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Transcriptomic And Metabolomic Analyses Of Acetaminophen Exposure In Human Ipscs: Potential Mechanisms Explaining Epidemiological Associations Between Prenatal Acetaminophen Exposure And Increased Risk Of Autism Spectrum Disorder

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Transcriptomic and Metabolomic Analyses of Acetaminophen Exposure in Human iPSCs:
Potential Mechanisms Explaining Epidemiological Associations Between Prenatal
Acetaminophen Exposure and Increased Risk of Autism Spectrum Disorder

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May 2021
Master of Public Health, 2021
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Committee Members: Dr. Abha Gupta, M.D., Ph.D.
Abstract
Acetaminophen (APAP) is the most common analgesic taken during pregnancy. Indeed, approximately 65% of pregnant women use the drug. Recent epidemiological studies suggest that prenatal exposure to APAP is associated with increased risk of autism spectrum disorder (ASD). Although this neurodevelopmental disorder affects 1 in 54 children in the U.S., no studies have investigated the molecular mechanism by which APAP may increase the risk of ASD. In vitro experiments in cell cultures have shown that high concentrations of APAP induces cell death. Through the use of a systems-level approach in human induced Pluripotent Stem Cells (iPSCs), the present study investigates the mechanisms by which prenatal exposure to physiologically relevant doses of APAP could increase the risk of ASD. Initial experiments examined the concentration-dependence of APAP toxicity in human iPSCs exposed for 6 days, as estimated by changes in cell counts. Thereafter, RNA-sequencing and metabolomic analyses were conducted on cells exposed to therapeutic (0.16 mM) and toxic (0.32 and 0.48 mM) APAP concentrations for 6 days. APAP concentration-dependent decreases in expression for COBL, CYP2B6, FZD5, GPC4, NCALD and FGFBP3, and increases in expression for ATP1A2, PROS1, HES1 and NRIP1 were observed. These changes in gene expression are identical to those described in the ASD literature. Metabolomic analyses revealed differential regulation of metabolites involved in aminoacyl-tRNA, and valine, leucine and isoleucine biosynthesis, both of which have been linked to ASD. Our integration of transcriptomics and metabolomics data has revealed an impact of APAP on biological processes that appear to parallel those seen in ASD. In so doing, the results of the present study provide an important first step in elucidating the potential mechanisms by which prenatal exposure to APAP may increase the risk of ASD and promote adverse neurodevelopmental outcomes.
Acknowledgements

I would like to thank Dr. Vasilis Vasiliou for all of his guidance throughout my time as a research assistant in the V-Lab. His support and mentorship have led to me to develop research and interpersonal skills that will remain with me throughout my career. I would like to thank Dr. Abha Gupta for her patient and prudent advice throughout my thesis research. I also would like to thank Rolando Garcia-Milian and Dr. Zeyan Liew for their assistance with bioinformatics processing, and interpretation of the epidemiological evidence, respectively. Finally, I would like to thank my fellow lab members, Brian Thompson, Dr. Georgia Charkoftaki, Emily Davidson, Yewei Wang, and Dr. Ying Chen for their support and mentorship throughout various components of this research.
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Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APAP</td>
<td>N-acetyl-para-aminophenol; Acetaminophen</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism Spectrum Disorder</td>
</tr>
<tr>
<td>ATP1A2</td>
<td>ATPase Na+/K+ transporting subunit alpha 2</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched-Chain Amino Acid</td>
</tr>
<tr>
<td>CASP3</td>
<td>Caspase 3</td>
</tr>
<tr>
<td>CAST</td>
<td>Childhood Autism Spectrum Test</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>COBL</td>
<td>Cordon-bleu WH2 repeat protein</td>
</tr>
<tr>
<td>COL6A3</td>
<td>Collagen Type VI alpha 3 Chain</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Cytochrome P450 Family 2 subfamily B member 6</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DEGs</td>
<td>Differentially Expressed Genes</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNBC</td>
<td>Danish National Birth Cohort</td>
</tr>
<tr>
<td>DPBS or PBS</td>
<td>Dulbecco's Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>FC</td>
<td>Fold Change</td>
</tr>
<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
</tr>
<tr>
<td>FGFBP3</td>
<td>Fibroblast Growth Factor Binding Protein 3</td>
</tr>
<tr>
<td>FZD5</td>
<td>Frizzled Class Receptor 5</td>
</tr>
<tr>
<td>GPC4</td>
<td>Glypican 4</td>
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<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>HSP90</td>
<td>Heat Shock Protein 90</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>HIF1A</td>
<td>Hypoxia Induced Factor A</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cells</td>
</tr>
<tr>
<td>IQ</td>
<td>Intelligence Quotient</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal Kinases</td>
</tr>
<tr>
<td>LC50</td>
<td>Lethal Concentration 50%</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MAP-2</td>
<td>Microtubule-Associated Protein 2</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOBA</td>
<td>Mother and Child Cohort study</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>NAPQI</td>
<td>N-acetyl-4-benzoquinone imine</td>
</tr>
<tr>
<td>NCALD</td>
<td>Neurocalcin Delta</td>
</tr>
<tr>
<td>NIM</td>
<td>Neural Induction Medium</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural Progenitor Cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear Factor Erythroid 2-Related Factor 2</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PLSDA</td>
<td>Partial Least-Squares Discriminant Analysis</td>
</tr>
<tr>
<td>PROS1</td>
<td>Protein 1</td>
</tr>
<tr>
<td>PYGL</td>
<td>Glycogen phosphorylase L</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations Per Minute</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RYR2</td>
<td>Ryanodine Receptor 2</td>
</tr>
<tr>
<td>SDQ</td>
<td>Strengths and Difficulties Questionnaire</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>QTof</td>
<td>Quadrupole time-of-flight</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-Performance Liquid Chromatography</td>
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</table>
Introduction

Epidemiological Evidence

Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting 1 in 54 children in the U.S. (Maenner et al., 2020). Several epidemiological studies conducted in various countries within the last decade associate maternal prenatal use of acetaminophen (APAP) with increased risk of autism spectrum disorder (ASD) and ASD-linked symptoms, such as internalizing- and communication-related behavioral problems, in offspring (Brandlistuen et al., 2013; Liew et al., 2014; Avella-Garcia et al., 2016; Liew et al., 2016; Ji et al., 2020). These studies raise concerns that prenatal APAP exposure may interfere with optimal fetal brain development. Liew’s 2014 study using the Danish National Birth Cohort (DNBC) was among the first to analyze 64,322 mothers and children enrolled in DNBC with 11 years of follow-up and found maternal APAP use during pregnancy to be associated with Attention-Deficit/Hyperactivity Disorder (ADHD) (Liew et al., 2014). A following study by Liew et al. from 2016 found that maternal APAP use during pregnancy was associated with ASD, specifically among children who had comorbid hyperkinetic symptoms (Hazard Ratio (HR) = 1.51; 95% CI: 1.19-1.92) but not with other ASD cases (HR = 1.06; 95% CI: 0.92-1.24) (Liew et al., 2016). The risks were nearly 2-fold higher for frequent users among mothers who took APAP for more than 20 weeks during gestation. A Spanish birth cohort study associated the Childhood Autism Spectrum Tests (CAST) scores of 5-year-old males with prenatal exposure to APAP (Avella-Garcia et al., 2016). The study reported elevated CAST scores (β=0.63; CI: 0.09-1.18) among males prenatally exposed to APAP with effect size increasing as frequency of prenatal APAP use increased. The Norwegian Mother and Child Cohort study (MOBA) used a same sex sibling-pairs study design and revealed through sibling-control analysis that prenatal exposure to APAP for more than 28 days was associated with worse gross motor development (β=0.24; 95% CI: 0.12-0.51), communication (β=0.20; 95% CI: 0.01-0.39), externalizing behavior (β=0.28; 95% CI: 0.15-0.42), and internalizing behavior (β=0.14; 95% CI: 0.01-0.28) (Brandlistuen et al., 2013).

Prenatal APAP exposure has also been linked to increased risk of ADHD in offspring (Thompson et al., 2014; Ystrom et al., 2017). The Auckland Birthweight Collaborative Study used Strengths and Difficulties Questionnaire (SDQ) scores from parents and children to assess the relationship between prenatal APAP use and ADHD-like symptoms in 7–11-year-old
children (Thompson et al., 2014). Researchers found that prenatal exposure to APAP was significantly associated with total higher SDQ scores in all three iterations of the questionnaire when compared to children who were not prenatally exposed to APAP: parent SDQ at age 7 (OR = 2.1; 95% CI: 0.0, 5.0), parent SDQ at age 11 (OR = 1.2; 95% CI: 0.6, 2.5), and child SDQ at age 11 (OR = 1.0; 95% CI: 0.6, 1.6). Another study using the same MOBA cohort as Brandlistuen et al. examined the association between prenatal maternal use of APAP and risk of ADHD and found a modest positive association in all trimesters (1st trimester: HR = 1.07; 95% CI: 0.96–1.19; 2nd trimester: HR = 1.22; 95% CI: 1.07–1.38; 3rd trimester HR = 1.27; 95% CI: 0.99–1.63) (Ystrom et al., 2017). If maternal APAP use surpassed 29 days, HR rose to 2.20 (95% CI: 1.50–3.24); however, short-term use (< 8 days) was negatively associated with ADHD (HR = 0.90; 95% CI: 0.81–1.00).

