMSO), BPAF (0.01–10  $\mu\text{M}$ ) and BPA (0.01–10  $\mu\text{M}$ ) for 9 days. Data are normalized to  $\beta$ -actin. Data represent mean  $\pm$  S.E.M. \* $P < 0.05$  ( $n = 3$ –12) relative to control calculated by One-Way ANOVA followed by Holm-Sidak or Kruskal-Wallis One-Way ANOVA on Ranks followed by Dunn’s Method.



**Figure 8. 24hr treatment with 10  $\mu$ M BPA or BPAF does not significantly increase lipid accumulation in mature adipocytes.** Differentiated 3T3-L1 cells at day 9 were treated with control (DMSO), 10  $\mu$ M BPA or 10  $\mu$ M BPAF for 24hrs. a) Representative images of control (DMSO), 10  $\mu$ M BPA and 10  $\mu$ M BPAF stained with Oil Red O (10x). b) Lipid accumulation was determined by Oil Red O staining. Data represent mean  $\pm$  SEM. \* $P < 0.05$  relative to control (n=3) calculated by One-Way ANOVA.

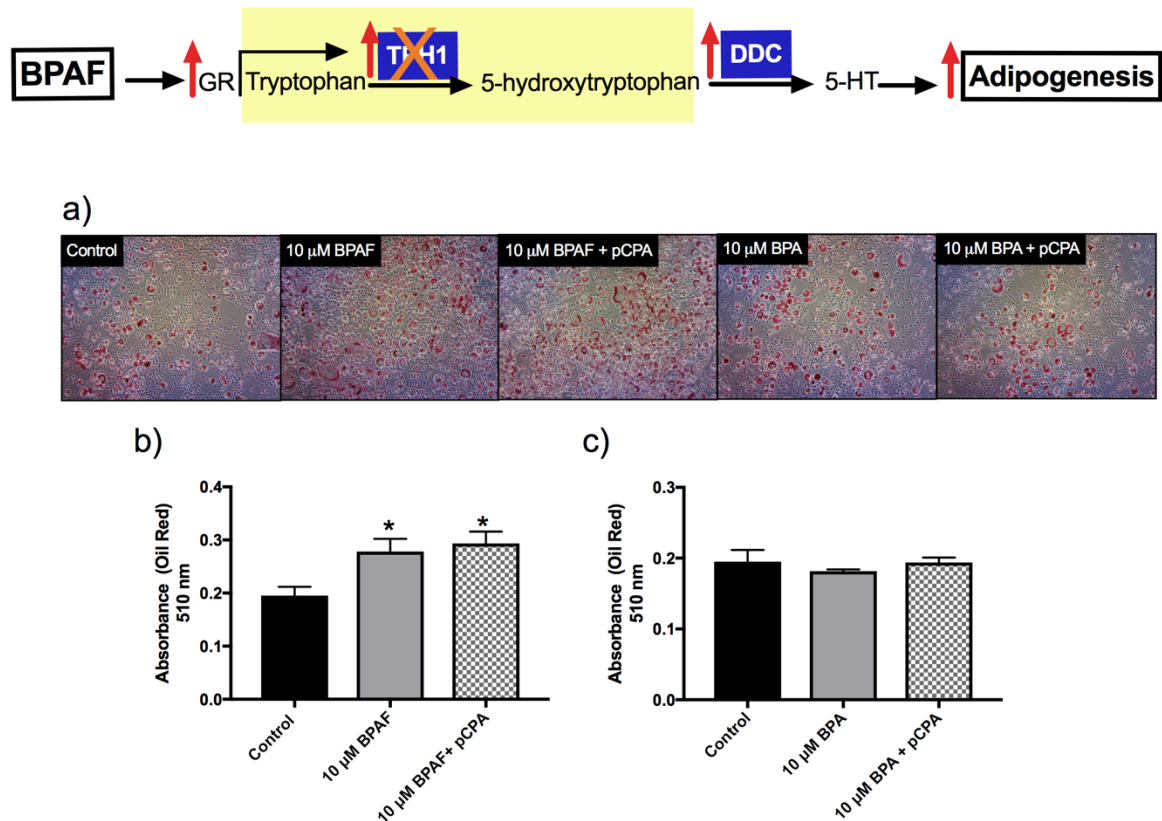


**Figure 9. BPAF is able to induce adipogenesis in the absence of the synthetic glucocorticoid dexamethasone (Dex).** 3T3-L1 cells were treated with 0.25 μM Dex (Day 0–Day 3), 0.25 μM Dex (Day 0–Day 9), 10 μM BPAF (Day 0–Day 3), 10 μM BPAF (Day 0–Day 9) and (-) Dex (DMSO control). a) mRNA expression levels of *Nr3c1* (*GR*) in 3T3-L1 cells treated with 10 μM BPAF. Data are normalized to  $\beta$ -actin. Data represent mean  $\pm$  S.E.M. \* $P<0.05$  (n=4–11) calculated by Student’s T-test. b) *Nr3c1* (*GR*) and *Tph1* gene transcripts are positively correlated ( $R^2=0.829$ ,  $P<0.0001$ ). c) Representative images of lipid accumulation as determined with Oil Red O staining (10x). d) Lipid accumulation in 3T3-L1 cells treated 0.25 μM Dex (Day 0–Day 3), 10 μM BPAF (Day 0–Day 3) or (-) Dex (DMSO control) by Oil Red O staining. e) Lipid accumulation in 3T3-L1 cells treated with 0.25 μM Dex (Day 0–Day 9), 10 μM BPAF (Day 0–Day 9) or (-) Dex (DMSO control) by Oil Red O staining. Data represent mean  $\pm$  S.E.M. \* $P<0.05$  (n=4) calculated by Student’s T-test (d) or Mann-Whitney Rank Sum test (e) versus (-) Dex (DMSO control).

