

Prenatal acetaminophen exposure as a risk factor for Attention Deficit Hyperactivity Disorder
(ADHD): underlying mechanisms in humans and mice

Brennan H. Baker

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
under the Executive Committee
of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2022

© 2022

Brennan H. Baker

All Rights Reserved

Abstract

Prenatal acetaminophen exposure as a risk factor for Attention Deficit Hyperactivity Disorder (ADHD): underlying mechanisms in humans and mice

Brennan H. Baker

Despite evidence of an association between prenatal acetaminophen exposure and attention deficit hyperactivity disorder (ADHD) in offspring, the causal role of prenatal acetaminophen exposure in child ADHD remains unclear owing to limitations of prior studies. Prior studies have relied on maternal self-report, failed to quantify acetaminophen dose, and lacked mechanistic insight. Chapter 1 formally introduces this topic and provides background information summarizing the high prevalence of ADHD, widespread use of acetaminophen during pregnancy, and potential molecular mechanisms through which the drug may harm fetal development.

In Chapter 2, we examined the association between prenatal acetaminophen exposure measured in meconium and ADHD in children aged 6 to 7 years, along with the potential for mediation by functional brain connectivity. Data came from a prospective birth cohort study from the Centre Hospitalier Université de Sherbrooke in Sherbrooke, Québec, Canada. We included 393 eligible children, of whom 345 had meconium samples collected at delivery and information on ADHD diagnosis. Mothers were enrolled from September 25, 2007, to September 10, 2009, at their first prenatal care visit or delivery. Acetaminophen levels were measured in meconium, and physician diagnosis of ADHD was determined at follow-up when children were

aged 6 to 7 years or from medical records. Additionally, when children were aged 9 to 11 years, resting-state brain connectivity was assessed with magnetic resonance imaging, and attention problems and hyperactivity were assessed with the Behavioral Assessment System for Children Parent Report Scale. Associations between meconium acetaminophen levels and outcomes were estimated with linear and logistic regressions weighted on the inverse probability of treatment to account for potential confounders. Causal mediation analysis was used to test for mediation of the association between prenatal acetaminophen exposure and hyperactivity by resting-state brain connectivity. Among the 345 children included in the analysis (177 boys [51.3%]; mean [SD] age, 6.58 [0.54] years), acetaminophen was detected in 199 meconium samples (57.7%), and ADHD was diagnosed in 33 children (9.6%). Compared with no acetaminophen, detection of acetaminophen in meconium was associated with increased odds of ADHD (odds ratio [OR], 2.43; 95%CI, 1.41-4.21). A dose-response association was detected; each doubling of exposure increased the odds of ADHD by 10% (OR, 1.10; 95%CI, 1.02-1.19). Children with acetaminophen detected in meconium showed increased negative connectivity between frontoparietal and default mode network nodes to clusters in the sensorimotor cortices, which mediated an indirect effect on increased child hyperactivity (14%; 95%CI, 1%-26%).

In Chapter 3, we used data from the same Canadian birth cohort to examine whether prenatal acetaminophen exposure is associated with adverse birth outcomes and/or pregnancy complications, and if birth outcomes may mediate the association of prenatal acetaminophen with child ADHD. This study included 393 children for whom acetaminophen was measured in meconium at delivery. We tested associations of prenatal acetaminophen with birthweight, preterm birth, gestational age, small and large for gestational age, gestational diabetes, preeclampsia, and high blood pressure. Using causal mediation analyses, we assessed whether

birth outcomes mediated the association of prenatal acetaminophen with ADHD. We imputed missing data via multiple imputation and used inverse probability weighting to account for confounding and selection bias. Prenatal acetaminophen exposure was associated with decreased birthweight by 136 g ($\beta = -136$; 95% CI [-229, -43]), 20% increased weekly hazard of delivery (hazard ratio = 1.20; 95% CI [1.00, 1.43]), and over 60% decreased odds of being born large for gestational age (odds ratio = 0.38; 95% CI [0.20, 0.75]). Prenatal acetaminophen was not associated with small for gestational age, preterm birth, or any pregnancy complications. Causal mediation effects were non-significant for all birth outcomes in both unadjusted and adjusted models, indicating no evidence that birth outcomes linked prenatal acetaminophen exposure with child ADHD.

In Chapter 4, we examined the effects of developmental acetaminophen exposure on mouse behavior and frontal cortex gene expression. Although prior studies have investigated neurodevelopmental effects of prenatal acetaminophen exposure in rodents, the results of these studies are not always in agreement. Additionally, no mouse studies of prenatal acetaminophen exposure have investigated offspring attention deficits in behavior tasks specifically designed to measure attention, and no prior rodent studies have utilized ‘omics’ technologies for an untargeted exploration of potential mechanisms. We randomly assigned pregnant mice (starting embryonic day 4-10) to receive acetaminophen (150 mg/kg/day) or vehicle control through postnatal day 14. We employed a battery of behavior tests for 111 mouse offspring, including pup ultrasonic vocalizations, elevated plus maze, open field test, CatWalk, pre-pulse inhibition, and 5-choice serial reaction time task. Frontal cortex was collected at birth from 24 pups for RNA-sequencing. Developmental acetaminophen treatment resulted in increased pup vocalizations after separation from the litter, as well as decreased ambulation and vertical

rearing in the open field task among male but not female offspring. Acetaminophen treatment was also associated with altered frontal cortex gene expression relating to glutathione and cytochrome p450 metabolism, DNA damage, and the endocrine and immune systems.

Together with the multitude of other cohort studies showing adverse neurodevelopment associated with prenatal acetaminophen exposure, this work suggests caution should be used in administering acetaminophen during pregnancy. In humans, we found that prenatal acetaminophen exposure was associated with child ADHD, altered resting-state brain connectivity, and adverse birth outcomes. Furthermore, our results suggest altered brain connectivity as a potential underlying mechanism linking prenatal acetaminophen use with child hyperactivity. While adverse birth outcomes such as preterm birth and reduced birthweight are known to be associated with ADHD, we found no evidence for mediation by birth outcomes of the association between prenatal acetaminophen exposure and ADHD. In mice, we found that developmental acetaminophen treatment resulted in elevated anxiety-like behaviors in male offspring, as well as gene expression changes in the frontal cortex. Future studies are needed to explore whether the altered molecular pathways revealed by RNA-sequencing directly link acetaminophen exposure with offspring behavior changes.

Table of Contents

List of Abbreviations	iii
List of Tables and Figures.....	v
Acknowledgments.....	viii
Chapter 1: Preface.....	1
Chapter 2: Association of Prenatal Acetaminophen Measured in Meconium with Risk of Attention-Deficit Hyperactivity Disorder (ADHD): Mediation by Frontoparietal Network Brain Connectivity	6
2.1 Introduction.....	7
2.2 Methods.....	9
2.3 Results.....	14
2.4 Discussion	15
2.5 Conclusions.....	18
2.6 Tables	20
2.7 Figures.....	24
2.8 Supplement	27
Chapter 3: Association of Prenatal Acetaminophen Exposure Measured in Meconium with Adverse Birth Outcomes in a Canadian Birth Cohort	36
3.1 Introduction.....	37
3.2 Methods.....	39
3.3 Results.....	43
3.4 Discussion	45

3.5 Conclusions.....	48
3.6 Tables.....	49
3.7 Figures.....	54
3.8 Supplement	55
Chapter 4: Sex-Specific Neurobehavioral and Frontal Cortex Gene Expression Alterations	
Following Developmental Acetaminophen Exposure in Mice.....	62
4.1 Introduction.....	63
4.2 Methods.....	65
4.3 Results.....	71
4.4 Discussion.....	74
4.5 Tables.....	80
4.6 Figures.....	81
4.7 Supplement	88
Conclusion	134
References.....	144

List of Abbreviations

5CSRTT	5-Choice Serial Reaction Time Task
ACME	Average causal mediation effect\
ADE	Average direct effect
ADHD	Attention-Deficit Hyperactivity Disorder
APAP	N-acetyl-p-aminophenol
ASD	Autism Spectrum Disorder
BASC3-PRS	Behavioral Assessment System for Children Parent Report Scale
BMI	Body mass index
CHUS	Centre Hospitalier Universitaire de Sherbrooke
CI	Confidence interval
CYP	Cytochrome P450
dB	Decibel
DMN	Default mode network
EGSEA	Ensemble of gene set enrichment analysis
EPM	Elevated plus maze
FDA	Food and Drug Administration
FDR	False discovery rate
fMRI	Functional magnetic resonance imaging
FP	Frontoparietal
GESTE	GESTation and the Environment cohort
GST	Glutathione S-transferase
HR	Hazard ratio
IPW	Inverse probability weighting
KEGG	Kyoto encyclopedia of genes and genomes
LGA	Large for gestational age
LOD	Limit of detection
LOQ	Limit of quantification
MICE	Multivariate imputation by chained equations
MSigDB	Molecular signatures database
NAPQI	N-acetyl-p-benzoquinoneimine
NCE	Negative control exposure
OFT	Open field test
OR	Odds ratio
PPI	Pre-pulse inhibition
RNA	Ribonucleic acid
ROI	Region of interest
SD	Standard deviation
SGA	Small for gestational age

SMFM

Society for Maternal-Fetal Medicine

USV

Ultrasonic vocalizations

List of Tables and Figures

Chapter 2

Table 1: Characteristics of study population stratified by meconium acetaminophen exposure in the GESTation and the Environment (GESTE) cohort.....	20
Table 2: Associations of meconium acetaminophen with child ADHD (whole study sample, N = 345)	22
Table 3. Functional Connectivity Differences between Exposed and Unexposed Groups	23
Figure 1. Differences in resting state functional connectivity related to prenatal acetaminophen exposure	24
Figure 2: Causal mediation by connectivity between the frontoparietal network and right precentral/fontal gyrus	25
eTable 1. Differences in demographic and head motion variables across exposed and unexposed children with MRI data	30
eTable 2: Characteristics of study population stratified by meconium acetaminophen weighted on measured covariates in the GESTation and the Environment (GESTE) cohort	31
eTable 3. Associations between resting state connectivity and hyperactivity and attention problems.....	32
eTable 4: Causal mediation by connectivity between the frontoparietal network and right precentral/fontal gyrus of the relationship between meconium acetaminophen and hyperactivity	33
eFigure 1. Non-linear association of meconium acetaminophen concentration with ADHD	34

Chapter 3

Table 1: Characteristics of study sample stratified by detection of acetaminophen in meconium in the GESTation and the Environment (GESTE) cohort (n = 393).....	49
Table 2: Association of meconium acetaminophen with birth outcomes.....	51
Table 3: Associations of meconium acetaminophen with pregnancy complications (N=393)	52
Table 4: Analysis of mediation of the association between prenatal acetaminophen and ADHD by birth outcomes (N = 345).....	53
Figure 1: GESTation and the Environment (GESTE) cohort flowchart.....	54
eTable 1: Characteristics of study sample stratified by meconium collection in the GESTation and the Environment (GESTE) cohort (n = 810).....	55
eTable 2: E-value sensitivity analysis for unmeasured confounding.....	57
eTable 3: Covariate adjusted analysis of mediation of the association between prenatal acetaminophen and ADHD by birth outcomes in 10 datasets imputed for missing covariates (N = 345).....	58

Chapter 4

Table 1: Acetaminophen treatment over control fold change for significantly differentially expressed genes.....	80
Figure 1: Pup Ultrasonic Vocalizations (USV)	81
Figure 2: Open Field Test (OFT).....	82
Figure 3: CatWalk.....	83
Figure 4: Elevated plus maze (EPM)	84
Figure 5: Pre-pulse inhibition (PPI).....	85
Figure 6: 5-Choice Serial Reaction Time Task (5CSRRT)	86

Figure 7: Ensemble of Gene Set Enrichment Analysis (EGSEA) 87

eTable 1: Beta estimates from linear regression of APAP versus control treatment on individual
CatWalk parameters 88

eTable 2: Top 20 APAP over control enrichments from EGSEA for all mice and stratified by sex
..... 111

Acknowledgments

I would like to express my deepest appreciation for my advisors, Drs. Andrea A. Baccarelli and Brandon L. Pearson, without whom my success and the completion of my dissertation would be impossible. Following my passion for the study of epigenetics and the developmental origins of health and disease, I chose the Mailman School of Public Health for my PhD in the hopes of working with Andrea, and he has certainly delivered. Beyond benefiting from his research experience and expertise, I have learned so much from Andrea in the realms of grant writing, lab management, presentation skills, and conducting impactful research. Brandon has been an invaluable mentor and friend. Whether he's working at the lab bench with students or in his office devising an ambitious research agenda, Brandon displays a contagious passion for science that I hope to emulate in my career. I've learned from Brandon that we *should* be ambitious: even when our goals seem out of reach, we can always find the experts or learn the skills we need. I am also grateful to the members of my defense committee, Drs. Julie B. Herbstman, Jonathan Posner, and Shanna H. Swan, for kindly offering their expertise, advice, and time towards the review and improvement of my dissertation.