The findings presented in these epidemiological studies appear to be specific to APAP as evidenced by the MOBA and the Auckland cohort which reported other painkillers, such as ibuprofen, to have no association with neurodevelopmental endpoints; the DNBC and Spanish studies adjusted for maternal use of aspirin or ibuprofen in all analyses. Furthermore, the associations between prenatal APAP use and behavioral problems appear to not be explained by unmeasured behavioral or social factors linked to APAP use (Stergiakouli et al., 2016). Hence, there is no evidence suggesting confounding.

A recent study from the Boston Birth Cohort found concentrations of biomarkers for fetal exposure to APAP to be associated with significantly higher risk of ASD and ADHD (Ji et al., 2020). The umbilical cord plasma of 996 mother-infant dyads (mean [standard deviation] age, 9.8 [3.9] years; 548 [55%] male) were tested for biomarkers of fetal exposure to APAP. The cohort included 257 children (25.8%) with ADHD only, 66 (6.6%) with ASD only, 42 (4.2%) with both ADHD and ASD, 304 (30.5%) with other developmental disabilities, and 327 (32.8%) who were neurotypical. Increased APAP biomarker concentrations were associated with significantly higher risks of ASD and ADHD. Umbilical cord APAP biomarker concentrations were stratified into three tertiles. Compared to the first tertile (lowest concentration of APAP biomarkers), placement in the second and third tertiles of cord APAP burden was associated with higher odds of ASD diagnosis (Odds Ratio (OR) for second tertile, 2.14; 95% CI, 0.93-5.13; OR for third tertile, 3.62; 95% CI, 1.62-8.60). While these findings are significant, they should be interpreted cautiously due to the geographically limited demographics of the study. Given the
research interests and expertise of the present study’s author and advisors, prenatal APAP exposure will be explored here with regards to ASD as the primary outcome of concern, while ADHD is mentioned here to hopefully inspire future research or prompt interpretation of the following results in the context of ADHD. Ultimately, prenatal exposure to APAP is associated with adverse neurodevelopment while the underlying biological mechanisms linking APAP and neurodevelopment remain unknown (Bauer et al., 2018). Given that APAP is the most commonly consumed medication during pregnancy with approximately 65% of pregnant women taking the drug, exploring the association between prenatal exposure to APAP and adverse neurodevelopment is of critical public health importance (Werler et al., 2005).

**Exposure Route and Assessment**

With ASD displaying a complex—and still unknown—etiology, it is necessary to describe the fetal APAP exposure route to understand a possible risk factor which may lead to the repeatedly observed association between prenatal exposure and increased risk of ASD. After ingestion, APAP, the active drug, is circulated in blood plasma with an 88% oral bioavailability (Wishart et al., 2018). The drug is primarily metabolized in the liver, as well as in the intestine and kidneys to a lesser extent (Mazaleuskaya et al., 2015). In therapeutic doses, APAP’s half-life in plasma ranges from 1.5 to 2.5 hours (McGill and Jaeschke, 2013). Importantly, it is documented that APAP can cross the placenta and the fetal blood-brain barrier (Levy et al., 1975; Horowitz et al., 1997; Nitsche et al., 2017). Suggested mechanisms for APAP’s potential influence on fetal development and neurodevelopment include APAP-induced oxidative stress from its major metabolite produced in the liver, N-acetyl-p-benzoquinone imine (NAQPI), or endocrine effects (Albert et al., 2013; Mazaud-Guittot et al., 2013; Kristensen et al., 2016; Liew and Ernst, 2021), as well as repression of angiogenesis and vascular remodeling genes in trophoblast (Burman et al., 2020). Ultimately, the risk fetal APAP exposure poses to developmental health remains largely unknown. That, in combination with the aforementioned prenatal vulnerability to APAP exposure, has prompted some researchers to push for the reconsideration of the safety and dosage of APAP during pregnancy (Thiele et al., 2013).

**Previous Cell-based Findings**
In various cell-based studies, iPSCs and iPSC-derived cells have been exposed to APAP and found that APAP exposure often leads to cell death. The main difference between the following studies lies in the concentration of APAP used and dosing timeframes. A study exposing developing mouse cortical neurons found that 10–250 μg/ml (0.066-1.65 mM) concentrations of APAP were not toxic; however, all tested concentrations (1-100 μg/ml) of p-aminophenol, a metabolite of APAP, resulted in significant loss of neuronal viability over a 24-hour period (Schultz et al., 2012). Another study also tested the effects of APAP on rat cortical neurons. Researchers found that APAP causes direct concentration-dependent neuronal apoptosis in vitro at concentrations of APAP observed in human plasma during APAP overdose (1 mM and 2 mM) (Posadas et al., 2010). While both cortical neuron models find toxic effects related to APAP, the former study finds no toxic effect of the parent compound while the latter determines equivalent concentrations to be toxic.

A study using human mesenchymal stem cells found high concentrations of APAP (7.94 mM) decreased cell viability, as determined by MTT assay, and induced apoptosis via the caspase-9/-3 signaling pathway, but not at 0.794 mM APAP (Yiang et al., 2015). In another study where APAP served as the negative control for testing chemicals with known neurodegenerative effects, 24-hour treatments with 0.1-100 μM APAP was shown to have no significant effects on neurite count and neurite length in human-iPSC-derived GABAergic and glutamatergic neurons (Cohen and Tanaka, 2018). The upper end of the APAP concentrations used in this study are analogous to the lowest concentration used in the present study (0.16 mM), and thus brings into question the effects of APAP exposure that are not immediately measurable through cytotoxic assays. Finally, a study examining the effects of 5 and 10 mM concentrations of APAP on human neural cells found that the lower concentration increased Jun N-terminal Kinases (JNK), hypoxia induced factor A (HIF1A), and Caspase 3 (CASP3), which indicate apoptotic cell death (Aleksandrova et al., 2016). Neuronal cells exposed to 10 mM APAP showed reduced expression of heat shock protein 90 (HSP90), which indicates activation of Caspase-3-independent mechanism of cell death.

Collectively, these cell models exposed to APAP demonstrate induced cell death often via apoptosis, and examine the effects of acute, high-concentration APAP exposures. While neuropathological evidence suggests that activation of apoptosis during development may be
mechanistically involved in the pathophysiology of ASD, other factors—some of which may remain unelucidated—are likely to be involved (Wei et al., 2014).

In the context of the recent epidemiological findings, these cell-based studies have little relevance nor relation to clinical implications on understanding potential mechanisms leading to an association between prenatal APAP exposure and ASD. Many of the concentrations tested on these cell models exceed what a pregnant woman would be exposing her fetus to after APAP ingestion. In reality, prenatal APAP exposure is more likely to be low in dose and long-lasting, say during chronic pain. This perspective is supported by the epidemiological studies that found greater effect sizes in their associations between prenatal APAP exposure and adverse neurodevelopmental outcomes as frequency of APAP consumption increased (Avella-Garcia et al., 2016; Ystrom et al., 2017). Therefore, examining more long-term, low concentration APAP exposures in cell models is more relevant to clinical practices and could be useful in understanding the potential mechanisms contributing to the development of the affected offspring; however, such low concentrations of APAP are unlikely to result in measurable changes of toxic markers, if any. Hence, there is a need to understand the effects of low-concentration APAP exposure not with regards to cellular toxicity, but rather more subtle, nuanced gene expression and metabolic changes in cells, to which a systems approach lends itself well.

A study using a developing human neural stem cell line derived from umbilical cord blood found that exposure to APAP at concentrations lower than 1 mM had no toxic effects and did not alter expression profiles of neuronal, astroglial and oligodendroglial markers, such as β-tubulin III, microtubule-associated protein 2 (MAP-2), and S100β (Buzanska et al., 2009). However, this study used a 48-hour exposure period, potentially too brief to detect any changes and to represent a chronic exposure as is needed in modeling APAP’s potential role in ASD. This study also uses a targeted approach which limits its discussion to predetermined readouts that cannot be interpreted with regards to ASD.