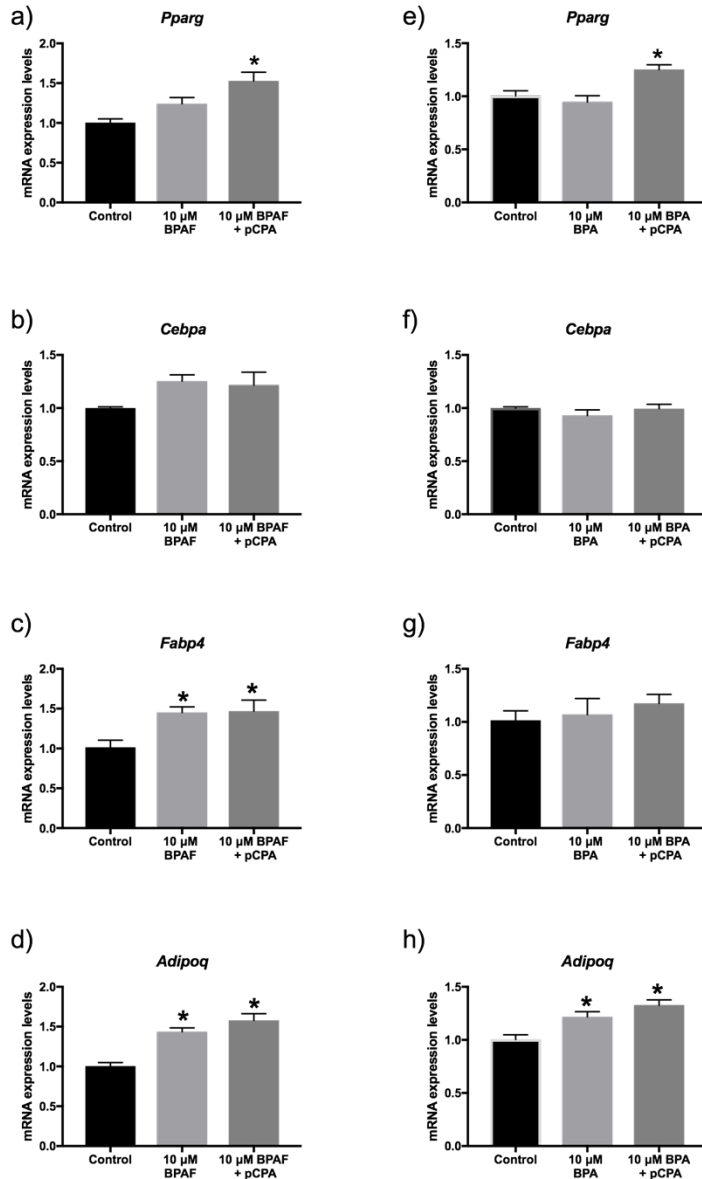


**Figure 10. mRNA expression of 5-HT synthetic enzyme, *Tph1*, is significantly increased with 10 μM BPAF treatment.** mRNA levels were determined by RT-qPCR in murine 3T3-L1 preadipocytes treated with control (DMSO) or 10 μM BPAF for 9 days. Data are normalized to  $\beta$ -*actin*. Data represent mean  $\pm$  S.E.M. \* $P < 0.05$  ( $n = 4-11$ ) calculated by Mann-Whitney Rank Sum Test.

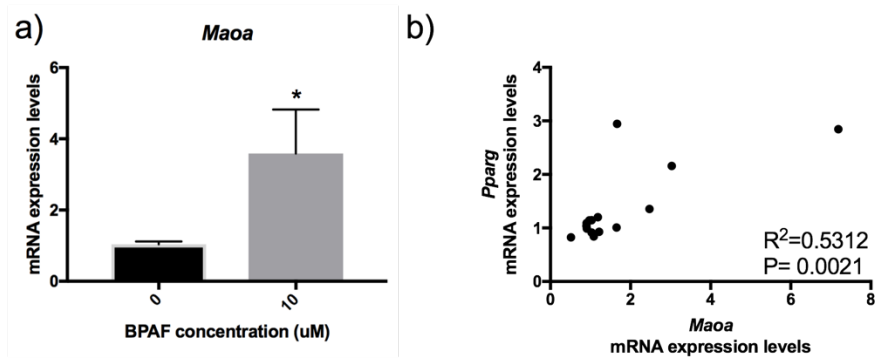
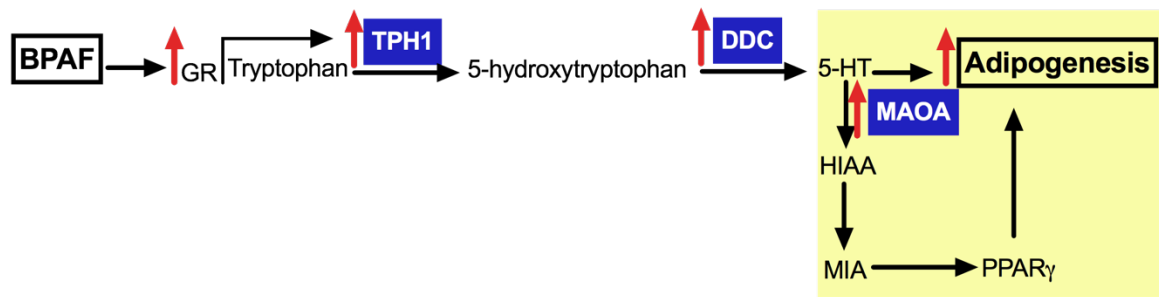




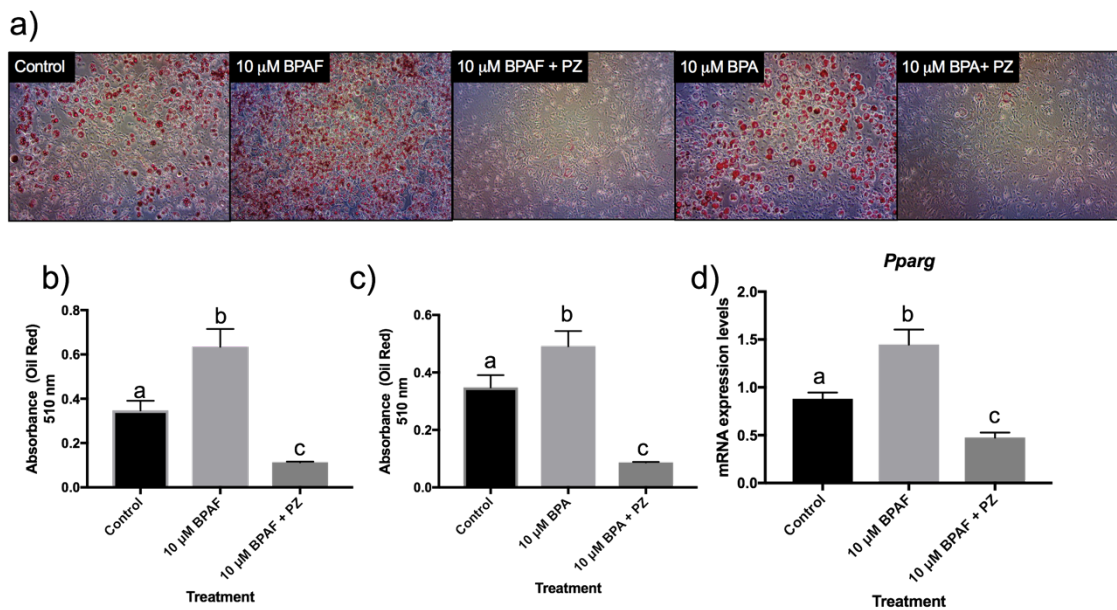
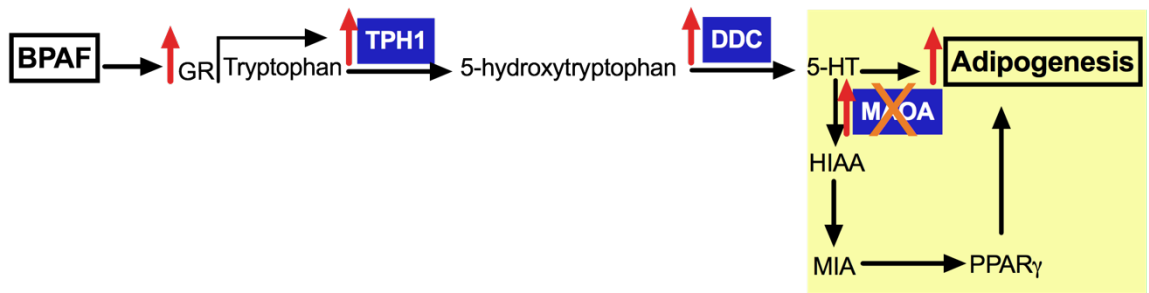
**Figure 11. Co-treatment with 10 μM BPAF or BPA with 50 μM pCPA does not significantly alter lipid accumulation in 3T3-L1 cells.** a) Representative images of control (DMSO), 10 μM BPAF, 10 μM BPAF + 50 μM pCPA, 10 μM BPA and 10 μM BPA + 50 μM pCPA following Oil Red O staining (10x). b) Lipid accumulation was determined by Oil Red O staining in cells treated with control (DMSO), 10 μM BPAF and 10 μM BPAF + 50 μM pCPA for 9 days. c) Lipid accumulation was determined by Oil Red O staining in cells treated with control (DMSO), 10 μM BPA and 10 μM BPA + 50 μM pCPA for 9 days. Data represent mean ± S.E.M. \*P<0.05 relative to control (n=5–6) calculated by One-Way ANOVA followed by Holm-Sidak.