I am extremely grateful to numerous collaborators, mentors, and colleagues for their help with my dissertation and beyond. In particular, many thanks to Jonathan Posner for his mentorship on Chapter 2 of this dissertation and on two other manuscripts. Jonathan helped to foster many of my collaborations and always exhibited a deft mentorship style that I will strive for in my future career. I am grateful to Larissa Takser and all the GESTE cohort researchers, without whom the birth cohort studies in Chapter's 2 and 3 would be impossible. The mouse behavior studies in Chapter 4 would not be possible without the kind help of Mu Yang and the technicians in the Mouse NeuroBehavior Core. I have learned so much from Howie Wu, who

was always around to answer my statistics, epidemiology, and programming questions. I would also like to extend my gratitude to every member of the Baccarelli and Pearson labs for your support over the years. Thanks to many other co-authors and colleagues, including Claudia Lugo-Candelas, Heather Burris, Hannah Laue, Allison Kupsco, Tamara Sussman, Jiok Cha, and Yoonjung Yoonie Joo.

Finally, thank you to my family, friends, and all the doctoral students in the Department of Environmental Health Sciences for your support over the years. I am so grateful to all the past and current students who have ensured structure within our program, fought for our rights, and taken on responsibilities such as leading ouR club and working with the union. I am thankful for my friendships with Tess, Sarah, Marisa, and Howie – New York City would not have been the same without you all. Thank you, A.J., for always being so welcoming and for being the first friendly face in the city. Dad, Mary Ann, Barrett, and Kathryn, thank you for always being there for me. Madeleine and Kitty, thank you for your love and incredible friendship, and for being the best roommates I could ever ask for.

Chapter 1: Preface

Attention deficit hyperactivity disorder (ADHD) is a major public health concern. The disorder has an estimated worldwide prevalence of 5.3% in children and 2.5% in adults,^{1,2} and is characterized by age-inappropriate levels of inattention, disorganization, hyperactivity, and impulsivity. ADHD is often comorbid with mood, anxiety, and substance abuse disorders,^{3,4} which may lead to long-term functional impairments and reduced quality of life. For instance, ADHD is associated with poorer long term outcomes in several domains, including addictive behavior, academics, antisocial behavior, social function, self-esteem, occupation, driving, services use, and obesity, and these poor outcomes can only be partially ameliorated via treatment.⁵ In recent decades, temporal and cultural heterogeneity in prevalence estimates have raised concerns about inconsistent, excessive, and inaccurate diagnoses of ADHD, and about the misuse and overprescription of pharmaceuticals used to treat the disorder.⁶ However, meta-regression analyses confirm that ADHD is not simply a product of competitive and capitalistic cultures, but that different prevalence estimates across studies are mainly explained by different methodologies, including the use of different sources of information and different diagnostic criteria.⁷ Owing to the high prevalence of ADHD and its associated comorbid medical problems and impairments, primary prevention of the disorder is an eminent public health concern.

Although ADHD is highly heritable,⁸ the recent World Federation of ADHD's International Consensus Statement highlights shared genetic and environmental risks for the disorder.⁹ Identifying preventable environmental risks could help reduce the worldwide burden of ADHD. One possible avenue of primary prevention could target prenatal exposures that may affect brain development, such as acetaminophen, the active ingredient in the brand name *Tylenol* (also known as paracetamol). Acetaminophen is one of the most commonly used drugs

during pregnancy, with use reported by over 50% of pregnant women in many populations.^{10,11} It is the only recommended over-the-counter pain reliever during gestation, as other analgesics like ibuprofen and aspirin may cause miscarriage or birth defects.¹²⁻¹⁶ Despite acetaminophen's widespread use and reputation as a safe drug during pregnancy, concerns over the long-term impacts of prenatal exposure on child health outcomes have risen over the past several decades.¹⁷⁻¹⁹ Recent meta-analyses of observational studies support an association of prenatal acetaminophen with ADHD and autism spectrum disorders (ASD).^{19,20} A full understanding of the potential harms of acetaminophen use will help doctors better manage pain during pregnancy in the safest way possible.

Despite studies showing associations of prenatal acetaminophen with child ADHD,^{19,20} the evidence remains limited by reliance on self-reported exposure. Most of the studies of prenatal acetaminophen have measured exposure with surveys administered during and shortly after pregnancy. Inaccurate maternal self-report in these surveys could result in misclassification of prenatal acetaminophen exposure. Because surveys are completed before children are old enough to be diagnosed, such misclassification is most likely nondifferential with respect to child ADHD. When measurement error for the exposure is nondifferential with respect to the outcome, bias toward the null typically results. Hence, the effect of prenatal acetaminophen on ADHD could be underestimated. A recent study in the Boston Birth Cohort addressed this limitation by showing that biomarkers of acetaminophen in cord plasma were associated with ADHD (odds ratio [OR] for second tertile, 2.26; OR for third tertile, 2.86, compared to lowest tertile of exposure).²¹ Indeed, this association was far stronger in magnitude compared to the pooled risk ratio for ADHD of 1.34 estimated in a meta-analysis of studies relying on maternal self-report.¹⁹

However, owing to differential placental transfer across individuals, even exposure assessment in blood or urine may result in misclassification of exposure to the fetus. Meconium, the first feces of a mammalian infant, is another biological substrate that can be analyzed for prenatal exposures. Chemicals in meconium accumulate throughout the last two thirds of pregnancy and are known to have passed through the fetus and into the fetal intestinal tract.²²⁻²⁶

Another limitation in prior work is that the mechanisms linking prenatal acetaminophen to health outcomes remain under studied. One potential mechanism is oxidative stress. In the brains of mice and rats, acetaminophen has been shown to induce mitochondrial dysfunction, oxidative stress, and even cortical neuron death.²⁷⁻²⁹ The toxicity of acetaminophen is mediated by its noxious metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). Cytochrome P450 (CYP) enzymes are responsible for the bioactivation of acetaminophen into NAPQI,^{18,30-33} which is detoxified at therapeutic doses via conjugation to glutathione by glutathione S-transferase (GST) enzymes. High doses of acetaminophen, however, may overload and deplete glutathione stores,³⁴⁻³⁶ resulting in the accumulation of NAPQI, along with exogenous xenobiotics and endogenous sources of oxidative stress that are normally conjugated to glutathione. The resulting overproduction of reactive oxygen and nitrogen species along with binding of NAPQI to mitochondrial proteins disrupts complex I and II of the electron transport chain, causing mitochondrial oxidant stress that ultimately leads to fatal liver necrosis.³⁷⁻³⁹ Even therapeutic doses of the drug may deplete glutathione and induce oxidative stress.^{28,40-45} Thus, variant polymorphisms in CYP and GST genes may alter the levels of oxidative stress produced during acetaminophen metabolism.

Another potential mechanism is endocrine disruption.⁴⁶ Acetaminophen may affect sex⁴⁷⁻⁵⁰ and thyroid^{51,52} hormone levels. Issues with hormone regulation, such as thyroid dysfunction

during pregnancy, could interfere with fetal brain development. A recent meta-analysis of 39 studies showed that maternal subclinical hypothyroidism more than doubled the odds of intellectual disability in offspring,⁵³ and several studies have shown that altered maternal thyroid hormone function may increase the odds of ADHD.⁵⁴⁻⁵⁶

The benefits of discovering the mechanisms underlying this exposure-disease relationship are twofold: (1) the FDA continues to recommend acetaminophen use during pregnancy, as the organization still requires more evidence of acetaminophen's prenatal harm. Insights into the mechanisms through which prenatal acetaminophen impairs child neurodevelopment could aid the FDA in future recommendations. (2) If acetaminophen must be used, an understanding of the mechanisms through which the drug harms the developing brain will be crucial. There may be no better alternatives for maternal pain and fever management during pregnancy.^{57,58} If this is the case, then pharmaceutical interventions to combat the harm of prenatal acetaminophen while still allowing its use will be needed. Interventions must be tested in animal models before humans, so mechanistic discoveries in both humans and animal models are necessary. For instance, if acetaminophen acts through oxidative stress, a future intervention study could expose an animal model prenatally to acetaminophen co-administered with N-acetylcysteine, the antioxidant used to treat acetaminophen overdose.⁵⁹

Mice are a common animal model in health studies, and could be a useful system for the study of prenatal acetaminophen exposure. Mouse models are well-established in pharmaceutical discovery and testing, and also a standard in the study of prenatal analgesic exposures, including acetaminophen.⁴⁶ Furthermore, the mechanisms mediating the toxicity of acetaminophen in humans and mice are similar.⁶⁰ Finally, mice are a long-standing model for neurodevelopmental disorders, including ADHD.⁶¹

In sum, the high prevalence of ADHD makes primary prevention of the disorder an eminent public health concern. The strong associations of prenatal acetaminophen with child ADHD reported in numerous studies, along with the fact that more than 50% of women take acetaminophen during pregnancy in western populations, presents an incredible opportunity for intervention. As mentioned above, human observational studies on the role of prenatal acetaminophen in ADHD are likely limited by inaccurate self-report. Additionally, the mechanisms linking prenatal acetaminophen exposure to child ADHD remain unknown, and uncovering these mechanisms is an important step in determining whether the association is causal. In this dissertation, I address these limitations by: 1) utilizing meconium as a substrate to measure prenatal acetaminophen exposure in a human observational study, thereby removing the possibility of inaccurate self-report; 2) exploring brain connectivity and birth outcomes as potential mechanisms linking acetaminophen to ADHD in humans; and 3) investigating the effects of prenatal acetaminophen on offspring behavior in a mouse model, and exploring mechanistic pathways through RNA-sequencing of the prefrontal cortex in both exposed and unexposed mice.