Systems Approach

Recent considerations to the importance of metabolites with regards to phenotype have prompted researchers to recognize the metabolome’s importance in linking the genotype and phenotype (Johnson et al., 2016; Zampieri and Sauer, 2017; Handakumbura et al., 2019). The
metabolome encompasses all the small biomolecules, typically less than 1500 Da in size, that comprises the energetic, structural and functional building blocks of all life (Harrison et al., 2020). Understanding the effects APAP exposure can have at the genomic, proteomic and metabolomic levels may elucidate potential mechanisms or pathways that could help explain the observed association between prenatal APAP exposure and increased risk of ASD. Such a systems approach, one in which data from various omics levels is integrated to improve our understanding of their interrelation and combined influence, has been recognized to be most applicable to complex diseases, such as ASD (Ahn et al., 2006). Additionally, a human iPSC line is an appropriate model for early developmental exposure to APAP and the resulting transcriptional and metabolic changes that result from APAP exposure (Bar and Benvenisty, 2020). Inspired by epidemiological associations, the present study is an attempt at proposing potential mechanisms that could serve as initial grounds for considering the previous studies’ exposure-outcome relation causally. To our knowledge, this is the first study that attempts to holistically examine the effects of APAP on iPSCs through a multi-omics, systems-level approach, and the work presented here establishes a model and methodology by which other compounds of concern can be assessed with regards to effects on development.

**Hypothesis**

We hypothesize that the association between prenatal exposure to APAP and increased risk of ASD in offspring can partially be explained through alterations in genetic and metabolic expressions. APAP exposure may significantly upregulate or downregulate genes, metabolites and pathways that have been associated with ASD in published literature.

**Methods**

*Y6 induced Pluripotent Stem Cell Line*

The Yale Stem Cell Center provided a line of induced Pluripotent Stem Cells (iPSCs), termed the Y6 line, with confirmed pluripotency derived from fibroblasts from a healthy, phenotypically normal, female donor without any family history of disease. Working with this cell line did not require approval by the Yale University Institutional Review Board as the line was previously established and de-identified; additionally, there was no interaction with human
subjects for this study. An iPSC line was well suited for our purposes of modeling prenatal exposure due to its high proliferation rate and its variably inducible genetic profile.

*Maintenance of iPSCs*

iPSCs were plated in 6-well plates (Corning® Costar®) coated in a 1:59 (v/v) mixture of Corning® Matrigel® and DMEM/F-12. Cells were fed every other day with StemCell Technologies mTeSR™1 Basal Media with 5X supplement, a serum-free, complete cell culture medium. Cells were passaged with the use of Dispase on a weekly basis once differentiated cells were manually removed via scraping in order to maintain a pluripotent line. iPSCs were incubated at 37 °C and 5% CO₂.

*Stock APAP Solution*

0.1889 g of APAP (Sigma-Aldrich) were dissolved in 500 µl of dimethyl sulfoxide (DMSO) to create a 2.5 M APAP solution, which is half the maximum concentration of APAP in DMSO to ensure none of the APAP crystalized once in solution. A sample of the highest concentration used in the concentration response curve, 1.28 mM of APAP (8 times higher than the lowest concentration) in mTeSR with 0.0512% DMSO (v/v), was prepared and examined under a microscope to ensure the complete solubility of APAP.

*Concentration Response*

An initial dilution was performed with the 2.5 M APAP in DMSO stock solution by adding mTeSR media to yield 1.28 mM of APAP in mTeSR with 0.0512% (v/v) DMSO. From this initial concentration, a serial dilution was performed by adding 0.0512% (v/v) DMSO in mTeSR media to obtain 0.96 mM, 0.64 mM, 0.48 mM, 0.32 mM, and 0.16 mM concentrations of APAP in mTeSR each with a constant 0.0512% (v/v) concentration of DMSO. The 0.16 mM APAP concentration corresponds to the upper end of the therapeutic concentration of APAP (10-25 mcg/ml) or the recommended maximum daily dosage in adults of 4 g of APAP per day (Table 1) (Winek et al., 2001; Healthcare, 2010). A vehicle control was also prepared by exposing cells to 0.0512% (v/v) DMSO in mTeSR media.

Before exposing the cells to APAP-containing media, iPSC colonies in a 6-well plate were incubated in 500 µl of Accutase® Cell Detachment Solution for 20 minutes to generate a single cell suspension that was then diluted with DMEM/F12 in a 50 ml falcon tube. The diluted single cell suspension was centrifuged at 800 rpm for 8 minutes, the supernatant was discarded, and the pellet was resuspended in mTeSR media. A 100 µl aliquot was created from which two
10 µl samples of single cell suspension were taken for counting via hemocytometer. Cell concentrations were determined and a sufficient aliquot of cells from the 50 ml falcon tube was taken and diluted such that 15 ml of cell suspension were obtained at a concentration of 100,000 cells/ml. Aliquots of cell suspension (500 µl) were then placed into each well of a 24-well plate (Corning® Costar®) coated in Corning® Matrigel® at a density of 500,000 cells per well.

After one day of incubation, triads of wells were dosed with one of the 5 APAP concentrations or the vehicle control for 6 days with daily media changes. A 6-day dosing period was utilized to model an exposure more biologically relevant to a pregnant woman taking APAP during a period of chronic pain or over the course of gestation. This 6-day approach also allows our assays to detect long-term transcriptional changes that may be occurring. Similar multi-day, chronic exposures of APAP dissolved in low concentrations of DMSO in media (0.1% v/v) have been utilized before in cell models for up to 10-day dosing regimens (Foster et al., 2019).

At the end of the 6-day period, cell morphology images were captured in bright-field on a ZEISS Axio Vert.A1 microscope with a ZEISS Axiocam 105 color camera attachment and processed on ZEISS ZEN (Blue Edition 2.3) (Figure 1). Cell counts for each well were determined by treating wells with Accutase, and two aliquots per well were diluted with AOPI Staining Solution and placed on a Cellometer slide before total cell counts were assayed using the Nexcelom Bioscience Cellometer K2. Total cell counts were used as wells were rinsed twice to remove any dead cells before adding Accutase. Therefore, any dead cell counts present in the Cellometer slide are due to Accutase exposure rather than APAP exposure. Assaying APAP concentration-dependent cell death, as measured by cell count, is an appropriate measure of cellular phenotypic changes relevant to ASD because APAP exposure leads to glutathione (GSH) depletion, which in turn leads to apoptosis, one of the major biological processes implicated in the etiology of ASD (Franco and Cidlowski, 2009; Wei et al., 2014; Mazaleuskaya et al., 2015; Manivasagam et al., 2020). Average cell counts for each well were plotted against APAP concentrations in R Studio (Version 1.2.5033) and fit to a concentration-response curve (Figure 2) using the Analysis of Dose Response Curves (drc) package with the non-linear function (fct) set to five-parameter log-logistic model (Ritz and Streibig, 2005; Ritz et al., 2015). The Lethal Concentration 50% (LC50) was determined based off the concentration response curve, and 0.16 mM, 0.32 mM, and 0.48 mM APAP were selected for further testing as they all represent the
concentrations around the inflection point of the concentration-response curve while the former is also representative of therapeutic concentrations.

Table 1: APAP concentration equivalents in mM and mcg/ml with equivalent of therapeutic concentration listed as a multiple.

<table>
<thead>
<tr>
<th>APAP Concentration [mM]</th>
<th>APAP Concentration [mcg/ml]</th>
<th>Equivalent of therapeutic concentration</th>
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<tbody>
<tr>
<td>0.16</td>
<td>25</td>
<td>1X</td>
</tr>
<tr>
<td>0.32</td>
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</tr>
</tbody>
</table>

**RNA Extraction**

The same 6-day dosing procedure, used in the concentration-response experiment, was used in 6-well plates, originally plated with 100,000 cells/well with dosing beginning the day after passage once wells had reached 5-10% confluency. Wells were treated in triplicate with either 0.16 mM, 0.32 mM or 0.48 mM APAP in mTeSR with 0.0512% DMSO (v/v) and three wells were treated with only 0.0512% (v/v) DMSO in mTeSR media as a vehicle control. At the end of the dosing period, wells were rinsed and treated with Accutase as to break up the colonies into single cell suspensions. Cells were then rinsed twice in ice cold Dulbecco's Phosphate-Buffered Saline (PBS) and centrifuged at 800 g and 4°C for 8 minutes. PBS was aspirated and the cell pellets were flash frozen in liquid nitrogen before storage at -80°C. About two weeks later, the cell pellets were retrieved from cold storage and thawed on ice and RNA extracted with the RNeasy Mini Kit (QIAGEN) following the manufacturer’s protocol with cell type-specific modifications including: the homogenization of samples via a 1 minute vortex, and an on-column DNase digestion was performed prior to RNA elution. The purity and quantity of eluted RNA were initially assessed with a ND-1000 spectrophotometer (NanoDrop). Only RNA samples with 260/230 and 260/280 of 2 ± 0.3 were stored in -80°C for sequencing.

**RNA Sequencing**

RNA samples were submitted to the Yale Center for Genome Analysis for RNA sequencing. First, the RNA integrity number (RIN) was determined with the Agilent 2100 Bioanalyzer RNA 6000 Pico assay. Only RNA samples with an RIN ≥ 9.5 were used for sequencing. Polyadenylated RNA was purified from the RNA samples using oligo-dT beads.
followed by random priming. 25 million pairwise 100 base pair reads were generated with NovaSeq S2. Due to an insufficient number of reads produced by the initial sequencing run, a second sequencing run was performed.