**Figure 12. Effects of co-treatment with 10  $\mu$ M BPAF or BPA  $\pm$  50  $\mu$ M pCPA on adipogenic gene markers in 3T3-L1 cells.** mRNA levels were determined by RT-qPCR in murine 3T3-L1 preadipocytes treated with vehicle (DMSO), 10  $\mu$ M BPAF or BPA  $\pm$  50  $\mu$ M pCPA for 9 days. Data are normalized to  $\beta$ -actin. Data represent mean  $\pm$  S.E.M. \*P<0.05 (n=4–5) relative to control calculated by One Way ANOVA followed by Holm-Sidak (a–d, f–h) or Kruskal-Wallis One-Way ANOVA on Ranks followed by Dunnett’s Method (e).



**Figure 13. mRNA expression of *Maa* is significantly increased with 10 μM BPAF treatment.** a) mRNA levels were determined by RT-qPCR in murine 3T3-L1 preadipocytes treated with control (DMSO) or 10 μM BPAF for 9 days. Data are normalized to  $\beta$ -*actin*. Data represent mean  $\pm$  S.E.M. \* $P<0.05$  (n=4–11) calculated by Mann-Whitney Rank Sum Test. b) *Pparg* and *Maa* gene transcripts are positively correlated ( $R^2=0.5312$ ,  $P=0.0021$ ).



**Figure 14. Co-treatment with 10 μM BPAF + phenelzine (PZ) significantly decreases lipid accumulation and *Pparg* gene expression in 3T3-L1 cells.** a) Representative images of control, 10 μM BPAF, 10 μM BPAF + PZ, 10 μM BPA and 10 μM BPA + PZ in 3T3-L1 cells stained with Oil Red O (10x). b) 3T3-L1 cells were treated with control (DMSO) or 10 μM BPAF ± 100 μM PZ for 9 days. Lipid accumulation was determined by Oil Red O staining (n=3). c) 3T3-L1 cells were treated with control (DMSO) or 10 μM BPA ± 100 μM PZ for 9 days. Lipid accumulation was determined by Oil Red O staining (n=3). d) mRNA expression of adipogenic gene marker *Pparg* by RT-qPCR (n=4–5). Data are normalized to *Rplp0*. Data represent mean ± S.E.M. Groups with different letters are significantly different from each other (P<0.05) calculated by One-Way ANOVA followed by Holm-Sidak.

## CHAPTER 5: DISCUSSION

### ***5.1 The effects of BPAF on lipid accumulation during adipogenesis***

As a known endocrine disruptor, BPA has been extensively studied for its role in the development of obesity. There is now considerable evidence ranging from cell culture and animal experiments to human epidemiological studies to support the hypothesis that BPA is an obesogen affecting adipogenesis and lipid homeostasis. Due to growing concerns surrounding the use of BPA, there is increasing use of BPA structural analogues as replacements for BPA in consumer products. However, there is currently little information available regarding their toxicity; this is even more notable in the context of their effects on obesity. The results of my work demonstrated that at the dose used in this study (i.e., 10  $\mu$ M), BPAF is a stronger inducer of adipogenesis than BPA. BPAF significantly increased lipid accumulation, as well as mRNA expression levels of adipogenic gene markers (*Pparg* and *Cebpa*) and markers of mature adipocytes (*Fabp4* and *Adipoq*). Although a range of doses was evaluated, these effects were only seen at the highest (i.e., 10  $\mu$ M) dose tested. The results of this study are in agreement with recent findings showing that BPAF, at the same dose, stimulated triglyceride accumulation in ATCC 3T3-L1 cells; the effect of BPAF to induce triglyceride accumulation was approximately 45–50% of the response seen relative to ROSI<sup>79</sup>, a known inducer of triglyceride accumulation. The authors observed minimal changes in triglyceride levels in the lower nanomolar ranges which is also consistent with the lack of

effects on adipogenic gene expression in the lower dose ranges in this study. Although BPAF has recently been shown to increase lipid accumulation in 3T3-L1 cells<sup>79</sup>, the results of this study, to my knowledge, are the first to evaluate its effects on mRNA expression of adipogenic gene markers.

The results of this study also support recent findings demonstrating the ability of BPS to increase murine adipogenesis *in vitro*<sup>76,77</sup>. Ahmed and Atlas (2016) showed that BPS significantly increased lipid accumulation (10–50  $\mu$ M) in 3T3-L1 cells<sup>77</sup>. Moreover, 25  $\mu$ M BPS significantly increased adipogenic gene markers (ie., *Fabp4*, *Pparg*, perilipin (*Plin*), *Lpl*, adiponectin (complement factor D; *cfD*) and *Cebpa*) and lipid accumulation to a greater extent than BPA at an equivalent dose. Halogenated bisphenols, tetrachlorobisphenol A (TCBPA) and tetrabromobisphenol A (TBBPA), have been shown to activate PPAR $\gamma$  and induce lipid accumulation in 3T3-L1 cells<sup>121</sup>. The fact that structurally similar derivatives of BPA have stronger ability to activate PPAR $\gamma$ <sup>121,122</sup>, the master regulator of adipogenesis, supports the notion that these alternatives may stimulate adipogenesis to a greater extent than BPA. Additionally, BPA was found to be the least potent chemical when compared to TCBPA, TBBPA and BPAF at inducing triglyceride accumulation (23% vs 45–50%) relative to cells maximally stimulated with ROSI<sup>79</sup>. Taken together, the results of this study add to the growing body of literature supporting the fact that structural analogues of BPA may be more obesogenic than BPA itself.