Chapter 2: Association of Prenatal Acetaminophen Measured in Meconium with Risk of Attention-Deficit Hyperactivity Disorder (ADHD): Mediation by Frontoparietal Network Brain Connectivity

Brennan H. Baker, MA¹; Claudia Lugo-Candelas, PhD^{2,3}; Haotian Wu, PhD¹; Hannah E Laue, ScD⁴; Amélie Boivin, M.Sc⁵; Virginie Gillet, PhD⁵; Natalie Aw, MS³; Tonima Rahman, BS³; Jean-François Lepage, PhD⁵; Kevin Whittingstall, MD^{6,7}; Jean-Philippe Bellenger, PhD⁸; Jonathan Posner, MD^{2,3,9}; Larissa Takser, MD;^{5,10} Andrea A. Baccarelli, MD, PhD¹

¹Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, New York

²Department of Psychiatry, Columbia University Medical Center, New York, New York

³New York State Psychiatric Institute, New York, New York

⁴Department of Epidemiology, Geisel School of Medicine, Dartmouth College, Hanover, New Hampshire

⁵Département de Pédiatrie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada

⁶Department of Nuclear Medicine and Radiobiology, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, Québec, Canada

⁷Department of Diagnostic Radiology, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, Québec, Canada

⁸Department of Chemistry, Faculty of Sciences, University of Sherbrooke, Sherbrooke, Quebec, Canada

⁹Sackler Institute for Developmental Psychobiology, Columbia University Medical Center, New York, New York

¹⁰Département de Psychiatrie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada

Published as: Baker BH, Lugo-Candelas C, Wu H, Laue HE, Boivin A, Gillet V, Aw N, Rahman T, Lepage JF, Whittingstall K, Bellenger JP. Association of prenatal acetaminophen exposure measured in meconium with risk of attention-deficit/hyperactivity disorder mediated by frontoparietal network brain connectivity. *JAMA pediatrics*. 2020 Nov 1;174(11):1073-81.

2.1 Introduction

Acetaminophen is one of the most commonly used drugs during pregnancy, with use reported by over half of pregnant women in some populations.^{10,11} It is the only recommended over-the-counter pain reliever during gestation, as other analgesics like ibuprofen and aspirin may cause miscarriage or birth defects.¹²⁻¹⁶ Despite acetaminophen's widespread use and reputation as a safe drug during pregnancy, concerns over the long-term impacts of prenatal exposure on respiratory and neurodevelopmental outcomes have risen over the past several decades.¹⁷⁻¹⁹

One major concern is that acetaminophen may impair fetal brain development, both directly by inducing oxidative stress and apoptosis in the brain,²⁷⁻²⁹ and indirectly via disruption of important developmental hormones such as testosterone.^{62,63} Indeed, recent meta-analyses of observational studies support an association between prenatal acetaminophen and three neurodevelopmental outcomes: attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD) and hyperkinetic disorder/hyperactivity symptoms.^{19,20} All eight studies in these meta-analyses, however, used maternal self-report of acetaminophen. Inaccurate maternal self-report may introduce information bias. Genetic and environmental factors may also affect acetaminophen metabolism, thereby altering the amount of the drug that reaches the fetus. Differential metabolism is not accounted for when acetaminophen is measured by maternal self-report.

Owing to limitations of prior studies, the U.S. Food and Drug Administration (FDA) and the Society for Maternal-Fetal Medicine (SMFM) have not changed their recommendations to reflect the potential harm of prenatal acetaminophen to neurodevelopment.^{64,65} The SMFM cited maternal self-report of acetaminophen, lack of quantification of acetaminophen dose, and

measurement of outcomes using questionnaires as three limitations of previous studies. A recent study in the Boston Birth Cohort addressed these limitations by finding a positive association between acetaminophen metabolites measured in cord plasma and physician diagnosis of ADHD.²¹

Despite growing evidence of an association between prenatal acetaminophen and increased risk for ADHD, several limitations in prior studies remain. First, the Boston Birth Cohort study is the only one that used a direct measurement of acetaminophen.²¹ No single observational study is sufficient for causal inference, and more observational studies using direct measurements of fetal acetaminophen exposure are needed. Second, owing to the < 3 hour half-life of acetaminophen,⁶⁶ a cord plasma measurement may only reflect acetaminophen use shortly before and immediately after birth.²¹ A direct measurement of fetal acetaminophen exposure that reflects longer term exposure throughout pregnancy is warranted. Third, no prior studies have examined the potential mechanisms mediating the association of prenatal acetaminophen with neurodevelopment, a key component for assessing the potential for causation.⁶⁷ Neuroimaging research has repeatedly documented altered connectivity in important brain networks (e.g., default mode, salience, frontoparietal) in individuals with ADHD,^{6,68,69} yet to date no studies have examined functional connectivity in relation to prenatal acetaminophen exposure.

In an ongoing prospective birth cohort, we addressed the first two limitations by evaluating the association between ADHD and acetaminophen measured directly in meconium, the first feces of newborn infants. Chemicals in meconium are known to have passed through the fetus and into the fetal intestinal tract.^{22,23,25} Additionally, meconium measurements reflect cumulative exposures during the last two thirds of pregnancy, as drugs and drug metabolites are deposited in meconium during that period.²⁶ We addressed the third limitation by conducting the

first study using neuroimaging to assess the potential mediating role of functional connectivity in the association between prenatal acetaminophen and child hyperactivity.

2.2 Methods

Study population

This analysis was conducted in the GESTation and the Environment (GESTE) cohort in Sherbrooke, Quebec, Canada. Women age ≥ 18 years and with no known thyroid disease enrolled at the Research Center of the CHUS (Centre Hospitalier Universitaire de Sherbrooke) between 2007 and 2009 at their first prenatal care visit or delivery and were followed up when children were 6-7 years old. Families are currently completing a fourth follow-up assessment (2017-2023; children ~9-11 years of age). As a part of this assessment, children are asked to undergo a magnetic resonance imaging (MRI) assessment. Parents were asked to not give their children ADHD medication on the day of the scan. On the day of the scan, parents were questioned to confirm adherence to this instruction. The eligible study sample were 394 individuals for whom meconium was collected at delivery. The final sample size was 345, as ADHD status was unknown for 49 individuals due to loss to follow-up. Thus far 76 subjects have undergone fMRIs, 48 of whom had meconium samples collected in infancy; only children with both meconium samples and MRIs were included in MRI analyses. Parents signed informed consent forms at each follow-up, and children provided written consent at the 9-11-year-old follow-up. All study protocols were approved by the Institutional Review Boards of the University of Sherbrooke, Harvard T.H. Chan School of Public Health, and Columbia University.

Exposure assessment

Meconium was collected from the diapers of newborn infants after delivery and stored at -80 °C until analysis. Acetaminophen was extracted from < 120 mg meconium and analyzed with ultraperformance liquid chromatography mass spectrometry following the methods described elsewhere.⁷⁰ Acetaminophen was detected in 58% of the 345 samples, with a recovery of 104% and repeatability of ±15%. The limit of detection (LOD) and limit of quantification (LOQ) were 2 ng/g and 5 ng/g respectively. Additionally, clinical files from the hospital database and medical charts were used to determine administration of acetaminophen during labor (yes/no).

Outcome assessment

At a scheduled cohort follow-up when children were 6-7 years old, parents were asked on a questionnaire if their child had physician-diagnosed ADHD. In total, 176 parents provided information at the 6-7 year follow up. For those that did not complete the 6-7-year-old follow-up visit (n=169), physician diagnosis of ADHD was obtained from reviewing medical charts from CHUS pediatric clinics, which are available in the hospital database. Additionally, among the 48 children in the MRI analysis subsample, 46 completed the Behavioral Assessment System for Children Parent Report Scale (BASC3-PRS) at ages 9-11 years. In the BASC3-PRS, parents answer a range of questions concerning the behavior of their children which are combined into various rating scales, including scales for attention problems and hyperactivity.⁷¹

MRI assessment

At ages 9-11 years, T1-weighted structural MRI and functional images were acquired on a Phillips Ingenia 3T whole-body scanner with a 32-channel head coil. The CONN toolbox⁷² was used for preprocessing and seed-based analyses were conducted. Forty-eight subjects had both

resting state MRI data and meconium samples collected. eMethods and eTable1 provide details on MRI acquisition, preprocessing, and head motion, as well as demographic characteristics of the scanned children.

Covariates

Covariate data were obtained from questionnaires given during pregnancy and after delivery. Covariates were child sex and familial income (dichotomized at the sample median), along with maternal characteristics including age at delivery, education status (College/University vs. no College/University), pre-pregnancy BMI, smoking during pregnancy (yes/no), and alcohol during pregnancy (yes/no). A sensitivity analysis including ADHD of the mother (self-reported, obtained from questionnaire) as an additional covariate was conducted, but this variable was excluded from the final models because data were only available for 155 individuals. Controlling for maternal ADHD in this subset altered the estimate for meconium acetaminophen by just 2%, and the shift was away from the null. Missing covariate data were imputed with the median of continuous variables and the mode of categorical variables.

Statistical analysis

To control for potential confounders, we employed inverse probability weighting (IPW) using propensity scores⁷³⁻⁷⁶ with the ‘CBPS’ R package.⁷⁷ Propensity scores (p, the likelihood of detectable meconium acetaminophen) were estimated using logistic regression models in which exposure (meconium acetaminophen detected vs not detected) was regressed on child sex and maternal covariates described above. Weights were estimated as $1/p$ for exposed individuals, and $1/(1 - p)$ for unexposed individuals. Study sample weighting creates a pseudo-population

balanced on measured baseline covariates.⁷³⁻⁷⁵ Standardized mean differences were computed to assess balance of covariates between the exposed and unexposed groups in both weighted and unweighted samples (eTable 2). In a sensitivity analysis, we excluded all mothers that were administered acetaminophen during delivery to account for potential confounding by indication for use during labor.

To explore a potential dose response, we repeated models with continuous meconium acetaminophen level, and with acetaminophen categorized into three levels: not detected, low (≤ 69.0 ng/g, the 50th percentile of exposure), and high ($> 50^{\text{th}}$ percentile of exposure). Continuous acetaminophen was $\log_2 n$ transformed, with 146 values below the LOD imputed with $\frac{(\text{LOD})}{(\sqrt{2})}$ And 13 values below the LOQ imputed with $\frac{(\text{LOQ})}{(\sqrt{2})}$. We modeled continuous acetaminophen with both a linear regression and a generalized additive model including a penalized spline term. A likelihood ratio test was used to compare these linear and non-linear models.

Based on the outcome of interest, i.e., child ADHD, resting state analyses focused on connectivity in three classical brain networks often implicated in ADHD: the default mode (DMN), salience/cingulo-opercular and frontoparietal/central executive networks.⁶⁸ Seed-based functional connectivity analyses were restricted to regions of interest (ROIs) comprising the aforementioned networks (from the CONN provided atlas) and compared subjects with ($n = 25$) and without ($n = 23$) prenatal acetaminophen exposure. Analyses controlled for confounders using propensity scores calculated specifically for the 48 subjects with MRI data. Scores included the previously detailed variables, as well as child age at scan. Analyses were thresholded at a voxel level $p < 0.001$ (uncorrected) and at a cluster level $p < 0.05$ (FDR corrected), and eMethods provide details on ROIs and data analyses. Following connectivity analyses, we tested associations between connections that differed between the prenatal acetaminophen-

exposed versus unexposed children and BASC-3 hyperactivity and attention problems scores at age 9-11 years. We performed logistic regressions on BASC-3 scores categorized as above or below the median.