**RNA-seq Analysis**

The raw reads (fastq.gz files) were uploaded into the Galaxy web platform (PMID: 27137889) for data processing. The reads from the two sequencing runs were concatenated using the Concatenate datasets tail-to-head tool (Galaxy Version 1.0.0). Low quality reads (phred ≤ 20) and ambiguous bases (N) were removed with the Trimmomatic tool (Bolger et al., 2014) (galaxy version 0.38.0). HISAT2 (Kim et al., 2015) (galaxy version 2.1.0+galaxy7) was used to map the trimmed reads to the *Homo sapiens* reference genome (hg19). Mapped reads were counted using the featureCounts tool (Liao et al., 2014) (galaxy version 2.0.1). Differentially expressed genes and a multivariate principal component analysis (PCA) were identified and created, respectively, with DESeq2 (Love et al., 2014) (galaxy version 2.11.40.6+galaxy1) (Figure 3). Differentially expressed genes (DEGs) were initially classified as ≥ ±0.5 log2 fold change (log2(FC), KO/CON) and adjusted p-value < 0.05 (Benjamini-Hochberg method) (Benjamini and Hochberg, 1995). DESeq2 was also used to generate normalized counts. Volcano plots indicating DEGs (blue, significantly downregulated; grey, non-significant; red, significantly upregulated) were generated using ggplot2 (Galaxy Version 0.0.3) (Figure 4).

**RNA-seq Bioinformatic Analysis**

DEGs were then analyzed with Ingenuity Pathway Analysis (IPA) (Krämer et al., 2014). Normalized counts of all genes from each sample were uploaded to Qlucore Omics Explorer (Qlucore AB, Lund, Sweden) to produce 3D PCA plots, box plots (p-values are adjusted by Benjamini Hotchberg method) (Figure 5), a hierarchical clustered heatmap (Appendix 1) and to perform gene set enrichment analysis (GSEA) (Subramanian et al., 2005). GSEA score plots were produced with FDR adjusted p-values (Figure 6).

**Proteomics Sample Preparation**

At the end of the 6-day dosing period, 6-well plates treated in triplicate with the same dosing regimen as in the RNA extraction procedure were rinsed with DMEM/F12 as to remove any dead cells before incubating with Dispase to detach cells from the Matrigel coating. Cell colonies from each well were transferred to their respective 15 ml falcon tubes and rinsed with DMEM/F12 twice. Cell pellets were suspended in ice cold DPBS with complete protease and...
phosphatase inhibitors. The suspension was then centrifuged at 15,000 rpm and 4°C for 15 minutes before the supernatant was discarded. This rinse was repeated twice more for a total of three washes. Cell pellets were then stored at -80°C until submission to the Yale Keck Biotechnology Research Laboratory Proteomics department for label free quantification analysis. Proteomics analysis will proceed if the present study’s results indicate a need to understand the steps between gene expression and metabolite production via protein expression analysis, and if so, once additional funding is acquired.

**Metabolomics Sample Preparation**

Wells were prepared and dosed on 6-well plates in the same manner as for RNA extraction and proteomics, however, for metabolomics sample preparation, each experimental and control group consisted of 8 wells, with an additional 3 wells containing the matrigel coating and 0.0512% DMSO in mTeSR media (v/v) without any cells to serve as extraction blanks. At the end of the 6-day dosing period, as described by Charkoftaki et al., wells were washed with ice cold PBS twice before quenching and metabolite extraction with 1 ml of cold ACN:MeOH:H2O (2:2:1 v/v) to cover the entire well (Charkoftaki et al., 2019). Cells were then harvested by gentle scraping and collected in Protein LoBind® Eppendorf tubes. Cell suspensions were then subject to three freeze-thaw cycles (freezing in liquid nitrogen for 1 minute, thawing at room temperature for 5 minutes) followed by 10 seconds of vortexing between each cycle. Quenched cell suspensions were then centrifuged at 15,000 rpm and 4°C for 15 minutes and the supernatant was transferred to a new 2 ml tube. Aliquoted supernatant samples were then dried overnight in a Speed Vac (SC210A Savant, Thermo Fisher Scientific) at room temperature before storage at -80°C. Cell pellet protein levels were quantified through Branford protein assay in order to perform sample-specific normalization for protein when reconstituting metabolites.

Metabolite samples were reconstituted in ACN:H2O (1:1 v/v), vortexed, and centrifuged at 15,000 rpm and 4°C for 15 minutes. 90 µl of the reconstituted sample was transferred into 2mL vials with glass inserts (Waters Corporation) and 10 µl aliquots from each sample were pooled to generate Quality Control (QC) samples. Additionally, extraction blanks were reconstituted, pooled, and analyzed alongside experimental samples to account for background metabolites. All experimental samples were randomized and analyzed by ultra-performance liquid chromatography (UPLC)-electrospray ionization quadrupole time-of-flight (QToF) mass
spectrometry (Xevo G2-XS QTof, Waters Corporation, Milford, Massachusetts) in negative mode. QC samples were analyzed every 5 injections.

**Metabolomics Analysis**

The .raw Waters files were converted to MzMine through MSConvert before peak deconvolution and identification processing was performed in MZmine. Resulting mass-to-charge ratio (m/z) features were then exported for the purposes of analyzing unidentified m/z features, which will henceforth be referred to as the unidentified approach, while the same original m/z features were compared against an internal reference library to yield tentatively identified metabolites, yielding an approach that will be referenced to as the identified approach. Both unidentified and identified datasets were then subjected to blank filtering to eliminate background m/z features and metabolites, and QC checks were done with a threshold cutoff of 30% so that the data was truncated to peaks with less than 30% missing values. Metabolites from the identified approach were identified with Level 2 chemical identification confidence (probable structure determined via comparison to library standards retention time (rt) and m/z features).

The significant level 2 metabolites were then analyzed in MetaboAnalyst 5.0 where quantile normalization of the dataset was performed along with glog data transformation and mean centered data scaling. The latter normalization is necessary in order to detect variances in the lower-concentration metabolites which is necessary due to the present study’s untargeted approach. After normalization, MetaboAnalyst was used for exploratory analysis to produce PCA plots (Figures 7A and B). PCA analysis revealed tight clustering of the QCs, but also two potential outliers (one in the 0.48 mM APAP experimental group and one in the DMSO control group). QC and extraction blank checks were visually performed in each plot and reassured that reliability of our methods through the QC’s tight clustering and the extraction blanks distinct separation. QCs, extraction blanks, and outliers were not taken into account in subsequent analyses. MetaboAnalyst was also used to generate pairwise Partial Least-Squares Discriminant Analysis (PLSDA) plots (Figure 8), a biased or forced method of separating groups that gives heavier weight to the metabolites that separate the groups the best. A one-way ANOVA analysis of identified metabolites was then completed in Quilcore to yield the concentration dependent metabolites that are differentially regulated as a result of APAP treatment (Table 2). Quilcore was also used to generate pairwise comparison Dendrogram heatmaps or hierarchical clustering analyses of significant (p < 0.05, log2(FC) > 1.5) differentially regulated metabolites (Figure 9).
**Enrichment Analysis**

All identified metabolites (n=36) from the untargeted approach were uploaded to MetaboAnalyst’s enrichment analysis annotation feature, and those that matched to MetaboAnalyst’s internal reference system during ID standardization (n=34) were compared to the 84 metabolite sets from KEGG human metabolic pathways through an over representation analysis to generate Figure 10. Metabolite sets were included in the enrichment analysis if at least 2 uploaded metabolites matched to that set.

**Integrated Pathway Analysis**

A pathway analysis was performed using IPA to integrate the results from RNA-seq and metabolomics with consideration to how they connect with ASD and related functions and diseases. Significant differentially regulated genes and metabolites from the pairwise comparisons (Figure 4 and 9, respectively) were compiled along with their p-value, FDR p-value, also known as q-value, and Log2(FC) into one common spreadsheet, which was then uploaded to IPA with a core expression analysis based on Log2(FC). The analysis was run with the reference set selected as Ingenuity Knowledge Base (Genes + Endogenous Chemicals). The resulting top 8 significant canonical pathways are displayed along with their Fisher’s Exact Test p-value (Figure 11). A comparison analysis was then conducted to find the differentially expressed genes and metabolites that overlapped with molecules associated with ASD in IPA. These common molecules were used to generate a preliminary network of diseases found to be significantly associated with these molecules (Fisher’s Exact Test p-value < 0.001). The resulting network was built out with biological functions significantly related to the original genes and diseases. As additional functions were added to the pathway analysis, additional differentially expressed genes and metabolites were also added (Figure 12).

**Results**

**Concentration Response**

Bright-field images of iPSCs taken in 24-well plates after 6 days of dosing (Figure 1). Images taken at 20X magnification. Apoptotic and necrotic blebs increase as APAP concentration increases. This has been confirmed through visual inspection of the iPSC colonies through the microscope lens without the camera attachment. Apoptotic blebs are typically indicated by cellular fragments smaller than the original cell, whereas necrosis leads to
inflammation and the swelling of cells. Therefore, necrotic blebs are larger than the original cell. Higher concentrations of APAP also reduce the frequency of visual markers of differentiation, especially on the edges of cell colonies.