While BPA has been shown to stimulate *in vitro* differentiation (i.e., increase adipogenesis) at 10  $\mu\text{M}$ <sup>122,123</sup>, other studies using 10  $\mu\text{M}$  BPA did not report increased adipogenesis similar to what is seen in my work<sup>124,125</sup>. However, BPA has been shown to affect adipogenesis at lower doses (fentomolar- nanomolar range)<sup>50,76,115</sup> as well as in higher micromolar ranges (20–80  $\mu\text{M}$ )<sup>41,44,48,77,124</sup>. Inconsistencies between studies, however, may be attributed to varied differentiation models (e.g., murine, human preadipocytes) differentiation protocols (e.g., in the absence/presence of dexamethasone; differing induction reagents) and timing/length of exposure. Therefore, comparisons across studies can be challenging when attempting to draw definitive conclusions of the most potent adipogenic dose of BPA. Regardless, in my study, BPAF and not BPA at the equimolar concentrations induced adipogenesis suggesting that BPAF may be a more potent obesogen than BPA.

### ***5.2 The effects of BPAF on lipid accumulation in mature adipocytes***

To determine whether the obesogenic actions of BPAF were specific to the process of adipogenesis, I evaluated the effects of BPAF on fully differentiated adipocytes. I did not observe changes in lipid accumulation in mature adipocytes when treated for 24hrs with 10  $\mu\text{M}$  BPAF. This suggests that although BPAF may act during adipogenesis to increase differentiation of preadipocytes, it may not play a role in regulating lipogenesis in fully differentiated fat cells. A previous study evaluating the effects of BPA in mature adipocytes isolated from subcutaneous adipose tissue, determined that 1 and 10 nM

BPA significantly increased mean lipid area after 24hr treatment<sup>126</sup>. Triglyceride levels were also increased at 1 and 10 nM, though only the 10 nM dose was significant<sup>126</sup>. Treatment with 10  $\mu$ M BPA in this study exhibited no effects on mature murine adipocytes. However, other metabolic parameters have been shown to be altered by BPA in mature adipocytes, such as adipokine secretion, insulin sensitivity and inflammatory markers<sup>45,46,51,126</sup>. Adiponectin plays a role in improving insulin sensitivity and its level has been shown to be reduced in obesity<sup>127</sup>. Moreover, BPA exposure has been associated with decreased adiponectin levels. Hugo et al. (2008) isolated mature adipocytes from subcutaneous tissue in women and observed significantly decreased adiponectin release with 0.1 and 10 nM BPA treatment<sup>45</sup>. Another study discovered that BPA, in addition to BPF, BPE and BPB at 80  $\mu$ M significantly decreased both adiponectin production and secretion in 3T3-L1 adipocytes<sup>46</sup>. Additionally, 10 nM and 100 nM BPA has been found to decrease insulin stimulated glucose utilization in differentiated 3T3-L1 cells and differentiated human adipocytes<sup>51</sup>. A role for inflammation has also been determined with dysregulation of IL-1B, IL18, IL-6, CCL20 (chemokine (C-C motif) ligand 20) and interferon gamma (IFN- $\gamma$ ) cytokines in BPA treated mature adipocytes<sup>51,126</sup>. As obesity is multifactorial, with dysregulation of multiple metabolic parameters, inflammation, insulin sensitivity in addition to lipid homeostasis may play a role in an EDC's deleterious effects. Therefore, although BPAF did not increase lipid accumulation in mature adipocytes, other deleterious metabolic effects cannot be ruled out such as its effects on insulin sensitivity and inflammatory markers.



Results from my study also suggest that exposure to BPAF *in utero* or in early life may be a more critical window of exposure. This hypothesis is supported by a number of lines of evidence. Firstly, based on current epidemiological studies higher levels of BPA in childhood and adolescence have most consistently been associated with an increased odds of obesity<sup>37</sup>. Secondly, from my study, BPAF appears to have more profound effects on adipogenesis; adipogenesis begins during the 14<sup>th</sup> week of development and formation of majority of new adipocytes occurs during childhood/ adolescence. This occurs predominately before the first two years of life and during puberty from approximately age 8 to 18<sup>5,38,40</sup>. Thirdly, it has been suggested from work on BPA that the BPA metabolite BPA-G may be unable to cross the placenta once metabolized by the fetus and that in fact BPA-G can be deconjugated back to free BPA by the fetus<sup>128</sup>. Importantly, BPA-G itself has been shown to have adipogenic capacity<sup>129</sup>. These data suggest that with increasing exposure to BPA during pregnancy, the fetus is exposed to these potent adipogenic stimuli.

There have been a number of animal studies which have evaluated the effects of prenatal exposure to BPA on the development of obesity in the offspring during postnatal life. Somm et al. (2009) found elevated body weight and an increase in genes regulating lipogenesis, lipolysis and hypertrophic adipocytes at postnatal day 21 (PND21) in female Sprague Dawley rats exposed to 1 mg/ml (70 µg/kg body weight) BPA *in utero* and lactation<sup>42</sup>. Another recent study in rats had similar finding (increased adipocyte

density in female offspring and alterations in metabolic markers (triglyceride levels, lipid homeostasis, adipokine secretion) using a much lower dose (0.5 µg/kg BPA)<sup>63</sup>, which was below the recently lowered tTDI set by the EFSA of 4 µg/kg/day. van Esterik et al. (2014) found dose dependent increases in body weight in male mice (0–3000 µg/kg body weight) from week 6 to week 21; however, in females a dose dependent decrease in body weight and adipocyte size was observed (week 8–week 21). Similarly, another study with male offspring by Angle et al. (2013) reported significant weight gain in addition to adipocyte number/volume at 19 weeks of age<sup>58</sup>. Conversely, Alonso-Magdalena et al. (2010) found male mice exposed *in utero* to 10 or 100 µg/kg body weight/day displayed no changes in body weight at 6 months of age; females however had significantly reduced body weight (10 µg/kg body weight) beginning at 3 months old which persisted until 6 months<sup>61</sup>. Studies evaluating the effects of *in utero* BPA exposure further into adulthood have reported an increase in weight gain in OF-1 male mice at 28 weeks of age (10 µg /kg)<sup>59</sup>.

Exposure to BPA in conjunction with a high fat diet may also exacerbate BPA's effects. When rats were exposed to 50 µg/kg bodyweight from gestational day (GD) 0 until PND21, both male and female offspring had significantly increased weight when fed a normal diet; an effect which began at week 19 and week 17 for males and females respectively<sup>43</sup>. This increase was more profound and appeared earlier in age in animals on a high fat diet. In addition, body fat percentage and adipocyte size were significantly

increased in males at 27 weeks. However, a significant increase in body fat percentage and adipocyte size was only observed in females when on a high fat diet when compared to control high-fat diet fed animals. This was also evident in a study by Somm et al. (2009) who only observed significant weight gain in males exposed to BPA *in utero*/lactation when they were fed a high fat diet<sup>42</sup>. Conversely, another study did not find differences in BPA exposed animals when they were placed on high fat diet<sup>64</sup>.

Additionally, BPA exposure during adolescence may represent a period of increased vulnerability as both male and female mice exposed to BPA (5–5000 ug/kg body weight/day) at 5 weeks of age for 30 days displayed significant weight gain and adiposity<sup>66</sup>. Moreover, in males, increased adipogenesis was evident with elevated mRNA levels of *Pparg*, *Cebpa* and *Fabp4* in inguinal white adipose tissue (iWAT), as well as increased adipogenic capacity of stromal vascular fraction (SVF) cells. Similarly, females treated perinatally (GD8–PND16) and during puberty from PND21–35 displayed augmented weight gain and dysregulated glucose homeostasis when compared to females only treated during pregnancy/lactation<sup>60</sup>. Therefore, sex, timing and dose dependent differences across *in vivo* studies are apparent.