Finally, we performed causal mediation analysis examining connectivity between the frontoparietal network and right precentral/fontal gyrus, as connectivity between these regions was (1) significantly different between exposed and unexposed children and (2) a significant predictor of hyperactivity. The purpose of our mediation analyses was not to investigate the total effect of acetaminophen on hyperactivity, but rather to investigate processes potentially underlying ADHD. Thus, we did not consider a significant total effect on hyperactivity as a requirement to test for indirect effects. Dropping this requirement reduces type II error associated with the Barron and Kenny causal steps approach.⁷⁸ We used the ‘mediation’ R package,⁷⁹ implementing a quasi-Bayesian Monte Carlo method with 1,000 simulations, to test whether connectivity mediated the relationship between meconium acetaminophen exposure and hyperactivity. This method computes the average direct effect (ADE) and average causal mediation effect (ACME), reflecting direct and indirect (i.e. mediated by connectivity) effects of meconium acetaminophen on hyperactivity. The ‘mediation’ package uses information from two models with: (1) connectivity as outcome and meconium acetaminophen as predictor, and (2) hyperactivity as outcome and both connectivity and meconium acetaminophen as predictors. To assess the potential impact of unobserved pre-treatment confounders, we introduced a sensitivity parameter ρ – the correlation between the residuals of the mediator and outcome regressions. We allowed ρ to vary from -0.9 to 0.9 by 0.05 increments to determine what level of confounder-induced correlation would bias results to the null.

Statistical analyses were conducted with R version 3.5.1.⁸⁰

2.3 Results

Among the total study sample of 345, acetaminophen was detected in the meconium of 199 individuals and ADHD was diagnosed in 33 individuals. This 10% ADHD prevalence was comparable to the lifetime 11.3% prevalence in Quebec.⁸¹ Baseline covariates stratified by acetaminophen detection are presented in Table 1. Standardized mean differences were < 0.1 for all covariates after inverse probability weighting, indicating balance between the exposed and unexposed groups (eTable 2).⁸²

Acetaminophen detection in meconium was associated with nearly 2.5-fold increased odds of ADHD at 6-7 years (OR = 2.43; 95% CI [1.41, 4.21]) (Table 2) in the weighted sample balanced on covariates. When acetaminophen exposure was categorized into three levels, low acetaminophen exposure did not significantly modify the risk of ADHD compared to no acetaminophen exposure (OR = 1.44; 95% CI [0.79, 2.63]). However, high levels of acetaminophen detected in meconium increased the odds of ADHD more than 4-fold (OR = 4.10; 95% CI [2.41, 6.95]) (Table 2). When meconium acetaminophen was linearly modeled, each doubling of exposure increased the odds of ADHD by 10% (OR = 1.10; 95% CI [1.02, 1.19]) (Table 2). Introducing a non-linear penalized spline for continuous acetaminophen did not improve the model fit (likelihood ratio test $p = 0.10$, eFigure1). Results did not differ in a sensitivity analysis excluding 44 mothers that were administered acetaminophen at delivery (OR = 2.38; 95% CI [1.35, 4.21]).

Functional connectivity analyses revealed that, compared to the unexposed group, children with detectable levels of acetaminophen in meconium demonstrated increased negative connectivity between the medial prefrontal cortex gyrus (DMN network seed) and six clusters covering regions of bilateral pre and postcentral gyri, superior parietal lobules, and

supramarginal gyri ($ts > 5.30$; $p_{s\text{fdr}} < 0.028$). Exposed children also demonstrated increased negative connectivity between the left lateral prefrontal cortex (frontoparietal network seed) and a cluster spanning portions of the right precentral and frontal gyrus ($t = 4.62$; $p_{\text{fdr}} = 0.018$; Table 3, Figure 1). There were no differences detected using salience network seeds.

Among these brain connections associated with meconium acetaminophen, connectivity between the frontoparietal network and right precentral/frontal gyrus was also associated with BASC hyperactivity score (eTable 3). Consistent with the potential for mediation, meconium acetaminophen detection was associated with decreased connectivity (Figure 2a), and children with decreased connectivity were more hyperactive (Figure 2b). Causal mediation analysis revealed no total or direct effect of meconium acetaminophen on hyperactivity (15% increase; 95% CI [-6%, 36%] and 1% increase; 95% CI [-200%, 26%] respectively), but a significant indirect effect on increased hyperactivity mediated through frontoparietal network and right precentral/frontal gyrus connectivity (14% increase; 95% CI [1%, 26%]) (Figure 2c, eTable 4). A sensitivity analysis for pre-treatment confounders revealed that in order to bias this result to the null, an unobserved confounder would need to induce a correlation between the residuals of the mediator and outcome regressions of $\rho = -0.3$.

2.4 Discussion

In this prospective Eastern Canadian cohort, children exposed to acetaminophen prenatally were at increased risk of ADHD at 6-7 years. Categorical and continuous models suggested that higher levels of meconium acetaminophen increased the risk of child ADHD in a linear manner. Prenatal acetaminophen exposure was also associated with increased negative connectivity between left prefrontal cortex (frontoparietal seed) and the right precentral/frontal

gyrus, which mediated the association of acetaminophen with hyperactivity. Several prior studies have implicated prenatal acetaminophen exposure in the etiology of neurodevelopmental diseases like ADHD and autism, yet none have examined brain function following acetaminophen exposure. Further, to the best of our knowledge, our results are just the second report of an association between child ADHD and prenatal acetaminophen measured not via questionnaire, but in a biological sample,²¹ and the first study of the association between acetaminophen measured in meconium and ADHD. A prior study in this cohort examined the relationship between meconium acetaminophen and the Wechsler Intelligence Scale for Children, finding no consistent associations.⁸³

A major strength of this study was the unbiased, biological measure of fetal acetaminophen exposure. All but one of the prior studies of the association between prenatal acetaminophen and child ADHD have relied on questionnaires requiring mothers to recall drug use in intervals greater than 3 months.⁸⁴⁻⁹⁰ Difficulty recalling drug use during pregnancy may result in non-differential misclassification bias toward the null. This source of bias may explain the smaller pooled risk ratio of 1.34 for ADHD from past cohort studies¹⁹ compared to the nearly 2.5-fold increased odds reported here. Supporting this hypothesis, the only other study not relying on maternal self-report, which measured acetaminophen in cord plasma, reported an odds ratio of 2.26 for the second tertile and 2.86 for the third tertile compared to the first tertile of exposure.²¹ However, it is possible that results in this population, which is highly educated and genetically homogeneous,⁹¹ are not generalizable to other populations with different characteristics.

This is the first study to examine associations of prenatal acetaminophen exposure with functional connectivity in childhood. Alterations in connectivity between the DMN and

frontoparietal networks to the sensorimotor cortices have been previously documented in both children⁹² and adults⁹³ with ADHD and have been linked to symptom severity. Here we offer a putative mechanistic insight into the relationship between prenatal acetaminophen and child ADHD. Causal mediation analysis revealed that altered frontoparietal network connectivity may link an association of prenatal acetaminophen exposure with increased child hyperactivity at ages 9-11 years. While this result suggests that brain connectivity may also mediate an indirect effect on ADHD, we were unable to explore this possibility, as ADHD diagnosis information was obtained when children were 6-7 years old. Studies have previously associated altered functional brain connectivity with environmental exposures including air pollution,⁹⁴ social stress,⁹⁵ and prenatal Selective Serotonin Reuptake Inhibitors,⁹⁶ although this is the first neuroimaging study of prenatal acetaminophen exposure. Taken together with the wide confidence interval of the mediation analysis indirect effect and the small MRI sample size, studies in larger and more diverse cohorts are needed to replicate these novel findings.

Confounding by unmeasured or unknown factors is always a possibility. While we did not control for indications for acetaminophen use in this study, prior cohort studies controlling for maternal fevers, infections, and other indications for acetaminophen use have reported lack of confounding by these factors.^{21,85-88,90} However, lack of confounding by indicators in prior cohort studies does not necessarily apply to the cohort studied here. We also considered the possibility that meconium acetaminophen concentrations were a reflection of acetaminophen administered during labor rather than throughout pregnancy. However, excluding women that were administered acetaminophen at delivery did not change our results. While meconium is known to accumulate drugs and drug metabolites throughout the last two thirds of pregnancy, we did not explicitly correlate maternal acetaminophen use with meconium acetaminophen concentrations, a

potential limitation that should be the subject of future work. Another possibility is confounding by unknown genetic, social, and familial factors related to acetaminophen use. This concern has been recently addressed with negative control exposure analysis: maternal acetaminophen use before pregnancy, after pregnancy, and partner's acetaminophen use were not associated with child ADHD in populations where maternal acetaminophen use during pregnancy did increase the risk.^{28,29} Furthermore, our study population has high genetic and sociodemographic homogeneity.⁹¹ Hence, confounding by unknown or unmeasured factors is unlikely. Finally, although children did not take ADHD medications on the day of the scan, we could not rule out prior medication use.

2.5 Conclusions

By using a direct measurement of prenatal acetaminophen exposure that is unbiased by maternal recall, our results add strong evidence in support of the association between prenatal acetaminophen and child ADHD. Taken together with the large odds ratios reported in the Boston Birth Cohort study,²¹ these results suggest that prior studies may have been biased toward the null by inaccurate maternal recall. Thus, there may be an even stronger association between prenatal acetaminophen and ADHD than previously estimated. This study additionally supports altered resting state brain connectivity as a potential underlying mechanism linking prenatal acetaminophen with child hyperactivity. Along with the multitude of other cohort studies drawing similar conclusions, this work joins the Boston Birth Cohort study as the second study addressing the FDA and SMFM's concerns of maternal self-report and lack of quantification of prenatal acetaminophen dose. These institutions should therefore consider reevaluating the evidence regarding the safety of fetal acetaminophen exposure.

Acknowledgements

This work was supported by the National Institute of Environmental Health Sciences (R21ES024841, R01ES027845, P30ES009089) and the Canadian Institutes of Health Research (MOP-84551). This work was also supported by a Canadian Research Chair (CRC-950-230570) Grant from the Natural Sciences and Engineering Research Council of Canada. Funding sources did not contribute to design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication. Brennan H Baker had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

2.6 Tables

Table 1: Characteristics of study population stratified by meconium acetaminophen exposure in the GESTation and the Environment (GESTE) cohort

	No acetaminophen (N=146)	Acetaminophen (N=199)	Total (N=345)	<i>P</i> value
Sex				0.68
Female	73 (50.0%)	95 (47.7%)	168 (48.7%)	
Male	73 (50.0%)	104 (52.3%)	177 (51.3%)	
Maternal age at delivery				0.45
Mean (SD)	29.2 (4.7)	28.8 (5.0)	29.0 (4.9)	
Range	17.0 - 43.0	0.0 - 41.0	0.0 - 43.0	
Maternal education				0.21
No College or university (<i>n</i> (%))	70 (47.9%)	82 (41.2%)	152 (44.1%)	
College or University (<i>n</i> (%))	76 (52.1%)	117 (58.8%)	193 (55.9%)	
Family income (Canadian dollars)				0.72
N-Miss	19	16	35	
< 60,000/year (<i>n</i> (%))	64 (50.4%)	96 (52.5%)	160 (51.6%)	
> 60,000/year (<i>n</i> (%))	63 (49.6%)	87 (47.5%)	150 (48.4%)	
Maternal BMI				0.10
N-Miss	10	5	15	
Mean (SD)	25.2 (6.4)	26.6 (7.8)	26.0 (7.3)	
Range	17.8 - 60.5	17.6 - 89.4	17.6 - 89.4	
Smoked during pregnancy				0.88
N-Miss	4	2	6	
No smoking (<i>n</i> (%))	121 (85.2%)	169 (85.8%)	290 (85.5%)	
Smoking	21 (14.8%)	28 (14.2%)	49 (14.5%)	
Alcohol during pregnancy				0.22

N-Miss	4	2	6	
No alcohol (<i>n</i> (%))	106 (74.6%)	158 (80.2%)	264 (77.9%)	
Alcohol (<i>n</i> (%))	36 (25.4%)	39 (19.8%)	75 (22.1%)	

Notes: Study sample data before imputation and inverse probability weighting shown. *P* values from chi-square goodness of fit tests for binary variables and two-sample t-tests for continuous variables.