Figure 1: Microscope slides of iPSCs at all 5 experimental concentrations (0.16 mM, 0.32 mM, 0.48 mM, 0.64 mM, 0.96 mM, and 1.28 mM) and vehicle control (0.0512% DMSO v/v) after 6-day dosing regimen. Images taken in bright-field at 20X magnification.

The 6-day concentration response curve created with the drc R package (Figure 2) graphs the Log of APAP concentrations against the percentage of total cell count as compared to vehicle treated control wells. This concentration response curve also defines the LC50 to be around 0.60 mM APAP. Cell counts are unaffected at therapeutic concentrations (0.16 mM) of APAP, but are drastically reduced at the highest APAP concentration (1.28 mM) to the point that the few cells remaining in the well (Figure 1) were counted as zero by the Cellometer once dilutions were performed. With the LC50, a fundamental value, defined, subsequent experiments focused on the concentrations below the LC50 that did not induce excessive cell death in order to assess the nuanced effects of APAP exposure on iPSCs through a multi-omics approach.
**Figure 2:** Concentration-response curve after 6 days of exposure created with the drc package in R with fct =LL.5(). Graph displays Log APAP concentrations versus iPSC count as a percentage of the DMSO vehicle-treated controls, mean of three independent wells.

**RNA-sequencing**

With the experimental APAP concentrations of interest defined via the concentration-response experiment, RNA-sequencing was conducted to determine the transcriptomic effects of low-concentration, chronic APAP exposures on iPSCs. In order to assess the performance of the RNA-seq in identifying distinct expression patterns between the DMSO control cells and the three experimental groups, PCA was completed (Figure 3). PCA is an unbiased comparison method that reduces the dimensions of the data under consideration to explain a certain amount of variability. Tight clustering and overlap of the controls, as well as the three treatment groups, apart from 0.16 mM, are observed along with general separation between groups. Principal component (PC) 1 explains 46% of the variance between samples, while PC2 explains 23%. Regarding the three treatment groups, there is quite a bit of variance observed on PC2, especially for the 0.48 mM group; however, this variance is negligible given the high percentage of variance explained by PC1.
Moving from the multivariate analysis of all groups (Figure 3) to a univariate analysis of the pairwise comparison between each experimental group and the DMSO control, volcano plots were produced and demonstrate the significantly upregulated and downregulated genes after APAP exposure (Figure 4). While few genes are significantly dysregulated due to the log2(FC) cutoff, as APAP concentration increases, more genes are found to be differentially expressed, suggesting a concentration-response relationship between APAP exposure and gene expression. Two genes in particular, hes family bHLH transcription factor 1 (HES1), which influences the maintenance of stem cells and plays a role in the nervous system, and nuclear receptor interacting protein 1 (NRIP1), which modulates transcriptional activity of the estrogen receptor, are significantly upregulated in the pairwise comparisons of DMSO with 0.32 mM APAP and 0.48 mM APAP (Figure 4B and C).
Figure 4: Volcano plots of differentially expressed genes from each pairwise comparison between APAP concentrations and DMSO control. (A) 0.16 mM APAP compared with DMSO control. (B) 0.32 mM APAP compared with DMSO control. (C) 0.48 APAP compared with DMSO control. P-values are Benjamini-Hochberg adjusted. Colors: blue, significantly downregulated (log2FC ≤ −0.5 and -log10(p-value) ≤ 0.05); grey, non-significant (log2FC − 0.5 ≥ and ≤ log2FC + 0.5 and -log10(p-value) ≤ 0.05; red, significantly upregulated (log2FC ≥ 0.5 and -log10(p-value) ≤ 0.05)

The 3D PCA plots of Figure 5A and B generated in Quircore offer a different perspective than Figure 3 on the clustering of the genes expressed by the various groups of iPSCs when a third component is added. Figure 5A shows the 3D PCA of the RNA-seq dataset after negligible genes (counts < 0.1 for at least 20% of samples) were removed, leaving 18,172 genes for analysis. Figure 5B plots the results of the one-way ANOVA that further filters the RNA-seq dataset to 917 genes (FDR p < 0.05) that separate the 4 groups well. To examine if any genes may be demonstrating concentration-response relationships similar to what was generally observed in the univariate volcano plots (Figure 4), the same methods are used to calculate the Benjamini-Hochberg p-values for each pairwise comparison; however, the results were not limited to any constraints based on significant FC values (log2(FC) = 0.5). The treatment groups were plotted against feature counts and visualized with boxplots (Figure 5C). Figure 5Ci-x correspond to the following genes which demonstrate concentration-response relationships, and their functions in humans: i) Cordon-bleu WH2 repeat protein (COBL) plays a role in reorganization of actin cytoskeleton, regulates neuron morphogenesis, and increases branching of axons and dendrites; ii) cytochrome P450 family 2 subfamily B member 6 (CYP2B6) is involved in drug metabolism; iii) ATPase Na+/K+ transporting subunit alpha 2 (ATPIA2) is one of the integral membrane proteins responsible for maintaining the electrochemical gradients of sodium and potassium across plasma membranes; iv) frizzled class receptor 5 (FZD5) encodes for transmembrane domain proteins that function as receptors for Wnt signaling proteins; v) Furnary 25
Glypican 4 (GPC4) codes for cell surface heparan sulfate proteoglycans that may be involved in cell division and growth regulation; vi) Neurocalcin delta (NCALD) codes for a member of the neuronal calcium sensor family of calcium-binding proteins; vii) Fibroblast growth factor binding protein 3 (FGFBP3) a chaperone that binds and mobilizes paracrine FGFs; viii) Protein 1 (PROS1) which encodes a vitamin K-dependent protein in plasma to function as a cofactor to anticoagulant protease; ix) HES1; and x) NRIP1. Each of these genes display an approximately linear upregulation or downregulation in response to APAP exposure. The observation that APAP exposure at different concentrations leads to differential gene expression is echoed by the heatmap of RNA-seq feature counts displayed in Appendix 1.
Figure 5: Analysis of RNA sequencing normalized counts. A) 3D PCA plot after removing genes with counts < 0.1 for at least 20% of samples with variance filtering, 18,172 genes were removed of the 25,702 original genes. B) One-way ANOVA of 917 genes (FDR p < 0.05) that separate the 4 treatment groups. C) Concentration-response box plots for genes, Benjamini-Hochberg adjusted p-values displayed for each pairwise comparison: i) COBL, ii) CYP2B6, iii) ATP1A2, iv) FZD5, v) GPC4, vi) NCALD, vii) FGFBP3, viii) PROS1, ix) HES1, and x) NRIPI. Samples are represented by dots. Colors: DMSO, turquoise; 0.16 mM APAP, red; 0.32 APAP, green; 0.48 APAP, blue.