Finally, exposure to BPS *in utero* has also been associated with increased weight gain. *In vivo*, male mice exposed to 1.5 and 50 µg /kg body weight/day *in utero* up to 22 weeks of age had significantly increased fat mass and body weight in conjunction with a high

fat diet<sup>78</sup>. Therefore, this demonstrated that a commonly used structural analogue of BPA is able to exert its effects during a critical window of exposure.

In human prospective studies, the association between *in utero* BPA exposure and obesity have mixed findings, however consistent with other cross-sectional studies in children<sup>33,37</sup>, BPA concentrations in childhood have mostly been associated with elevated BMI in some<sup>34,35</sup> but not all<sup>36</sup> studies. In the CHAMACOS longitudinal study, urinary BPA concentrations during pregnancy were associated with decreased BMI-z, percent body fat and reduced odds of overweight/obesity (OR = 0.37; 95% CI 0.16, 0.91); an effect only observed among girls at 9 years of age<sup>34</sup>. Interestingly, urinary BPA concentrations in both girls and boys measured at age 9 were positively associated with BMI, WC, percent body fat and overweight/obesity (OR = 4.20; 95% CI 1.60, 11.02; urinary concentrations > median)<sup>34</sup>. A similar finding in females was also observed in a study by Vafeiadi et al. (2016) which found a negative association between BMI-z score at ages 1 through 4 and BPA concentrations in the 1<sup>st</sup> trimester of pregnancy; a positive association was observed for boys at all at ages 1 through 4<sup>35</sup>. Childhood urinary BPA levels at age 4 were cross-sectionally associated with BMI-z score, WC and skinfold thickness. Hoepner et al. (2016) found fat mass index, percent body fat and waist circumference were positively associated with urinary BPA concentration during pregnancy, with no association with BMI-z score for both sexes<sup>36</sup>. After taking sex into account, FMI was the only parameter positively associated with prenatal urinary BPA

concentrations and this association was only observed for females. Further, Valvi et al. (2013) found urinary BPA levels in pregnant women were weakly associated with higher BMI and WC in their children when they were 4 years old<sup>130</sup>. Conversely, Braun et al. (2014) found no association for BMI or WC between BPA urinary levels in pregnant women and their children at 2–5 years of age<sup>131</sup>. However, studies examining this association into adulthood have not been reported in the literature. Therefore, as pregnant women and children have detectable amounts of BPA, it remains unknown whether detrimental effects associated with BPA exposure extend into adulthood, when obesity and metabolic syndrome are most likely to manifest. The results of my study demonstrate that BPAF may specifically act during adipogenesis, not on already fully differentiated adipocytes, therefore early life exposures to this BPA replacement compound may have the most profound effects on disruption of lipid homeostasis. Because I showed that BPAF primarily affects adipogenesis, and both glucocorticoid and 5-HT signaling play an important role during this process, I wanted to determine whether these signaling pathways mediate BPAF-induced adipogenesis.

### ***5.3 The potential role of glucocorticoid signaling in BPAF-induced adipogenesis***

It has been suggested that BPA-induced adipogenesis may be mediated via activation of GR signaling. Therefore, I evaluated whether BPAF may also be acting through this pathway. Treatment with dexamethasone in 3T3-L1 cells has been shown to increase both CEBP $\delta$  (*Cebpd*) and *Pparg* expression levels<sup>112,132</sup>. Additionally, C/EBP $\delta$  and C/EBP $\beta$

stimulates C/EBP $\alpha$  in 3T3-L1 cells<sup>113</sup>. Moreover, in NIH 3T3 fibroblasts, overexpressing C/EBP $\beta$  and C/EBP $\delta$  provides maximal induction of *Pparg* mRNA levels, however this response is only observed with the addition of glucocorticoids<sup>132</sup>. Steger et al. (2010) found that in 3T3-L1 cells GR and C/EBP $\beta$  binding results in an epigenomic transition state involving histone H3 acetylation, PPAR $\gamma$  activation (which results in a positive feedback loop with C/EBP $\alpha$ ) and subsequent induction of adipogenic genes<sup>8</sup>. Therefore, glucocorticoid signaling plays an important role in regulating adipogenesis through its association with C/EBPs and subsequent PPAR $\gamma$  activation.

Many studies evaluating adipogenesis *in vitro* use an induction cocktail with dexamethasone (a synthetic glucocorticoid) to activate the GR and stimulate glucocorticoid signaling pathways<sup>44,111</sup>. To evaluate whether BPAF alone can stimulate adipogenesis via GR signaling, dexamethasone was omitted from the induction cocktail. In the absence of dexamethasone, BPAF significantly increased lipid accumulation (Day 0–Day 3) when compared to control treated cells. This suggests that BPAF is able to induce adipogenesis in the absence of dexamethasone and is also able to potentially act in the early phases of adipogenesis (Day 0–Day 3) to accelerate differentiation. This suggests that BPAF may be acting like a GR agonist, although the response was not as strong as seen with dexamethasone, a potent GR agonist, alone. Using a luciferase binding assay, Sargis et al. (2010) reported that BPA could directly activate GR, however BPA was only able to induce adipogenesis under submaximal conditions with DHC in

3T3-L1 cells<sup>115</sup>. Conversely, the authors of another study did not observe GR stimulation with BPA treatment using luciferase assays, however BPA was able induce significant lipid accumulation in the absence of dexamethasone<sup>116</sup>. However, it was shown that 25  $\mu$ M BPA upregulated transcriptional activity of GR and C/EBP $\delta$  specifically at the FABP4 promoter<sup>116</sup>. Therefore, the adipogenic effects of BPA may be mediated through co-activators/co-repressors with promoter specificity<sup>116,133</sup>. Additionally, BPA may also be acting synergistically with GR to increase adipogenesis, as evident with submaximal GR activation or low dose dexamethasone exposure<sup>115,116</sup>. Of note, it is interesting that GR activation has been found to accelerate adipogenesis, however cells are able to accumulate lipid to the same extent when adipogenesis is continued to three weeks in GR knockdown cells<sup>134</sup>. The authors concluded that although GR accelerates preadipocyte differentiation, it is not absolutely necessary to stimulate lipid accumulation in 3T3-L1 cells.

In this study, mRNA steady-state level of GR was significantly increased in BPAF-treated cells. While the results of this study point to a possible link between BPAF and GR signaling, further studies are needed to determine whether BPAF is acting directly via the GR to increase adipogenesis. Recently, BPAF has been shown to bind to GR however, it only displayed weak binding activity, whereas BPA showed more potent binding activity<sup>117</sup>. Similarly, in another study both BPAF and BPA were not shown to stimulate GR activity in luciferase assays<sup>135</sup>. Sargis et al. (2010) reported that BPA treatment did

not affect GR protein expression despite the fact that there was an increase in GR transcriptional activity<sup>115</sup>. Therefore, it is unknown whether the changes in *Nr3c1* (*GR*) mRNA expression levels in this study are indicative of activation of GR transcriptional activity. Although I would like to investigate the role of GR signaling in BPAF-mediated adipogenesis, there are some technical limitations associated with using 3T3-L1 cells to address this question. It has been reported that using GR antagonists in 3T3-L1 cells is challenging. For example, RU486, a GR antagonist, has been reported to act as an inducer of adipogenesis<sup>136,137</sup>, therefore alternative methods such as luciferase assays or knockdown cell model should be used. Finally, it is also possible that the association between BPAF exposure, GR expression and increased adipogenesis may be indirect and involve 5-HT signaling pathways.