Table 2: Associations of meconium acetaminophen with child ADHD (whole study sample, N = 345)

Meconium acetaminophen exposure	Outcome		Odds Ratios			
	ADHD	No ADHD	Crude	<i>P</i> value	Weighted ^a	<i>P</i> value
Binary						
No acetaminophen (<i>n</i> (%))	8 (5.5)	138 (94.5)	-	-	-	-
Acetaminophen (<i>n</i> (%))	25 (12.6)	174 (87.4)	2.48 [1.08, 5.67]	0.03	2.43 [1.41, 4.21]	<0.01
Categorical						
No acetaminophen (<i>n</i> (%))	8 (5.5)	138 (94.5)	-	-	-	-
Low acetaminophen (<i>n</i> (%))	9 (8.5)	97 (91.5)	1.60 [0.60, 4.30]	0.35	1.44 [0.79, 2.63]	0.23
High acetaminophen (<i>n</i> (%))	16 (17.2)	77 (82.8)	3.60 [1.47, 8.76]	<0.01	4.10 [2.41, 6.95]	<0.01
<i>P</i> value for trend				<0.01		<0.01
Continuous						
Log2(acetaminophen)	-	-	1.10 [1.02, 1.20]	0.02	1.10 [1.02, 1.19]	0.01

^a Inverse probability weighted for maternal age at birth, maternal BMI, maternal smoking and alcohol during pregnancy, maternal education, family income, and child sex

Table 3. Functional Connectivity Differences between Exposed and Unexposed Groups

Network	Seed	Region	MNI coordinates			Hemisphere	Cluster size (mm ³)	Size p-FDR	Peak t value	Peak Z value
			x	y	z					
Default Mode	Medial Prefrontal cortex (1,55,-3)	Postcentral Gyrus, Superior Parietal Lobule, Lateral Occipital Cortex (superior division), Supramarginal Gyrus, Angular Gyrus	36	-52	58	R	1358	<0.01	5.73	4.94
		Postcentral Gyrus, Superior Parietal Lobule, Lateral Occipital Cortex (superior division), Supramarginal Gyrus	-38	-52	64	L	1104	<0.01	5.89	5.04
		Inferior and Middle Temporal Gyrus (temporooccipital and posterior divisions), Cerebellum Crus 1 and 6, Lateral Occipital Cortex, (inferior division), Temporal Occipital Fusiform Cortex	60	-52	-12	R	905	<0.01	5.75	4.95
		Precentral Gyrus, Middle and Superior Frontal Gyrus	26	-14	46	R	638	<0.01	5.73	4.94
		Precentral Gyrus, Superior and Middle Frontal Gyrus	-22	-14	48	L	536	<0.01	5.48	4.77
		Cerebellum 8 and 9	-28	-46	-56	L	241	0.03	5.30	4.64
Frontal Parietal	Left Lateral Prefrontal Cortex (-43,33,28)	Superior Frontal, Middle Frontal, and Precentral Gyrus	30	2	60	R	452	0.02	4.62	4.16

Notes: R = right, L = left, FDR = false discovery rate.

2.7 Figures

Figure 1. Differences in resting state functional connectivity related to prenatal acetaminophen exposure

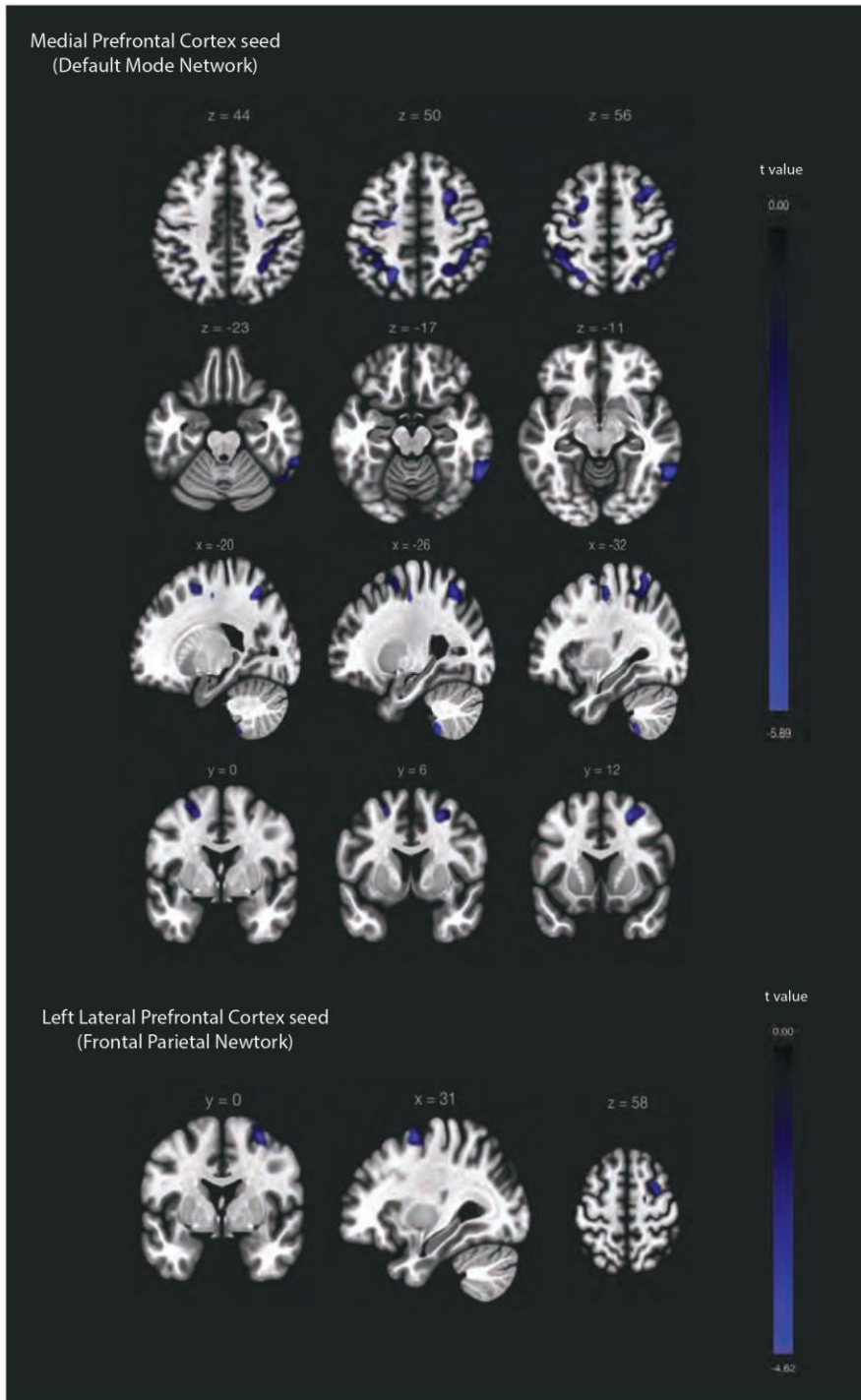


Figure 2: Causal mediation by connectivity between the frontoparietal network and right precentral/fontal gyrus

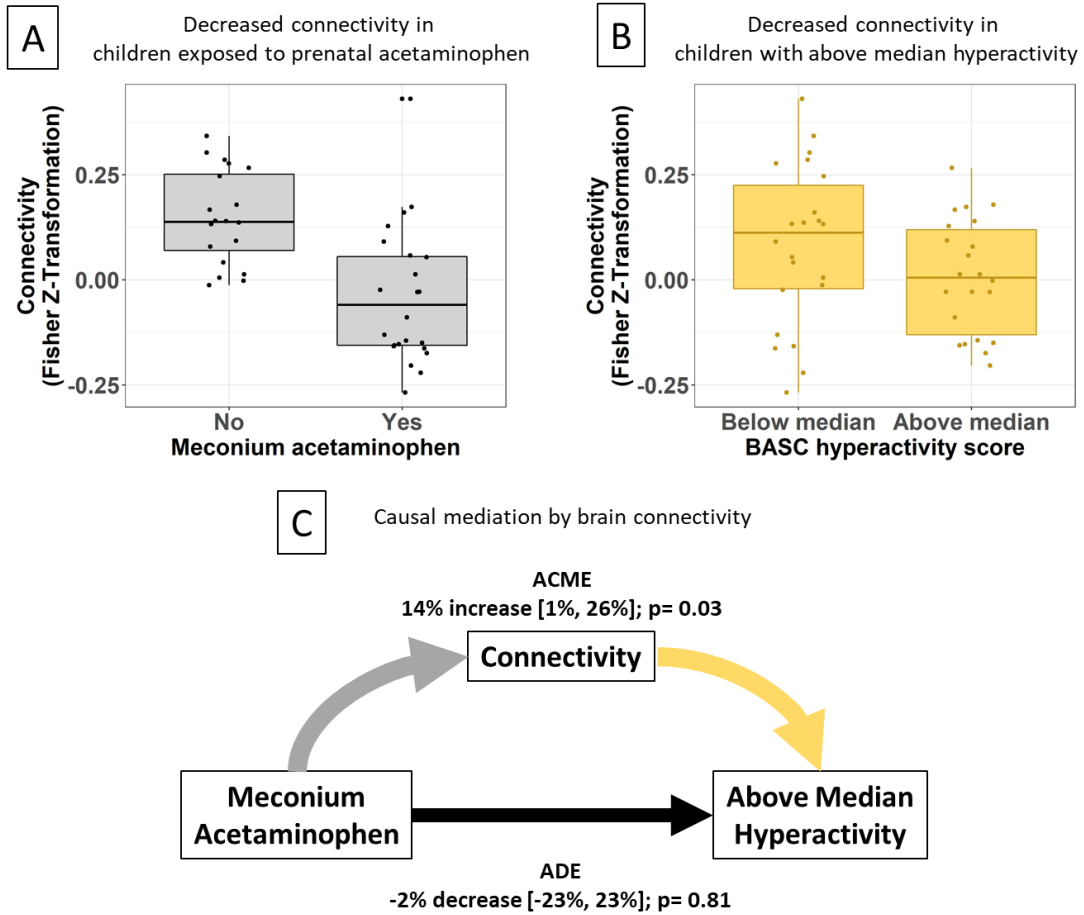


Figure legends

Figure 1. Differences in resting state functional connectivity related to prenatal acetaminophen exposure

Seed-based functional connectivity contrasts detected differences in resting state connectivity between prenatally acetaminophen exposed (n=25) and unexposed (n=23) children. Compared to the unexposed group, prenatally acetaminophen-exposed children demonstrated increased negative connectivity between the medial prefrontal cortex gyrus (DMN network seed) and six clusters covering regions of bilateral pre and postcentral gyri, superior parietal lobules, and supramarginal gyri as well as increased negative connectivity between the left lateral prefrontal cortex (frontoparietal network seed) and a cluster spanning portions of the right precentral and frontal gyri. Analyses were thresholded at a voxel level $p < 0.001$ (uncorrected) and at a cluster level $p < 0.05$ (FDR corrected). Colored areas show increases in negative (blue) connectivity between the groups. Values in top of each image indicate the brain slice being displayed.