While the dysregulation of certain genes is pertinent to understanding how APAP exposure may affect gene expression in iPSCs, GSEA in Quicore was completed to find upregulated and downregulated pathways as determined by differences in the groups of genes in each control-experimental pairwise comparison. GSEA is a non-parametric computational...
methodology that elucidates whether an a priori set of genes corresponding to a particular gene ontology shows statistically significant and concordant differences between the two biological groups being compared, the DMSO control and the APAP exposed experimental groups. The resulting GSEA score curves with the lowest FDR p-values for functions that pertain to neurodevelopment—and other processes of interest—provide graphical representation of the enrichment score for each pairwise comparison between the DMSO control and experimental group gene sets (Figure 6). Figures 6Ai-viii display the gene ontologies with the lowest FDR p-values (0.13 < q < 0.20) for the GSEA of the pairwise comparison between the DMSO control and 0.16 mM experimental groups: i) Modulation of excitatory postsynaptic potential, ii) Hindbrain morphogenesis, iii) Dendrite morphogenesis, iv) Solute sodium symporter activity, v) Anion cation symporter activity, vi) Neuron neuron synaptic transmission, vii) Cerebral cortex morphogenesis, and viii) Neurotransmitter transport, all of which show downregulation. Figures 6Bi-vi show the gene ontologies with the lowest FDR p-values (0.32 < q < 0.35) for the GSEA of the pairwise comparison between the DMSO control and 0.32 mM experimental groups: i) Meiotic chromosome segregation, ii) Protein localization to chromosome, iii) Protein sumoylation, iv) Ubiquitin like protein specific protease activity, v) Cerebral cortex development, and vi) Brain morphogenesis, all of which showed upregulation. Figure 6Ci-vi display the gene ontologies with the lowest FDR p-values (0.25 < q < 0.25) for the GSEA of the pairwise comparison between DMSO control and 0.48 mM experimental groups: i) Cytosolic large ribosomal subunit, ii) Positive regulation of neuron death, iii) Positive regulation of developmental growth, iv) Growth factor binding, v) Astrocyte development, and vi) Axonal growth cone, all of which demonstrated downregulation. This broader approach allows for the dysregulation of groups of genes to be interpreted with regards to more general gene ontology functions.
Figure 6: GSEA score curves providing graphical representation of the enrichment score for each pairwise comparison between the DMSO control and experimental group gene sets. Presented score curves represent gene ontologies with the lowest FDR p-values relative to each pairwise comparison. Selected score curves are those with evidently interpretable score plots (i.e. nonlinear trends or inflections towards one edge of the plot) that pertain to neurodevelopment. A) Gene ontologies with the lowest FDR p-values (0.13 < q < 0.20) for the GSEA of the pairwise comparison between the DMSO control and 0.16 mM experimental groups, i) Modulation of excitatory postsynaptic potential (q = 0.1302) showing downregulation, ii) Hindbrain morphogenesis (q = 0.1359) showing downregulation, iii) Dendrite morphogenesis (q = 0.1363) showing downregulation, iv) Solute sodium symporter activity (q = 0.1394) showing downregulation, v) Anion cation symporter activity (q = 0.1429) showing downregulation, vi) Neuron neuron synaptic transmission (q = 0.1576) showing downregulation, vii) Cerebral cortex morphogenesis (q = 0.1619) showing downregulation, and viii) Neurotransmitter transport (q = 0.1630) showing downregulation. B) Gene ontologies with the lowest FDR p-values (0.32 < q < 0.35) for the GSEA of the pairwise comparison between the DMSO control and 0.32 mM experimental groups, i) Meiotic chromosome segregation (q = 0.3238) showing upregulation, ii) Protein localization to chromosome (q = 0.3253) showing upregulation, iii) Protein sumoylation (q = 0.3265) showing upregulation, iv) Ubiquitin like protein specific protease activity (q = 0.3298) showing upregulation, v) Cerebral cortex development (q = 0.3309) showing upregulation, vi) Brain morphogenesis (q = 0.3373) showing upregulation. C) Gene ontologies with the lowest FDR p-values (0.25 < q < 0.25) for the GSEA of the pairwise comparison between DMSO control and 0.48 mM experimental groups, i) Cytosolic large ribosomal subunit (q = 0.2439) displaying downregulation, ii) Positive regulation of neuron death (q = 0.2448) showing downregulation, iii) Positive regulation of developmental growth (q = 0.2469) showing downregulation, iv) Growth factor binding (q = 0.2475) showing downregulation, v) Astrocyte development (q = 0.2476) showing downregulation, and vi) Axonal growth cone (q = 0.2483) showing downregulation.

Metabolomics

PCA plots generated with the identified metabolites in MetaboAnalyst show clustered QCs and extraction blanks grouping away from the control and treatment groups (Figure 7A) indicating that the methods used in both extraction and analysis are sound. With QCs and extraction blanks removed, two outliers could be visually identified in the PCA plot, one from the 0.48 mM APAP experimental group and one in the DMSO control group. These outliers were removed and the PCA plot was reconstructed with the remaining 30 samples (Figure 7B). In this PCA plot, PC1 explains approximately 39% of the variance while PC2 explains 22% of the observed variance. While there is not a large amount of separation between groups in this
PCA plot, the small amounts of variance between groups will be examined more closely through pairwise comparisons later on. Of the 34 level 2 identified metabolites, one-way ANOVA revealed 15 to be significantly differentially expressed as a result of APAP treatment (Table 2).

![PCA plots generated through MetaboAnalyst for identified metabolites from iPSCs A) with QC samples and extraction blanks, and B) and without QC samples and extraction blanks. Both plots exclude the two outliers. Extraction blanks (labeled EB) are represented by light blue diamonds, QCs by yellow squares, and DMSO controls represented with pink circles, while 0.16 mM, 0.32 mM, and 0.48 mM treatment groups are denoted with red, green, and blue circles, respectively.](image)

**Table 2:** One-way ANOVA showing differentially regulated, level 2 confidence (m/z, rt) identified metabolites with FDR $p < 0.05$. Metabolites listed in order of ascending FDR $p$-value.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>p-value</th>
<th>FDR p-value</th>
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<tbody>
<tr>
<td>ARABITOL</td>
<td>6.1e-06</td>
<td>2.2e-04</td>
</tr>
<tr>
<td>L-GULONOLACTONE</td>
<td>5.5e-05</td>
<td>9.8e-04</td>
</tr>
<tr>
<td>1-HYDROXY-2-NAPHTHOATE</td>
<td>2.1e-04</td>
<td>2.5e-03</td>
</tr>
<tr>
<td>METHYL VANILLATE</td>
<td>7.3e-04</td>
<td>4.4e-03</td>
</tr>
<tr>
<td>PYRIDOXINE</td>
<td>7.1e-04</td>
<td>4.4e-03</td>
</tr>
<tr>
<td>RIBOFLAVIN</td>
<td>5.0e-04</td>
<td>4.4e-03</td>
</tr>
<tr>
<td>INOSINE</td>
<td>1.1e-03</td>
<td>5.9e-03</td>
</tr>
<tr>
<td>5-HYDROXYINDOLEACETATE</td>
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<td>1.7e-02</td>
</tr>
<tr>
<td>DEOXYGUANOSINE</td>
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<td>1.7e-02</td>
</tr>
<tr>
<td>INOSINE MONOPHOSPHATE</td>
<td>5.0e-03</td>
<td>1.7e-02</td>
</tr>
<tr>
<td>TRYPTOPHAN</td>
<td>4.5e-03</td>
<td>1.7e-02</td>
</tr>
<tr>
<td>THYMIDINE-MONOPHOSPHATE</td>
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<td>1.8e-02</td>
</tr>
<tr>
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<tr>
<td>GUANOSINE</td>
<td>1.1e-02</td>
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</tr>
<tr>
<td>THYMIDINE</td>
<td>1.1e-02</td>
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</table>
To represent the metabolic differences between the DMSO vehicle control group and treatment groups, PLSDA plots were used to visually display the biased or forced separation between groups of the pairwise comparisons (Figures 8A-C). Ultimately, these plots suggest that there are some metabolic features that, when weighted more heavily than others, can drive separation between groups; however, there is not complete separation as the nodes slightly overlap. These plots give some indication that at each APAP treatment concentration—0.16 mM, 0.32 mM, and 0.48 mM—there are metabolic features that differentiate the exposed groups from the vehicle control.

![Figure 8: PLSDA plots of pairwise comparisons for level 2 identified metabolites extracted from iPSCs. A) DMSO control vs 0.16 mM APAP B) DMSO control vs 0.32 mM APAP C) DMSO control vs 0.48 mM APAP. Colors: DMSO, pink circles; 0.16 mM APAP, red; 0.32 APAP, green; 0.48 APAP, blue.](image)

In order to understand the exact metabolites that may be driving the differences in the pairwise PLSDA plots (Figure 8), pairwise hierarchical clustering analyses were performed to identify differentially expressed metabolites (p < 0.05, log2(FC) > 1.5) control-treatment comparison (Figures 9A-C). Comparing the DMSO control with metabolites from iPSCs exposed to 0.16 mM APAP (Figure 9A) reveals isoleucine and 5-hydroxyindoleacetate to be significantly upregulated while N-acetylglycine, tyrosine, deoxyguanosine and L-gulonolactone to be significantly downregulated. The pairwise comparison between the control and 0.32 mM APAP treated groups (Figure 9B) also reveals 5-hydroxyindoleacetate to be upregulated along with inosine. Again, N-acetylglycine, tyrosine, deoxyguanosine and L-gulonolactone were found to be downregulated as well as galactitol and 4-acetamidobutanoate. The dendograms of both Figures 9A and B confirm the separate clustering of upregulated and downregulated metabolites. Finally, comparing metabolites from the DMSO controls with those form the 0.48 mM treated cells revealed both galactitol and arabitol to be significantly downregulated.
Figure 9: Hierarchical clustering analysis of significant (p < 0.05, log2(FC) > 1.5) metabolites; A) DMSO control vs 0.16 mM APAP, B) DMSO control vs 0.32 mM APAP, C) DMSO control vs 0.48 mM APAP.

Similar to the GSEA approach used as a broader approach of assessing the RNA-seq data (Figure 6), the 34 level 2 identified metabolites were used in an over representation analysis in MetaboAnalyst’s enrichment analysis annotation feature to reveal metabolite sets significantly related to the metabolites identified through our approach (Figure 10). Aminoacyl-tRNA synthesis and valine, leucine and isoleucine biosynthesis metabolite sets were found to have the most significant p-values (p < 0.0001) of the enriched metabolite sets, while the latter had the greater enrichment ratio of the two.
Figure 10: Enrichment results from MetaboAnalyst using the 34 level 2 confidence metabolites extracted from iPSCs that matched to MetaboAnalyst’s internal reference system. Overview of the top 25 enrichment metabolite sets with position along the x-axis indicating -log(p-value), color indicating p-value, and circle size indicating the enrichment ratio of each metabolite set.