In rats, dexamethasone treatment significantly increased *Tph1* and *Ddc* mRNA and protein expression in intra-abdominal adipose tissue, as well as 5-HT levels in both intra-abdominal adipose tissue and serum<sup>118</sup>, suggesting that activation of the GR may transcriptionally regulate TPH1 and DDC. Interestingly, in my study I found a positive correlation between *Nr3c1* (*GR*) and *Tph1* mRNA levels. These data suggest that the effect of BPAF to increase adipogenesis may be mediated, in part, via alterations in 5-HT pathways.

#### **5.4 The effects of TPH1 inhibition on BPAF-induced adipogenesis**



*In vitro*, 5-HT levels have been shown to be produced during adipocyte differentiation<sup>94</sup> and 5-HT stimulates lipid accumulation when added to 3T3-L1 cells<sup>96</sup>. In TPH1 mutant cells, important adipogenic gene regulators, *Ppar $\gamma$* , *Cebpa* and *Fabp4* were significantly reduced<sup>96</sup>. Blocking Htr2a has also been shown to inhibit lipid accumulation in differentiated 3T3-L1 cells and treatment with an Htr2a agonist stimulates expression of genes involved in lipogenesis<sup>94</sup>. These studies suggest that both 5-HT and TPH1 play an integral role in regulating adipogenesis and lipogenesis in 3T3-L1 cells. However, Htr2B signaling in adipocytes has been shown to increase lipolysis<sup>98</sup> and microRNA-448 located on Htr2c plays a role in suppressing kruppel-like factor 5 (KLF5) expression<sup>96</sup>, an important regulator of adipogenesis. Therefore, 5-HT seems to act differentially in adipose tissue depending on its actions on a specific receptor subtype.

Additionally, although inhibiting peripheral 5-HT synthesis has been shown to play a protective role against obesity, in contrast, another study has pointed to beneficial effect of 5-HT in mice fed a high fat diet<sup>138</sup>. Mice injected intraperitoneally with 5-HT gained less weight, had lower intra-abdominal lipid accumulation and improved insulin/glucose tolerance, however this effect was only seen when combined with a high fat diet<sup>138</sup>. The authors postulated that an increase in oxidative metabolism in skeletal muscle, as well as an upregulation in mRNA levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha-b, c (*Ppargc1a-b, -c; PGC-1 $\alpha$* ) may play a role.

In the current study, although treatment with 10  $\mu\text{M}$  BPAF significantly increased *Tph1* levels in 3T3-L1 cells, blocking its activity with pCPA, a TPH inhibitor, did not impair adipogenesis as hypothesized. This was evident as there were no alterations in lipid accumulation with Oil Red O staining, as well as no significant changes in mRNA levels of adipogenic gene markers in cells treated with BPAF and pCPA. These results do not support the hypothesis that BPAF-induced adipogenesis is mediated via increased 5-HT production.

One limitation of this study is that only *Tph1* mRNA levels were reported and 5-HT levels were not measured. Therefore, it is unknown whether the 50  $\mu\text{M}$  pCPA dose chosen in this study sufficiently blocks TPH1 at a level to effectively reduce 5-HT levels in these cells. Previous studies in 3T3-L1 cells have shown significant reductions in lipid accumulation with pCPA at doses ranging from 8–200  $\mu\text{M}$ <sup>96</sup>. Therefore, the dose used in this study of 50  $\mu\text{M}$  was within the range reported to inhibit lipid accumulation in 3T3-L1 cells. Additionally, a 30  $\mu\text{M}$  dose was used to determine the effects of 5-HT in buffalo rat liver 3A cells (BRL-3A)<sup>118</sup>. However, pCPA seems to be a much less potent inhibitor of TPH1 than other TPH inhibitors such as LP-533401. In one study measuring inhibition of human TPH1 *in vitro*, the  $\text{IC}_{50}$  of pCPA was 250  $\mu\text{M}$  whereas the  $\text{IC}_{50}$  for LP-533401 was reported to be 0.7  $\mu\text{M}$ <sup>139</sup>. Additionally, inhibition of 5-HT levels displayed  $\text{IC}_{50}$  values of 43  $\mu\text{M}$  and 0.4  $\mu\text{M}$  for pCPA and LP-533401 respectively. Interestingly, in the brain, *Tph* gene transcripts and 5-HT levels were shown to differ with pCPA treatment, with an

increase in *Tph* mRNA levels corresponding with reduced 5-HT levels<sup>140</sup>. Therefore, these results presented in the current study may not reflect alterations in enzyme activity and interpretation of data should be made with careful consideration. Moreover, it is possible that pCPA may have a short half-life in culture conditions, and adding it in more frequent intervals may block TPH1 activity to a fuller extent. Furthermore, experiments evaluating the efficacy of pCPA to inhibit TPH1 activity and 5-HT production in 3T3-L1 adipocytes are needed.

### ***5.5 The effects of MAO inhibition on BPAF-induced adipogenesis***

While 5-HT has been shown to act on receptors, most notably Htr2a, Htr2b and Htr2c in adipocytes<sup>94,96,98</sup>, a possible role for its metabolites has also been reported. Waku et al. (2010) discovered that MIA, a metabolite of 5-HT, is able to increase lipid accumulation in 3T3-L1 cells. Moreover, both HIAA and MIA, indole-acetate molecules can activate PPAR $\gamma$  via binding to activation function 2 helix H12. When 5-HT is oxidized in 3T3-F442A cells by MAO, there is enhanced lipid accumulation and stimulation of phosphoenolpyruvate kinase (*Pck1*; *PEPCK*) and *Fabp4* expression, both PPAR $\gamma$  responsive genes<sup>103</sup>. Interestingly, indole-acetate can be produced from tryptophan itself, a pathway independent of 5-HT metabolism<sup>141</sup>. Therefore, a role for indole-acetate molecules, which have been shown to directly bind to PPAR $\gamma$  and stimulate adipogenesis is likely.

In this study, 10  $\mu$ M BPAF significantly increased mRNA expression of *Maoa*, the enzyme responsible for the breakdown of 5-HT to its metabolites. To determine whether BPAF-induced adipogenesis occurs via altered 5-HT metabolism, I investigated whether the adipogenic effects of BPAF were due in part to its actions on MAO. Phenzelzine, a non-selective irreversible MAO inhibitor, is used for treating depression<sup>142</sup>. Treatment with phenzelzine has been reported to be associated with increased weight gain in humans<sup>143</sup>, however the mechanisms underlying this effect are poorly understood. Interestingly, pharmacologically blocking MAO in adipocytes with 100  $\mu$ M phenzelzine has been shown to significantly decrease triglyceride accumulation during adipogenesis<sup>104</sup> and reduce weight gain in obese rats<sup>144</sup>. Moreover, in my study I observed a positive correlation between *Maoa* and *Pparg* gene transcripts. Co-treatment with 100  $\mu$ M phenzelzine in this study significantly decreased lipid accumulation in 3T3-L1 cells and was able to block the adipogenic effects of BPAF. A dose of 100  $\mu$ M was used in the present study was chosen as it has been reported to sufficiently inhibit amine oxidase activity in rat adipocytes<sup>144</sup>. Therefore, these results suggest a role for increased MAOA activity and potentially 5-HT metabolites in mediating the adipogenic effects of BPAF. However, there still remain some questions regarding the role of 5-HT metabolites in the adipogenic actions of BPAF seen in this study.