Figure 2: Causal mediation by supramarginal gyrus-frontal cortex connectivity

Individual values (points), median, and interquartile range (boxplots) depict significant differences in (A) connectivity between the frontoparietal network and right precentral/frontal gyrus in children prenatally exposed versus unexposed to acetaminophen, and (B) connectivity between the frontoparietal network and right precentral/frontal gyrus in children with above versus below the median hyperactivity. (C) Mediation by connectivity of the relationship between meconium acetaminophen and child hyperactivity at ages 9-11 years. Average causal mediation effect (ACME) and average direct effect (ADE) from causal mediation analysis shown. Connectivity expressed as a Pearson correlation between the two brain regions with Fisher z-transformation.

2.8 Supplement

eMethods. Detailed Methodology

eTable 1. Differences in demographic and head motion variables across exposed and unexposed children with MRI data

eTable 2: Characteristics of study population stratified by meconium acetaminophen weighted on measured covariates in the GESTation and the Environment (GESTE) cohort

eTable 3. Associations between resting state connectivity and hyperactivity and attention problems

eTable 4: Causal mediation by connectivity between the frontoparietal network and right precentral/frontal gyrus of the relationship between meconium acetaminophen and hyperactivity

eFigure 1. Non-linear association of meconium acetaminophen concentration with ADHD

eMethods:

Participants

MRI assessments are ongoing in this sample. At the time of this publication, 76 children had completed MRIs. Of these, 48 had meconium samples collected in infancy and are thus the sample analyzed here. Out of the 48 participants with MRIs and meconium samples, acetaminophen was detected in the meconium of 25 individuals. Subjects with and without prenatal acetaminophen exposure did not significantly differ in age at the time of scan, sex, or head motion parameters (eTable 1).

MRI Acquisition

Images were acquired on a Phillips Healthcare Ingenia 3T whole-body scanner with a 32-channel head coil. For the T1-weighted structural scans, the imaging parameters were: T1 3D TFE (Turbo Field Echo) pulse sequence, 8° flip angle, FOV 240mm, matrix size 240x240, slice thickness 1mm. For resting state MRI, echoplanar images with the following parameters were collected: (TR= 1075 ms, TE= 30 ms, 55° flip angle, single excitation per image, FOV 240mm, matrix size 80x80, slice thickness 3 mm, 48 slices. One resting run of 575 volumes was collected for each participant.

Resting-state Image Processing and Head Motion During Scanning

Several steps were taken to limit the influence of in-scanner head motion. First, a trained researcher (TR) visually examined every run. For every functional run, a separate 5 volume run was collected with reversed phase-encoded blips, resulting in a sequence of images with magnitude distortions

in the opposite direction¹. FSL's topup² was used to estimate and correct the susceptibility-induced off-resonance field. Images were then preprocessed using the CONN toolbox standard preprocessing pipeline. Briefly, images were coregistered with an anatomical scan, realigned, unwarped, normalized into standard MNI space, segmented, and smoothed using spatial convolution (8mm full width half maximum Gaussian kernel). Outlier scans (framewise displacement above 0.5mm or global BOLD signal changes above 3 SDs) were flagged as potential outliers using the conservative CONN setting³. Scrubbing and temporal band-pass filtering (0.008–0.09 Hz) was applied, the percentage of valid volumes (post-scrubbing) included in analyses did not significantly differ between the groups.

Seed-based functional connectivity

Resting fMRI time series were correlated region of interest (ROI) by voxel for each participant. Fisher-z transformation was applied. Whole brain connectivity maps were generated with the seed ROIs of the Default Mode Network (4 ROIs: medial prefrontal cortex [1,55,-3], left lateral parietal [-39,-77,33], right lateral parietal [47,-67,29], posterior cingulate gyrus [1,-61,38]), Salience/Cingulo-Opercular (7 ROIs: Anterior cingulate cortex [0, 22, 35], left anterior insula [-44, 13, 1], right anterior insula [47, 14, 0], left rostral prefrontal cortex [-32, 45, 27], right rostral prefrontal cortex [32, 46, 27], left superior marginal gyrus [-60, -39, 31], and the right superior marginal gyrus [62, -35, 32]), and FrontoParietal/Central Executive (4 ROIs; left lateral prefrontal cortex [-43,33,28], left posterior parietal cortex [-46,-58,49], right lateral prefrontal cortex [41,38,30], right posterior parietal cortex [52,-52,45]) derived from the CONN- provided atlas (all of were defined from CONN's Independent Component Analyses of Human Connectome Project dataset [derived from 497 subjects])³.

eTable 1. Differences in demographic and head motion variables across exposed and unexposed children with MRI data

Characteristic	Prenatally	Unexposed	Test	<i>p</i> Value
	exposed (25)	(23)	Statistic (df)	
Sex				
Male	10	14	$X^2_1 = 2.09$	0.15
Female	15	9		
Age	10.32 (0.54)	10.16 (0.68)	$F_{1,47} = 0.89$	0.35
Mean FD pre-preprocessing	0.31 (0.18)	0.45 (0.32)	$F_{1,47} = 3.58$	0.07
Mean post-preprocessing	0.05 (0.02)	0.06 (0.03)	$F_{1,47} = 2.28$	0.14
Percentage valid volumes (post scrubbing)	94.3 (9.15)	88.6 (12.90)	$F_{1,47} = 3.13$	0.08

eTable 2: Characteristics of study population stratified by meconium acetaminophen weighted on measured covariates in the GESTation and the Environment (GESTE) cohort

	No acetaminophen (N=146)	Acetaminophen (N=199)	Standardized mean difference before weighting	Standardized mean difference after weighting
Sex			0.045	0.000005
Female	166 (48.2%)	166 (48.2%)		
Male	179 (51.8%)	179 (51.8%)		
Maternal age at delivery			0.083	0.000001
Mean (SD)	29.1 (4.7)	29.1 (4.6)		
Range	17.0 - 43.0	19.0 - 41.0		
Maternal education			0.136	0.000009
No College or University	154 (44.8%)	154 (44.8%)		
College or University	191 (55.2%)	191 (55.2%)		
Family income (Canadian dollars)			0.011	0.000004
< 60,000/year	197 (57.2%)	197 (57.2%)		
> 60,000/year	148 (42.8%)	148 (42.8%)		
Maternal BMI			0.190	0.000025
Mean (SD)	27.0 (7.2)	27.0 (6.3)		
Range	15.7 - 60.5	17.4 - 89.1		
Smoked during pregnancy			0.009	0.000002
No smoking	295 (85.6%)	295 (85.6%)		
Smoking	50 (14.4%)	50 (14.4%)		
Alcohol during pregnancy			0.122	0.000004
No alcohol	270 (78.3%)	270 (78.3%)		
Alcohol	75 (21.7%)	75 (21.7%)		

eTable 3. Associations between resting state connectivity and hyperactivity and attention problems

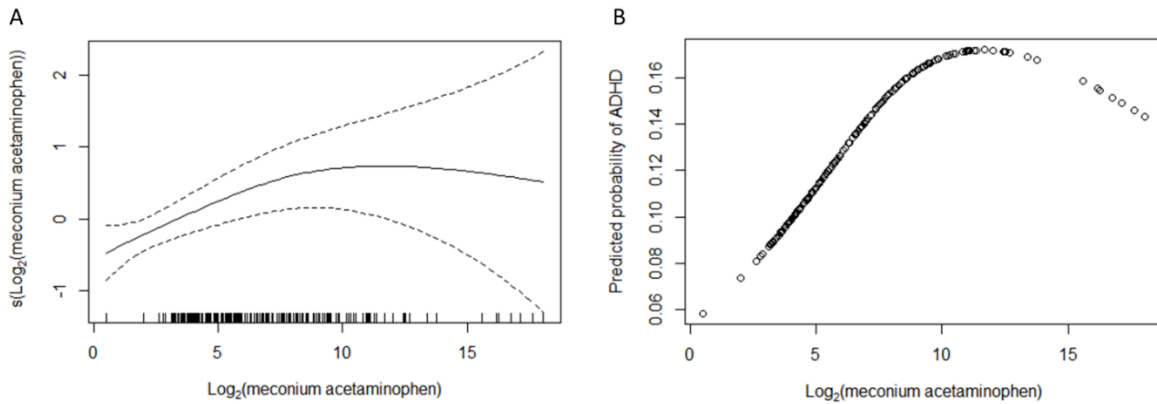
	Hyperactivity		Attention problems	
	Odds Ratio	P value	Odds Ratio	P value
DMN1	0.63 [0.04, 10.04]	0.75	5.81 [0.33, 101.66]	0.23
DMN2	0.44 [0.03, 6.51]	0.55	2.33 [0.16, 34.84]	0.54
DMN5	0.24 [0.01, 8.15]	0.43	2.04 [0.06, 66.87]	0.69
FP	0.04 [0.00, 0.68]	0.03	2.14 [0.15, 30.11]	0.57

Note: Odds ratios for a 1-point increase in the Fisher z-transformed Pearson correlation between brain regions. DMN1: connectivity between the Medial Prefrontal Cortex Gyrus and regions covering the Postcentral Gyrus, Superior Parietal Lobule, Lateral Occipital Cortex (superior division), Supramarginal Gyrus, Angular Gyrus. DMN2: connectivity between the Medial Prefrontal Cortex Gyrus and regions covering the Postcentral Gyrus, Superior Parietal Lobule, Lateral Occipital Cortex (superior division), Supramarginal Gyrus. DMN5: connectivity between the Medial Prefrontal Cortex Gyrus and regions covering the Precentral Gyrus, Superior and Middle Frontal Gyrus. FP: connectivity between the Left Lateral Prefrontal Cortex and the Superior Frontal, Middle Frontal and Precentral Gyrus.

eTable 4: Causal mediation by connectivity between the frontoparietal network and right precentral/frontal gyrus of the relationship between meconium acetaminophen and hyperactivity

Effect	Estimate [95% CI]	P value
Total effect	0.1543 [-0.0589, 0.36]	0.114
Average direct effect	0.0124 [-2.008, 0.26]	0.976
Average causal mediation effect	0.1419 [0.0165, 0.27]	0.024

eFigure 1. Non-linear association of meconium acetaminophen concentration with ADHD



Legend: Generalized additive model for the non-linear effect of meconium acetaminophen on ADHD ($p = 0.0383$). (A) Log odds of ADHD as a function of a smooth penalized spline term for \log_2 transformed meconium acetaminophen concentration. (B) Predicted probability of ADHD as a function of \log_2 transformed meconium acetaminophen concentration.

Supplemental references

1. Andersson JL, Skare S, Ashburner J. How to correct susceptibility distortions in spin-echo echo-planar images: application to diffusion tensor imaging. *NeuroImage* 2003; **20**(2): 870-88.
2. Smith SM, Jenkinson M, Woolrich MW, et al. Advances in functional and structural MR image analysis and implementation as FSL. *NeuroImage* 2004; **23 Suppl 1**: S208-19.
3. Whitfield-Gabrieli S, Nieto-Castanon A. Conn: a functional connectivity toolbox for correlated and anticorrelated brain networks. *Brain connectivity* 2012; **2**(3): 125-41.

Chapter 3: Association of Prenatal Acetaminophen Exposure Measured in Meconium with Adverse Birth Outcomes in a Canadian Birth Cohort

**Brennan H. Baker,¹ Heather H. Burris,² Tessa R. Bloomquist,¹ Amélie Boivin,³ Virginie
Gillet,³ Annie Larouche,³ Jean-Philippe Bellenger,⁴ Jean-Charles Pasquier,⁵ Andrea A.
Baccarelli,¹ Larissa Takser.^{3,6}**

¹ Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, New York

² Department of Pediatrics, University of Pennsylvania, Perelman School of Medicine, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

³ Département de Pédiatrie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada

⁴ Département de Chimie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada

⁵ Département d'Obstétrique et Gynécologie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada

⁶ Département de Psychiatrie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada

Published as: Baker BH, Burris HH, Bloomquist TR, Boivin A, Gillet V, Larouche A, Takser L, Bellenger JP, Pasquier JC, Baccarelli AA. Association of Prenatal Acetaminophen Exposure Measured in Meconium With Adverse Birth Outcomes in a Canadian Birth Cohort. *Frontiers in pediatrics*. 2022;10.