Pathway Analysis

To integrate the results from RNA-seq and metabolomics, IPA was used to perform pathway analysis. The differentially expressed genes and metabolites previously identified (Figures 4 and 9) were analyzed through a core expression analysis to yield significant canonical pathways, the top 8 of which are displayed in Figure 11 (Fisher’s exact test p-value < 0.01). The canonical pathway with the lowest Fisher’s exact test p-value is nuclear factor erythroid 2-related factor 2 (NRF2) mediated oxidative stress response, which induces the expression of antioxidants and cytoprotective genes (Vomund et al., 2017). Another canonical pathway of interest is serotonin receptor signaling as it plays an important role during early development of the brain (Yang et al., 2014). The pathway analysis with prediction overlaid (Figure 12) highlights key diseases and biological functions for further consideration. Each arrow of Figure 12 indicates a significant relationship between the connected features supported by findings in published literature. Three differentially expressed genes from our analysis (Collagen type VI alpha 3 chain (COL6A3), Ryanodine receptor 2 (RYR2), and Doublecortin (DCX)) overlapped with genes and metabolites from IPA’s ASD reference set and demonstrated significant connections with three diseases of interest: ASD or intellectual disability, cognitive impairment, and glucose metabolism disorder. Functions elucidated from the pathway analysis (Fisher’s exact test (right tailed) p-value) include: proliferation of neuronal cells (2.98e-04), growth of neurites
(1.08e-03), growth of axons (1.95e-03), dendritic growth/branching (6.81e-04), branching of neurites (1.08e-05), morphogenesis of neurons (6.83e-05), and quantity of neurons (6.17e-05).

Figure 11: Top 8 significant canonical pathways related to genes and metabolites found to be differentially expressed as a result of APAP exposure. -log(p-value) for Fisher’s Exact Test graphically displayed. Grey bars indicate that there is no activity pattern available identified in IPA, despite highly significant association of the genes and metabolites within the pathway, white bars indicate a z-score of zero. Threshold, indicated by the vertical yellow line, set to -log(1.33).
Figure 12: Pathway analysis results from IPA with prediction overlayed. Octagons correspond to biological functions; crosses correspond to diseases; the one oval represents the metabolite inosine; the remaining shapes correspond to certain classes of proteins: Ellipse: transcription regulator; rectangle: G-protein coupled receptor; diamond: enzyme; square: growth factor; and circle: other. Each arrow indicates a significant relationship between the connected features supported by findings in published literature. Genes and metabolites present in the diagram are those found to be differentially regulated as a result of APAP treatment (Figures 4 and 10) that also connect to the diseases and biological functions of interest. The differentially expressed genes and metabolites from our analysis that overlapped with genes and metabolites from IPA’s ASD reference set (COL6A3, RYR2, and DCX) have significant connections with the three diseases of interest: ASD or intellectual disability, Cognitive impairment, and Glucose metabolism disorder. Fisher’s Exact Test (right tailed) p-values for functions clockwise from top: Proliferation of neuronal cells = 2.98e-04, Growth of neurites = 1.08e-03, Growth of axons = 1.95e-03, Dendritic growth/branching = 6.81e-04, Branching of neurites = 1.08e-05, Morphogenesis of neurons = 6.83e-05, and Quantity of neurons = 6.17e-05.

Discussion

To our knowledge, this is the first study that holistically examines the effects of APAP on iPSCs through a multi-omics approach as a model for prenatal exposure. The results of the present study provide an important first step in elucidating the potential mechanisms by which prenatal exposure to APAP may increase the risk of ASD and promote adverse
neurodevelopmental outcomes. iPSCs chronically exposed to low-concentrations of APAP demonstrate concentration-dependent decreases in expression for COBL, CYP2B6, FZD5, GPC4, NCALD and FGFBP3, and increases in expression for ATP1A2, PROS1, HES1 and NRIP1. These changes in gene expression are identical to those described in the ASD literature. Metabolomic analyses revealed differential regulation of metabolites involved in aminoacyl-tRNA, and valine, leucine and isoleucine biosynthesis, both of which have been linked to ASD. Integrating significantly differentially expressed genes and metabolites in IPA revealed several functions and pathways closely related to neurodevelopment. Ultimately, the present study’s integration of transcriptomics and metabolomics data has revealed an impact of APAP on biological processes that appear to parallel those seen in ASD.

Taken together, the concentration response curve and cell images presented here indicate that iPSCs chronically exposed to high concentrations of APAP (1.28 mM) experience large amounts of cell death, while the cells treated with sub-LC50 concentrations of APAP appear to experience less of a toxic response to APAP. This sub-toxic exposure is of critical importance in the present study as the iPSC model employed is intended to represent prenatal exposure. It is highly unlikely that a pregnant woman ingests an amount of APAP that leads to toxic or lethal plasma concentrations similar to those that have been shown to cause cell death in the aforementioned cell-based studies discussed in the introduction. Therefore, the 0.16 mM, 0.32 mM and 0.48 mM concentrations of APAP were chosen for chronic exposure experiments in order to elucidate the sub-toxic effects of APAP as they may relate to ASD. However, given the high cellular proliferation rates of the iPSC model, these chronic, low-concentration exposure methods likely lead to minor, insignificant alterations in the transcriptome and metabolome. This is likely the reason why few differentially regulated genes were observed in the univariate analysis volcano plots (Figure 4). Still, there is an observable effect due to the APAP treatment as the number of differentially regulated genes increased in an approximately concentration-response manner as APAP concentrations increased.

In order to understand nuanced differential expression that may be occurring within the non-significant, grey region of the volcano plots (Figure 4) our analysis focused on broader trends observed as APAP concentrations increased or on methods such GSEA and metabolite enrichment analysis. For example, log2(FC) thresholds were omitted from consideration when creating the concentration-response relationships of differentially expressed genes with
significant Benjamini-Hochberg p-values (p < 0.05) in Figure 5C. This concession is necessary in order to assess the differences between APAP concentrations representative of therapeutic and toxic doses, which only vary from one another by at most a factor of 3 (Table 1).

Interestingly, each concentration-response relationship in the gene expression of iPSCs exposed to APAP (Figure 5C) displays similar trends to what is observed in the literature regarding the same genes and ASD. For example, the deletion of COBL has been shown to cause defects in neuronal cytoskeleton morphogenesis in model vertebrates and has been implicated in case-control ASD datasets as a novel candidate gene in ASD-associated pathways (Griswold et al., 2012). This deletion can be viewed as analogous to the downregulation of COBL due to APAP exposure (Figure 5Ci). APAP has been shown to downregulate CYP2B6, which matches the concentration-response relationship in Figure 5Cii (Yu et al., 2018), and in a cohort of Thai children and adolescents with ASD, several single nucleotide polymorphisms (SNPs) in CYP2B6 were found to be clinically relevant to ASD (Medhasi et al., 2016); however, there is no indication as to what effect, if any, these patients’ SNPs have on the expression of CYP2B6. ATP1A2 has been found to be upregulated in a mouse model for ASD as it is in Figure 5Ciii (Mychasiuk and Rho, 2017). FZD5 deletion has been observed in patients with ASD and is downregulated in Figure 5Civ (Roberts et al., 2014). Decreased expression of GPC4 has been linked to ASD and is also downregulated in Figure 5Cv (Dowling and Allen, 2018). Allelic expression imbalance of NCALD caused by SNPs has been associated with ASD, and thus somewhat matches the downregulation of NCALD due to APAP exposure (Figure 5Cvi) (Ben-David et al., 2011). FGFBP3 has been identified as a novel candidate gene for ASD (Figure 5Cvii) (Salyakina et al., 2011). PROS1 has been shown to be upregulated in children with ASD compared to typically developing children, which mirrors the upregulation in Figure 5Cviii (Qin et al., 2017). Dysregulation of HES1 has been shown to contribute to the etiology of ASD as the gene is important in the differentiation and maintenance of neural progenitor cells (Figure 5Cix) (Ghahramani Seno et al., 2011). Finally, dysregulation of NRIP1 has been linked to ASD through network functional analysis stemming from data on ASD cases (Figure 5Cx) (Cirnigliaro et al., 2017). Taken all together, the fact that the observed concentration-response relationships in Figure 5C consistently support previous observations in the literature of genetic dysregulations suggests that prenatal exposure to APAP may be associated with increased risk of ASD by acting on various combinations of these 10 genes and potentially other APAP-inducible genes.
GSEA was anticipated to be an incredibly beneficial tool for our analysis as it does not employ any cutoffs in its methodology, which theoretically would lend itself well to our RNA-seq data as few genes were significantly differentially regulated at the lower APAP treatments (Figure 4A and B). The GSEA score curves implicate gene ontologies relevant to ASD, such as dendrite morphogenesis (Figure 6Aiii), neuron neuron synaptic transmission (Figure 6Avi), cerebral cortex development (Figure 6Bv), and positive regulation of neuron death (Figure 6Cii); however, the GSEA is greatly underpowered, as indicated by the insignificant q-values of each plot. As a result, the GSEA score curves may be displaying general upregulation in all gene ontologies in the pairwise comparisons of the DMSO control group with the 0.16 mM and 0.48 mM treatment groups (Figures 6A and C), and downregulations in all gene ontologies from the DMSO control and 0.32 mM comparison group (Figure 6B). For example, two very similar gene ontologies, cerebral cortex morphogenesis (Figure 6Ai) and cerebral cortex development (Figure 6Bv), are respectively upregulated and downregulated in their score curves which conflicts with the general concentration-response relationships observed in the results. Therefore, while the GSEAs yield interesting gene ontologies for discussion, the present limitations restrain our discussion to speculation only rather than implications.