Chiche et al. (2009) questioned whether phenzelzine's lipid depleting effects were in fact due to a direct inhibition of amine oxidase activity<sup>104</sup>. When other amine oxidase

inhibitors were examined for their ability to regulate triglyceride levels during adipogenesis, no apparent relationship was observed in 3T3-F442A cells; not all amine oxidase inhibitors inhibited triglyceride accumulation to the same degree. However, it is apparent that other inhibitors block amine oxidase activity at differing concentrations, some more potent than others<sup>144,145</sup>. Moreover, it is plausible that if phenelzine's antiadipogenic effects were due its amine oxidase inhibitory ability and 5-HT metabolites directly bind to PPAR $\gamma$ , adding a PPAR $\gamma$  agonist should rescue phenelzine's antiadipogenic effects. However, the authors concluded that phenelzine's actions are not mediated through PPAR $\gamma$  signaling as troglitazone, a PPAR $\gamma$  agonist, was unable to rescue phenelzine's inhibitory effects on adipogenesis in 3T3-F442A cells and in cells constitutively expressing PPAR $\gamma$ . Moreover, these metabolites have not been shown to alter *Pparg* mRNA expression levels<sup>103</sup> but they may act as PPAR $\gamma$  agonists altering PPAR $\gamma$  receptor activity<sup>101</sup>. In my study, although mRNA expression of *Pparg* was significantly decreased when cells were treated with BPAF and phenelzine, phenelzine's effects may not be mediated through alterations in PPAR $\gamma$  activity.

It also must be noted that phenelzine is not only an inhibitor of MAOA and MAOB, but also of primary amine oxidase (SSAO/PrAO). SSAO/PrAO is also involved in modulating adipose tissue function<sup>144,146,147</sup>. Grès et al. (2013) reported that oxidation of 5-HT in adipocytes was predominantly through a MAO dependent mechanism<sup>103</sup>. Therefore, if phenelzine acts largely through SSAO/PrAO, this may represent an additional

mechanism of phenelzine to inhibit adipogenesis which is independent of 5-HT catabolism.

It is also possible that MAO and GR may be linked. Boucher et al. (2014) found MAOA to be the third highest upregulated gene in their microarray analysis upon 48hr treatment with 1  $\mu$ M dexamethasone in human preadipocytes<sup>148</sup>. Therefore, if BPAF does play a role in glucocorticoid signaling, as outlined above, it may also play a role in the increased *Maoa* gene expression levels that were observed in the current study. Nonetheless, this study demonstrates that BPAF is not able to overcome the inhibitory actions of phenelzine, pointing to a possible role for MAO in BPAF-induced adipogenesis.

### **5.6 Implications**

Although my work demonstrated BPAF as a potential obesogen, altering adipogenesis, it is only one of several known replacement chemicals (i.e., Bisphenol AP, Bisphenol E, BPSIP, Bisphenol E, Bisphenol PH, Bisphenol Z) that has been implicated in obesity<sup>67</sup>. These structurally similar substitutes have been detected in sediment, food, receipts, resin and municipal sewage sludge (Reviewed in: <sup>67,68</sup>). BPA structural analogues are also increasingly being detected in urine samples in humans<sup>68,83,84</sup>. Though limited toxicological data exists, these replacements have been shown to target many of the same endocrine pathways as BPA such as estrogen, androgen, pregnane X receptor and glucocorticoid signaling<sup>149</sup>. Additionally, several analogues (BPAF, BPB, BPF and BPS) are

suspected to display equivalent or greater toxicity than BPA<sup>68</sup>. Moreover, the metabolic consequences of these replacements, including their effects on adipocyte number, lipid storage and satiety/food intake are limited. This is especially concerning in regard to their potential effects during critical windows of exposure (i.e., during pregnancy, early childhood, adolescence) when exposure to these chemicals can permanently predispose an individual to obesity and metabolic disruption later in life.

As previously mentioned, adipocyte number remains relatively stable in adulthood. Therefore, increasing the number of fat cells through exposure to an EDC during development may create changes to an individual's metabolic set point<sup>17,40,150</sup>. Ultimately, this leads to an increase in stem cells committed to becoming adipocytes or more fat cells that have the ability to expand and be filled with lipid. This, coupled with the fact that adipocyte number cannot be diminished through diet, physical activity or surgery creates an environment favoring energy storage<sup>5,150</sup>. Additionally, a “second-hit”, such as a high fat diet may further exacerbate the effects on an individual who had previously been exposed to an EDC early in life<sup>150</sup>.

Secondly, while *in vivo* studies have shown BPA stimulates adipogenesis and adipocyte hypertrophy<sup>42,63</sup>, its exposure has also been associated with dysregulation of glucose homeostasis and impaired insulin sensitivity<sup>43,58,61</sup>. Therefore, obesogens may also be implicated in altering a wide range of metabolic factors so their effects on whole body

metabolic homeostasis need to be assessed. Thirdly, adipogenic EDCs have also been shown to possess transgenerational effects<sup>151</sup> which can permanently alter metabolic outcomes in subsequent generations.

Taken together my work has identified a new potential obesogen which in my study, has been shown to be even more potent than the chemical it is replacing (i.e., BPA). The fact that more than one BPA replacement compound has been shown to have similar or more profound effects than BPA on metabolic outcomes raises significant concerns about their increasing use in consumer products.



## CHAPTER 6: FUTURE DIRECTIONS

For future investigation into the role BPAF plays during adipogenesis, protein expression levels of PPAR $\gamma$ , C/EBP $\alpha$ , FABP4 and ADIPOQ should be assessed. Moreover, as gene and protein expression do not reflect activity, transcriptional binding assays to determine whether BPAF can directly bind to and alter specific promoter regions of GR or other transcriptional regulators such as PPAR $\gamma$  needs to be examined. Additionally, to further determine the role of 5-HT signaling in BPAF-induced adipogenesis, 5-HT levels need to be measured. An alternate MAO inhibitor could be used to delineate whether the antiadipogenic effects observed in this study are due to its actions on amine oxidase activity. Additionally, as BPA plays a role in glucose homeostasis, it would be interesting to observe whether BPAF also alters glucose transport and insulin sensitivity in adipocytes.

Finally, although the 3T3-L1 model has been extensively used for studying adipogenesis *in vitro*, using human preadipocytes may more closely resemble a human model. As BPA has been studied for its effects during development, the effects of BPAF in rodents exposed perinatally could also be examined.