3.1 Introduction

Acetaminophen (also known as paracetamol) is the only analgesic recommended by doctors for pregnant women, as prenatal exposure to other drugs commonly used to treat pain and fever such as aspirin and non-steroidal anti-inflammatory drugs (e.g. ibuprofen, indomethacin) have previously been associated with birth defects, premature ductus arteriosus closure, and miscarriage.^{12-15,97-100} Accordingly, acetaminophen is the most commonly used over-the-counter pain medication taken during pregnancy, with use reported by over half of pregnant women in many populations.^{10,11} During the last two decades, however, research from a multitude of diverse birth cohort studies has revealed consistent associations of prenatal acetaminophen exposure with adverse childhood outcomes including asthma, attention deficit hyperactivity disorder (ADHD), and autism.¹⁷⁻¹⁹ The severity of these adverse outcomes combined with such high rates of prenatal acetaminophen exposure makes further research an urgent public health priority. Indeed, a recent consensus statement supported by 91 scientists, clinicians and public health professionals from across the globe calls on health professionals to caution against the indiscriminate use of acetaminophen during pregnancy.¹⁰¹

One possibility is that the development of childhood disorders associated with prenatal acetaminophen exposure may be mediated via adverse birth outcomes, such as reduced birth weight and preterm birth. For instance, birth cohort studies have shown associations of pre-pregnancy and prenatal acetaminophen exposure with low birthweight¹⁰² and preterm birth¹¹ respectively, and large meta-analyses have shown associations of low birth weight and preterm birth with asthma,^{103,104} ADHD,^{105,106} and autism.^{107,108}

The limited number of studies reporting associations of prenatal acetaminophen with birth outcomes may be a consequence of inaccurate exposure assessment. To the best of our knowledge, all but two cohort studies investigating the effects of prenatal acetaminophen exposure on children's health have relied on mothers to self-report their acetaminophen use during pregnancy.^{21,109} Furthermore, the only studies to show associations of prenatal acetaminophen with birth outcomes administered questionnaires during pregnancy and postpartum to assess maternal acetaminophen use.^{11,102} Consequently, adverse birth outcomes could have influenced maternal responses in the postpartum interviews; when outcomes are suboptimal, women might be more likely to recall any potential explanatory behavior.¹¹⁰⁻¹¹² Self-reported exposure assessment could also result in misclassification bias towards the null.

The possibility of misclassification bias can be eliminated by measuring prenatal acetaminophen exposure in a biological sample rather than relying on maternal self-report. Measuring chemicals in meconium, the first feces of newborn infants, has proven to be an effective, non-invasive method to assess cumulative prenatal exposures.^{70,109,113} Chemicals in meconium are known to have passed through the fetus and into the fetal intestinal tract,^{22,23,25,26} making meconium an ideal substrate for measuring *in utero* exposures. Furthermore, meconium measurements reflect cumulative exposures during the 2nd and 3rd trimesters of pregnancy, as xenobiotics and their metabolites are deposited in meconium throughout that period.^{22,23,25,26} Approximately 12% of all deliveries show evidence of meconium stained amniotic fluid, indicating that meconium was passed *in utero*.¹¹⁴ Whether meconium is passed *in utero* is likely non-random: risk factors for meconium stained amniotic fluid include advanced gestational age and prolonged labor.¹¹⁵ It is therefore important to account for selection bias when relying on exposures measured in this substrate. The primary aim of this study was to evaluate the

association of acetaminophen measured in meconium with birth outcomes and pregnancy complications using inverse probability weighting methods to account for confounding and selection bias. The secondary aim was to explore the hypothesis that adverse birth outcomes may link prenatal acetaminophen with ADHD using causal mediation methods.

3.2 Methods

Cohort selection

This analysis was conducted in the GESTation and the Environment (GESTE) cohort in Sherbrooke, Quebec, Canada. The cohort was initially designed to examine the effects of environmental contaminants on endocrine disruption. Women age ≥ 18 years without chronic medical conditions and with no known thyroid disease enrolled at the Research Center of the CHUS (Centre Hospitalier Universitaire de Sherbrooke) from September 25, 2007, to September 10, 2009, at their first prenatal care visit or delivery. Recruitment at delivery excluded very preterm births before 33 weeks completed gestation. Among the 800 women recruited, 37 were excluded due to loss to follow up or miscarriage and 10 gave birth to twins, resulting in 773 live births (Figure 1). All study protocols were approved by the institutional review boards of the University of Sherbrooke and Columbia University.

Exposure

Meconium was collected from diapers of infants after delivery and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Acetaminophen was extracted from < 120 mg meconium and analyzed with ultraperformance liquid chromatography mass spectrometry following the methods described elsewhere.⁷⁰ Among all 773 live births, the eligible study sample was 393 individuals for whom meconium was collected at delivery (Figure 1). Meconium was not always collected, for instance

if the infant passed it in utero, if the diaper was thrown in the trash before the technician was able to collect it, or if we were unable to finance technician time for collecting the sample. These potential sources of selection bias were addressed by weighting on the inverse probability of selection (see statistical analysis for details). Acetaminophen was measured with a recovery of 104% and repeatability of $\pm 15\%$. The limit of detection (LOD) and limit of quantification (LOQ) were 2 ng/g and 5 ng/g respectively.

Outcome

Data for birthweight and gestational age were obtained from CHUS medical records. Infants were weighed on Scale-tronix pediatrics scale 4802 by the obstetric team. Preterm birth was defined as birth before 37 completed weeks of gestation. Small for gestational age (SGA) and large for gestational age (LGA) were defined as birthweights below the 10th percentile and above the 90th percentile for gestational age, respectively. We assigned SGA and LGA categories and computed birthweight for gestational age z-scores in accordance with the Fenton international growth chart.¹¹⁶ Data on pregnancy complications, including gestational diabetes, preeclampsia, and high blood pressure, were obtained from CHUS medical records. Complete birth outcome data were available for all 393 individuals with meconium collected at delivery.

Data on physician diagnosis of ADHD were obtained at a cohort follow-up when children were 6-7 years old or from medical records. These data were available for 345 individuals among the 393 individual study sample (Figure 1).

Statistical analysis

Covariate data were obtained from CHUS medical records and questionnaires administered after delivery. Covariates were child sex, familial income, and maternal characteristics including age at delivery, education status (College/University vs. no

College/University), pre-pregnancy BMI, smoking during pregnancy (yes/no), and alcohol during pregnancy (yes/no).

Differences in baseline characteristics between 1) individuals with and without prenatal acetaminophen exposure, and 2) individuals with and without meconium collected at delivery were determined using chi-square goodness of fit tests for binary variables and two-sample t-tests for continuous variables. Using linear regression, we estimated associations of meconium acetaminophen detection (yes vs. no) with birthweight in grams and birthweight for gestational age z-score. Using logistic regression, we estimated associations of meconium acetaminophen detection with SGA, LGA, preterm birth, and maternal gestational diabetes, preeclampsia, and high blood pressure. Using cox proportional hazards models with birth as the event and gestational age as the time to event, we estimated the association of meconium acetaminophen detection with the hazard for giving birth. Because birth (the event) occurred for all individuals, increased hazards in these models indicate shorter gestational age (the time to event). Confidence intervals for cox models were calculated with robust standard errors. Coefficients from logistic regressions and cox models were exponentiated into odds ratios and hazard ratios respectively. To account for missing covariate data, all models were employed on 10 datasets imputed using the 'MICE' R package.¹¹⁷ Estimates and standard errors from imputed datasets were combined using Rubin's Rule.^{118,119}

In addition to unadjusted models, we report models adjusted for the covariates described above. We controlled for covariates by inverse probability of exposure weighting (IPW) using propensity scores.⁷³⁻⁷⁶ Propensity scores (p, the likelihood of detectable meconium acetaminophen) were estimated using logistic regression models in which exposure (meconium acetaminophen detected vs. not detected) was regressed on the covariates described above.

Weights were estimated as $1/p$ for exposed individuals, and $1/(1 - p)$ for unexposed individuals. Study sample weighting creates a pseudo-population balanced on measured baseline covariates.⁷³⁻⁷⁵

We additionally addressed potential selection bias related to meconium collection by weighting models on both the inverse probability of exposure described above and the inverse probability of selection. We first created weights for the probability of selection using logistic regression models in which selection (meconium collected vs. not collected) was regressed on the covariates described above for the entire 810 individual cohort (800 recruited women, 10 sets of twins). Then we removed those without meconium data and created weights for the probability of exposure as described above before fitting weighted models of the effect of meconium acetaminophen exposure on birth outcomes. Weighting the 393 individual selected sample on the probability of selection creates a pseudo-population that is comparable on measured covariates to the entire 810 individual cohort.

We computed E-values to assess the potential for unmeasured confounding.¹²⁰ E-values indicate the minimum strength of association, on the risk ratio scale, of an unmeasured confounder with both the exposure and outcome that would confound a null effect to the observed effect estimate (i.e. completely explain the observed association between the exposure and outcome). In calculating E-values, all effects are converted to the risk ratio scale, with continuous outcomes dichotomized based on the exposure effect size on the outcome (Cohen's d). E-values were calculated for birth outcomes that were significantly associated with meconium acetaminophen based on covariate adjusted models.

In this cohort, we have previously shown an association of prenatal acetaminophen with increased odds for ADHD,¹⁰⁹ and associations of ADHD with adverse birth outcomes including

preterm birth and lower birthweight.¹¹³ Here, we additionally assessed whether birth outcomes mediated the relationship between prenatal acetaminophen exposure and ADHD, in separate models for each birth outcome, using the ‘mediation’ R package,⁷⁹ which implements a quasi-Bayesian Monte Carlo method with 1,000 simulations. To test for exposure-mediator interaction, we modeled interactions between meconium acetaminophen and birth outcomes on ADHD in separate models for each birth outcome. Because we found no significant interaction terms, we assumed no exposure-mediator interaction in the mediation models. We estimated the total and direct effects of prenatal acetaminophen on ADHD, as well as the causal mediation (i.e., natural indirect) effects through each birth outcome. We included the covariates discussed above as terms in the mediation models to control for confounders of the exposure-mediator, exposure-outcome, and mediator-outcome relationships. Because quasi-Bayesian confidence intervals cannot be combined with Rubin’s Rule, we report separate covariate adjusted mediation models for each imputed dataset. Statistical analyses were conducted with R, version 3.5.1.⁸⁰

3.3 Results

Baseline covariates stratified by meconium acetaminophen detection are presented in Table 1. Acetaminophen was detected in the meconium of 222 individuals (56.5%) among the total study sample of 393 (Table 1).