While there were significant differences in metabolic expression between the DMSO control and experimental groups due to APAP treatment (Table 2, Figure 9), these differentially regulated metabolites do not appear to play a major role in elucidating potential mechanisms that help delineate the association between prenatal exposure to APAP and increased risk of ASD as few metabolites are incorporated into the pathway analyses (Figures 11 and 12). This is likely due in part to the low power of the present study’s methods originating from the low APAP concentrations and small differences in APAP concentrations between experimental groups. However, the still developing metabolomics research on ASD may also explain the difficulty in relating differentially expressed metabolites to ASD. Metabolomics research regarding ASD has great potential and has been progressing steadily, but it is not yet robust in its identification of metabolic mechanisms (Ruggeri et al., 2014; Shen et al., 2020).

Still, APAP treatment was driving differences in metabolic expression profiles to some degree (Figure 8). Therefore, enrichment analysis in MetaboAnalyst was used to conduct over representation analysis in the level 2 confidence metabolites in order to elucidate the metabolic sets that the identified metabolites are involved in. This approach is very similar to that of GSEA.
(Figure 6), but appears to have generated more selective results (Figure 10). One of the most significant metabolite sets, aminoacyl-tRNA synthesis, has been identified via a cross-sectional case-control study as a modified canonical pathway and predictive element of ASD (Rangel-Huerta et al., 2019). Significantly lower aminoacyl-tRNA levels were observed in the brain of mice with Shank3 knockout, a leading candidate gene for ASD, compared to control mice (Torossian et al., 2021). Finally, an in silico approach found that, by constructing fetal and adult cortex specific protein-protein interactions, ASD-associated SNPs had an impact on 5 canonical pathways including aminoacyl-tRNA synthesis (Golovina et al., 2021). Another significant metabolite set, valine, leucine and isoleucine biosynthesis, also known as branched-chain amino acid (BCAA) synthesis, has been linked to ASD through the Children’s Autism Metabolome Project. The study identified different groups of amino acids that were negatively correlated with BCAs in ASD cases (Smith et al., 2019). Additionally, deficient levels of BCAAs have been reported in children with ASD (Novarino et al., 2012). Finally, the same cross-sectional case-control study referenced earlier found possible alterations of BCAA metabolism in ASD (Rangel-Huerta et al., 2019). Ultimately, over representation analysis of the level 2 identified metabolites highlights two significant and enriched metabolite sets that relate to ASD.

Analyzing the differentially expressed genes and metabolites concurrently with IPA via core expression analysis led to two canonical pathways of particular interest, NRF2 mediated oxidative stress, and serotonin receptor signaling. NRF2 mediated oxidative stress has been observed to be downregulated in the monocytes of individuals with ASD and show suppressed response to stimulation with lipopolysaccharides (Nadeem et al., 2020). Considering the downregulation of NRF2 associated with ASD, NRF2 activators are being considered as therapeutic interventions for those with ASD (Yang et al., 2020). Regarding serotonin signaling, developmental disruptions of this canonical pathway are shown to be involved in ASD while deficiency of serotonergic system leads to mis-wiring of neural networks (Yang et al., 2014). Therefore, the genes and metabolites that are differentially expressed as a result of chronic, low-concentration APAP exposure in iPSCs are highly involved in two canonical pathways with direct implications to ASD.

While IPA did not have enough power from the three differentially expressed genes that overlapped with the tool’s internal, ASD-associated genes and metabolites (COL6A3, RYR2, and DCX) to predict more diseases and functions, other differentially expressed genes and one
metabolite, inosine, were included in Figure 12 through the addition of biological functions relevant to ASD. These biological functions show both predicted activation and inhibition of processes relating to neuronal, axonal, and dendritic growth and maturation. This mixture of activation and inhibition of functions related to neuron development imply that some of these functions may relate more to quantitative aspects of neurons while others may relate more to qualitative factors. For the most part, the genes in Figure 12 are predicted to have decreased measurements, or be downregulated, which is an atypical response for genes after exposure to a xenobiotic such as APAP. Inosine, the one metabolite in Figure 12, is predicted to be upregulated. Currently, only one study based in Italy finds a significant association between inosine metabolite levels and ASD, observing that children with ASD secrete significantly higher urinary concentrations of inosine (Gevi et al., 2016).

The three diseases initially identified by connections between the three overlapping genes were ASD or intellectual disability, cognitive impairment, and glucose metabolism disorder. It should be noted that recent in silico research found carbohydrate metabolism to be involved in the etiology of ASD when prenatal exposure to APAP and pesticides are considered in tandem (Furnary et al., 2021). Ultimately, the pathway analysis presented in Figure 12 attempts to connect the differentially expressed genes and metabolites with the identified diseases and selected functions of interest to offer insight into potential mechanisms that may partially explain the association between prenatal APAP exposure and increased risk of ASD. Taken together, these enrichment analyses both suggest how the findings relate to the published literature on ASD, as evidenced by the aforementioned studies, and may also elucidate areas or new perspectives for future research. The present study’s results, when interpreted collectively, have built a case for further research examining the potential mechanism by which prenatal exposure to APAP may lead to increased risk of ASD and adverse neurodevelopment as the epidemiological evidence suggests (Bauer et al., 2018).

Some limitations of the present study include the insignificant results due to the chosen low APAP concentrations, as previously discussed; however, these concentrations are most representative of real-life exposures and therefore justify their use in the present study. Another limitation of this study’s design is the 6-day dosing. As the metabolomics data shows, there may be a loss of information due to the prolonged dosing period given that the highest concentration of APAP is most similar to the vehicle control group (Figure 7B). This unexpected result may be
due to higher concentrations of APAP lending themselves to a more immediate shock in the exposed iPSCs, which then die off leaving relatively fewer viable cells that may also carry greater resistance to the APAP exposure. Thus, throughout the remainder of the 6-day dosing period, the higher concentration wells may continue to be populated with APAP-tolerant iPSCs that have similar metabolic responses to the APAP as the control cells have to the vehicle DMSO treatment. Another limitation of this study is the fact that an in vitro iPSC model is being used to infer the effects of prenatal APAP exposure at the in vivo level. Therefore, it is yet to be determined how APAP exposure affects the developing fetus in vivo.

A few strengths of the present study are the use of a multi-omics approach, which is justified given the epidemiological associations pertaining to APAP-ingestion at therapeutic levels in combination with toxic adverse effects of APAP observed in cellular and animal models at high concentrations. Hence, it is reasonable to deduce that the effects of prenatal exposure to therapeutic levels of APAP are much more nuanced and require detailed techniques to understand, to which the omics-level approach lends itself well. This study is also novel as it exemplifies several steps that can be taken to understand the effects of certain exposures at concentrations that are much more physiologically relevant, such as increasing the dosing period and ensuring that concentration-response relationships align with clinically relevant exposure concentrations.

Future work stemming from the findings of this research include manually improving metabolite identification confidence levels to Level 1 via incorporation of MS2 features and comparison with internal C13 reference standards. Future analyses may also include performing proteomics analysis on the prepared samples to give a more holistic perspective of the central dogma as it relates to APAP exposure in iPSC model. Because many of the results presented here are insignificant, potentially other methods for evaluating cellular responses are needed, or potentially a different cell type that can better model fetal exposure to APAP needs to be explored. It should be noted that the present research has also served as a template for conducting similar assessments but with neuronal progenitor cells (NPCs) which the author initially planned to use in this study; however, the differentiation required to develop such a model was delayed due to the COVID-19 pandemic and is currently being pursued for future research. The multi-omics methods of this paper, combined with the NPC model currently under development, can also serve as an effective toot by which other chemicals of
neurodevelopmental concern may be assessed, such as ortho-phthalates, in order to prompt policy reforms (Engel et al., 2021).

Future research and clinical implications stemming from the methods employed in this study could include developing iPSC lines from individual patient samples for precision medicine. Such techniques would allow health care workers to understand before fertilization how individual mother-offspring dyads may be affected by APAP exposure during gestation or how ASD cases consider treatment options (Genova et al., 2018).
References


Appendix 1: Heatmap of RNA-seq feature counts with negligible genes (counts $< 0.1$ in at least 20% of samples) removed via variance filtering and the most significant genes identified through one-way ANOVA, resulting in 917 genes (FDR $p < 0.05$) displayed with hierarchical clustering of Gene IDs.