## **CHAPTER 7: CONCLUSIONS**

Due to the growing concern from scientists, regulators and the public surrounding the use of BPA, increasing attention has been placed on its structural analogues as replacements. However, little is currently known about how these substitutes may affect human health. The data from this study suggest that both BPAF and BPS, common replacements for BPA, may also contribute to metabolic perturbations associated with the development of obesity. Moreover, the results of this study add to the mounting evidence that EDCs have the ability to modulate adipose tissue physiology. The findings of this study also identify peripheral 5-HT signaling as a potential novel mechanism through which EDCs may exert their obesogenic effects. While exposure to BPA has been shown to alter key components of the central serotonergic signaling pathway, no study has attributed this pathway as a casual linkage between exposure to BPA and its structural analogues and metabolic dysfunction. Therefore, further studies evaluating the link between how EDCs might modulate their obesogenic effects through the peripheral serotonergic network are needed. As obesity is a serious global public health challenge representing one of the leading causes of morbidity, mortality and health care expenditure worldwide, there is an urgent need to determine which chemicals pose the greatest health risk to society.

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## CHAPTER 9: APPENDIX

**Table 1.** Summary of BPA Concentrations Inducing Significant Lipid Accumulation *In Vitro*.

Author	Model	Window of Exposure	[ ] Tested	Effect [ ] Lipid Accumulation
Sargis et al. <sup>115</sup>	3T3-L1	Day 0–Day 3	100 nM	100 nM
Masuno et al. <sup>152</sup>	3T3-L1	Day 2–Day 8	4–80 µM	20 µM, 40 µM, 80 µM
Masuno et al. <sup>41</sup>	3T3-L1	Day 0–Day 2	20 µg/ml (87.6 µM)	20 µg/ml (87.6 µM)
		Day 0–Day 11	20 µg/ml (87.6 µM)	20 µg/ml (87.6 µM)
Wada et al. <sup>123</sup>	3T3-L1	Day 0–Day 5/6	10 µM	10 µM
Atlas et al. <sup>116</sup>	3T3-L1	Day 0–Day 8	0.01–10 nM	0.01–10 nM
Chamorro-García et al. <sup>122</sup>	3T3-L1	Day 2–Day 7	100 pM–10 µM	100 nM, 1 µM, 10 µM
	mouse MSCs human MSCs	Day 0–Day 14	1 nM–100 µM	(↔)
Héliès-Toussaint et al. <sup>76</sup>	3T3-L1	Day 2–Day 10	1 fM–1 µM	1 fM, 1 pM, 1 nM
Taxvig et al. <sup>124</sup>	3T3-L1	Day 3–Day 6	5–20 µM	20 µM
Ariemma et al. <sup>50</sup>	3T3-L1	3 weeks (preadipocytes) & Day 0–Day 8	1 nM	1 nM
Wang et al. <sup>153</sup>	human visceral preadipocytes	Day 4–Day 18	10 nM, 1 µM, 80 µM	10 nM, 1 µM, 80 µM
Ohlstein et al. <sup>125</sup>	human adipose stromal cells	Day 0–21	100 pM–10 µM	100 nM, 1 µM
		Day 0–14		10 nM–1 µM
Boucher et al. <sup>44</sup>	primary human preadipocytes	Day 2–Day 14	25 µM, 50 µM	25 µM, 50 µM
Biemann et al. <sup>154</sup>	MSCs	Day 0–Day 12	10 nM, 10 µM	10 nM (↔)
				10 µM (↓)

**Table 2.** Comparison of Urinary Concentrations (ng/ml) of Total BPA and BPA Structural Analogues Across Human Studies.

Study	Author	Location	N	Urine (ng/ml) GM*
<b>BPA</b>				
NHANES 2003–2004	Lakind and Naiman <sup>155</sup>	USA	2517 (age 6–60+)	2.4
CMHS 2007–2009	Lakind et al. <sup>25</sup>	Canada	5476 (age 6–79)	1.2
NHANES 2005–2006	Lakind and Naiman <sup>22</sup>	USA	2548 (age 6–60+)	2.0
NHANES 2007–2008	Lakind et al. <sup>25</sup>	USA	2489 (age 6–79)	2.1
NHANES 2011–2012	Lakind and Naiman <sup>156</sup>	USA	2489 (age 6–60+)	1.5
NHANES 2013–2014	Liu et al. <sup>84</sup>	USA	1521 (age 20+)	1.3 <sup>a</sup>
	Ye et al. <sup>83</sup>	USA	141	0.67
			42	0.36
	Yang et al. <sup>157</sup>	China	94 <sup>b</sup>	0.886
<b>BPS</b>				
NHANES 2013–2014	Ye et al. <sup>83</sup>	USA	141	0.22 (2013)
			42	0.25 (2014)
	Liao et al. <sup>158</sup>	USA	31	0.299
	Yang et al. <sup>157</sup>	China	94 <sup>b</sup>	0.028
NHANES 2013–2014	Liu et al. <sup>84</sup>	USA	1521 (age 20+)	0.4 <sup>a</sup>
<b>BPAF</b>				
NHANES 2013–2014	Ye et al. <sup>83</sup>	USA	141	N/C (2013)
			42	N/C (2014)
	Yang et al. <sup>157</sup>	China	94 <sup>b</sup>	0.018
<b>BPF</b>				
NHANES 2013–2014	Ye et al. <sup>83</sup>	USA	141	0.18 (2013)
			42	0.41 (2014)
	Yang et al. <sup>157</sup>	China	94 <sup>b</sup>	0.228
	Liu et al. <sup>84</sup>	USA	1521 (age 20+)	0.3 <sup>a</sup>

\*GM – geometric mean

<sup>a</sup> urinary median<sup>b</sup> residents near BPAF manufacturing plant

**Table 3.** Primer Sequences for RT-qPCR.

<b>Gene</b>	<b>Forward (5'-3')</b>	<b>Reverse (5'-3')</b>
<i>Pparg</i>	GCGGAAGAAGAGACCTGGG	GTGACTTCTCCTCAGCCCG
<i>Cebpa</i>	CCGTGGTGGTTTCTCCTTGA	TTTTTGCTCCCCCTACTCGG
<i>Adipoq</i>	ATCTGGAGGTGGGAGACCAA	TGGGCTATGGGTAGTTGCAG
<i>Fabp4</i>	ATTCCTTCAAACCTGGGCGTG	CTTCCATCCCACCTTCTGCAC
<i>Tph1</i>	GACCATCTCCGAGAGCTAAACAA	AGCAAAGGGAGGTTTCTGAGGTA
<i>Ddc</i>	TCTTCGCTTACTTCCCCACG	AGGAGAAACCAATGCAGCCA
<i>Maoa</i>	GGCTGTCATCAAGTGCATGG	CATGATGGCAGGCATTGACC
<i>Nr3c1 (GR)</i>	GGACCACCTCCCAAACCTCTG	ATTGTGCTGTCCTTCCACTG
$\beta$ -actin	GCAAGCAGGAGTACGATGAG	GTGTAAAACGCAGCTCAGTAACA
<i>Rplp0</i>	CCAGCAGGTGTTTGACAACG	TCCAGAAAGCGAGAGTGCAG