Accounting for all covariates, prenatal acetaminophen exposure was associated with decreased birthweight by 136 grams ($\beta = -136$; 95% CI -229, -43) and decreased birthweight for gestational age z-score ($\beta = -0.17$; 95% CI -0.34, 0.00) (Table 2). Consistent with these associations with decreased birthweight, prenatal acetaminophen was also associated with over 60% decreased odds of LGA (odds ratio [OR] = 0.38; 95% CI 0.20, 0.75) (Table 2). In addition to their decreased birthweight and lower likelihood of LGA, the mean time of gestation was on

average 0.3 weeks shorter among individuals exposed to acetaminophen *in utero* compared to the unexposed group (39.0 vs. 39.3 weeks). Accordingly, prenatal acetaminophen exposure was associated with a 20% increased weekly hazard of delivery (hazard ratio = 1.20; 95% CI 1.00, 1.43), indicating a higher likelihood for earlier delivery and thus reduced gestational age in exposed individuals (Table 2). Our data suggest that prenatal acetaminophen was not associated with SGA or preterm birth (Table 2).

We conducted sensitivity analyses for selection bias and unmeasured confounding. Mothers of children for whom meconium was collected were significantly older and more educated (eTable 1), indicating the potential for selection bias. Controlling for selection bias by weighting models to account for these covariate differences, however, did not appreciably impact the estimates of the effects of meconium acetaminophen detection on any birth outcome, suggesting minimal bias related to meconium sampling in this cohort (Table 2). Sensitivity analyses for unmeasured confounding show that an unmeasured confounder would need to increase the risk of both prenatal acetaminophen exposure and low birthweight by 93% in order to explain away the association of prenatal acetaminophen with reduced birthweight (E-value = 1.93, eTable 2). E-values were greater than 1.5 for all other birth outcomes that were significantly associated with meconium acetaminophen (eTable 2).

Meconium acetaminophen was not associated with pregnancy complications including gestational diabetes, preeclampsia, or high blood pressure (Table 3).

In this cohort, we previously reported an association of prenatal acetaminophen exposure with more than two-fold increased odds of child ADHD.¹⁰⁹ Consistent with prior work, we found significant direct and total effects of meconium acetaminophen on ADHD in all mediation models (see Table 4 for unadjusted models, see eTable 3 for adjusted models in 10 datasets

imputed for missing covariates). Causal mediation effects, however, were non-significant for all birth outcomes in both unadjusted and adjusted models.

3.4 Discussion

Principal findings

In this Eastern Canadian birth cohort, detection of acetaminophen in meconium was associated with decreased birthweight, decreased gestational age, and decreased odds of LGA. Although meconium was only collected for approximately half of our eligible births, we found no evidence that selection bias impacted effect estimation: associations of meconium acetaminophen with outcomes were nearly identical in both models adjusted and not adjusted for selection bias. While adverse birth outcomes such as preterm birth and reduced birthweight are known to be associated with ADHD, we found no evidence for mediation by birth outcomes of the association between prenatal acetaminophen exposure and ADHD in this cohort.

Strengths of the study

Our study has several strengths. First, the high genetic and sociodemographic homogeneity in the GESTE cohort limits the likelihood of confounding by unknown genetic or sociodemographic factors. Second, the prospective nature of the cohort limits sources of bias common in retrospective designs, including selection and recall bias. Third, we explicitly controlled for known sources of confounding and selection bias. Finally, our measurement of prenatal acetaminophen exposure in meconium eliminates the possibility of recall bias.

Limitations of the data

This study has limitations. First, while the homogeneity of the GESTE cohort may limit confounding, it also lowers the generalizability of results. Second, this study had a relatively small sample size of just under 400 mother child pairs. Consequently, there were few events for

several outcomes, including early prematurity and severe preeclampsia. Third, our conclusions regarding preterm birth may be limited by the exclusion of some extremely preterm deliveries. Although unlikely, this could be a source of selection bias if there are different effects of prenatal acetaminophen exposure on the risk for preterm birth before 33 weeks versus before 37 weeks. Fourth, we lacked the information necessary to control for indications for acetaminophen use, such as chronic pain, fever, and infections during pregnancy. However, when controlling for indications for acetaminophen in the Danish National Birth Cohort study, Rebordosa and colleagues still observed associations of prenatal acetaminophen with increased risk of preterm birth.¹¹ However, results from other cohorts may not be generalizable to this Eastern Canadian population. Therefore, confounding by indication remains a possibility. Another possibility is that acetaminophen was more easily detected in the meconium of smaller infants owing to less efficient metabolism. However, acetaminophen pharmacokinetics in the fetus parallels that in the mother, with fetal and maternal acetaminophen reaching comparable levels as early as 30 minutes after maternal administration.¹²¹ Inverse causality is thus unlikely. Finally, we did not ask women to self-report their use of acetaminophen during pregnancy, so we were unable to correlate acetaminophen intake with levels of acetaminophen in meconium. Future studies are needed to determine the dosage and timing of acetaminophen required to have detectable levels in meconium.

Interpretation

A small number of studies have previously shown associations between maternal self-reported acetaminophen use during pregnancy and adverse birth outcomes. In the Danish National Birth Cohort, there was an increased risk of preterm birth among women using acetaminophen during the third trimester of pregnancy, but there were no associations of

acetaminophen use with miscarriage, stillbirth, low birth weight, or SGA, or with common preterm birth complications including bronchopulmonary dysplasia, intracranial hemorrhage, retinopathy of prematurity, perinatal infections and anemia of prematurity.¹¹ In the Ontario Birth Study, maternal acetaminophen use in the 3 months before pregnancy was associated with low birthweight and increased risk for SGA, but maternal acetaminophen use during pregnancy was not.¹⁰² However, both of those studies were prone to substantial recall bias, as they assessed maternal acetaminophen use via questionnaires administered during pregnancy and postpartum. When self-report occurs in postpartum interviews, mothers of infants with adverse birth outcomes may rack their brains for an explanation, thereby overreporting exposures.¹¹⁰⁻¹¹² Our study, on the other hand, utilized a direct measurement of prenatal acetaminophen exposure measured in meconium that is unbiased by inaccurate recall.

While the associations of prenatal acetaminophen exposure with adverse birth outcomes found here may be concerning, more studies in a diverse range of cohorts are needed before suggesting a change in clinical practice. Additionally, mechanisms underlying the associations of prenatal acetaminophen exposure with adverse birth outcomes remain unknown. Acetaminophen may inhibit prostacyclin synthesis and thereby promote pre-eclampsia,¹²² which has previously been associated with intrauterine growth restriction and reduced gestational age.¹²³ Acetaminophen exposure may also trigger the immune system and upregulate oxidative stress response pathways¹²⁴ that may underlie adverse birth outcomes. A better understating of the mechanisms through which prenatal acetaminophen exposure may affect birth outcomes is needed, not only to better assess causality, but also to serve as potential targets in future intervention studies.

3.5 Conclusions

While this study may add evidence that support questioning the safety of acetaminophen use during pregnancy, more work is needed to rule out confounding by indication and to assess generalizability before a change in clinical practice is recommended. Additionally, our data do not support adverse birth outcomes as the pathway through which acetaminophen may affect the risk of ADHD. Thus, further work to delineate the pathophysiology of prenatal acetaminophen and brain development is warranted.

3.6 Tables

Table 1: Characteristics of study sample stratified by detection of acetaminophen in meconium in the GESTation and the Environment (GESTE) cohort (n = 393)

	No acetaminophen (N=171)	Acetaminophen (N=222)	Total (N=393)	P value
Sex				0.968
Female	82 (48.0%)	106 (47.7%)	188 (47.8%)	
Male	89 (52.0%)	116 (52.3%)	205 (52.2%)	
Maternal age at delivery (years)				0.986
Mean (SD)	28.9 (4.7)	28.9 (4.5)	28.9 (4.6)	
Range	18.0 - 43.0	19.0 - 41.0	18.0 - 43.0	
Maternal education				0.980
No College or University	68 (39.8%)	88 (39.6%)	156 (39.7%)	
College or University	103 (60.2%)	134 (60.4%)	237 (60.3%)	
Family income (Canadian dollars)				0.596
N-Miss	21	18	39	
Mean (SD)	69,874 (47,968)	67,235 (44,858)	68,353 (46,153)	
Range	2,600 – 500,000	8000 – 450,000	2,600 – 500,000	
Maternal BMI (kg/m ²)				0.020
N-Miss	0	1	1	
Mean (SD)	24.9 (5.2)	26.2 (5.9)	25.7 (5.6)	
Range	17.9 - 45.2	17.7 - 49.1	17.7 - 49.1	
Smoked during pregnancy				0.927
N-Miss	8	5	13	
No	141 (86.5%)	187 (86.2%)	328 (86.3%)	
Yes	22 (13.5%)	30 (13.8%)	52 (13.7%)	
Alcohol during pregnancy				0.161
N-Miss	8	5	13	

No	120 (73.6%)	173 (79.7%)	293 (77.1%)	
Yes	43 (26.4%)	44 (20.3%)	87 (22.9%)	

Table 2: Association of meconium acetaminophen with birth outcomes (N=393)

^a Coefficients from linear regression shown for birthweight and birthweight for gestational age z-

	Mean or n (%)		Coefficient, Hazard Ratio, or Odds Ratio ^a		
	Acetamino phen	No Acetaminophen	Unadjusted	Covariate adjusted ^b	Selection bias adjusted ^c
Birthweight (g)	3338	3459	-121 [-213, -28]	-136 [-229, -43]	-141 [-232, -49]
Birthweight for gestational age z-score	-0.129	0.000	-0.13 [-0.3, 0.04]	-0.17 [-0.34, 0]	-0.18 [-0.35, -0.01]
Gestational age (weeks)	39.0	39.3	1.23 [1, 1.5] ^d	1.2 [1, 1.43] ^d	1.21 [1.01, 1.45] ^d
Small for gestational age	15 (6.8)	13 (7.6)	-0.13 [-0.9, 0.64]	-0.08 [-0.63, 0.46]	0.01 [-0.37, 0.39]
Large for gestational age	7 (3.2)	12 (7.0)	0.43 [0.17, 1.12]	0.38 [0.2, 0.75]	0.36 [0.22, 0.58]
Preterm birth	8 (3.6)	9 (5.3)	0.67 [0.25, 1.78]	0.7 [0.35, 1.41]	0.62 [0.38, 1.02]

score. Odds ratios from logistic regression shown for small for gestational age (SGA), large for gestational age (LGA), and preterm birth. Hazard ratios from cox proportional hazard models shown for gestational age.

^b Adjusted for covariates by inverse probability of exposure weighting using child sex, familial income, and maternal age, education, BMI, smoking during pregnancy, and alcohol during pregnancy to predict exposure.

^c Adjusted for covariates by inverse probability of exposure weighting and for selection bias by weighting on the inverse of the probability of selection.

^d Ratio for instantaneous hazard of delivery.

Table 3: Associations of meconium acetaminophen with pregnancy complications (N=393)

	Mean or n (%)		Odds Ratio		
	Acetaminophen	No Acetaminophen	Unadjusted	Covariate adjusted ^a	Selection bias adjusted ^b
Gestational diabetes	30 (13.5)	20 (11.7)	1.18 [0.64, 2.16]	1.05 [0.69, 1.6]	1.02 [0.76, 1.37]
Preeclampsia	3 (1.4)	2 (1.2)	1.16 [0.19, 7.01]	1.01 [0.29, 3.53]	0.88 [0.36, 2.16]
High blood pressure	19 (8.6)	11 (6.4)	1.36 [0.63, 2.94]	1.08 [0.64, 1.83]	1.13 [0.77, 1.64]

^a Adjusted for covariates by inverse probability of exposure weighting using child sex, familial income, and maternal age, education, BMI, smoking during pregnancy, and alcohol during pregnancy to predict exposure.

^b Adjusted for covariates by inverse probability of exposure weighting and for selection bias by weighting on the inverse of the probability of selection.

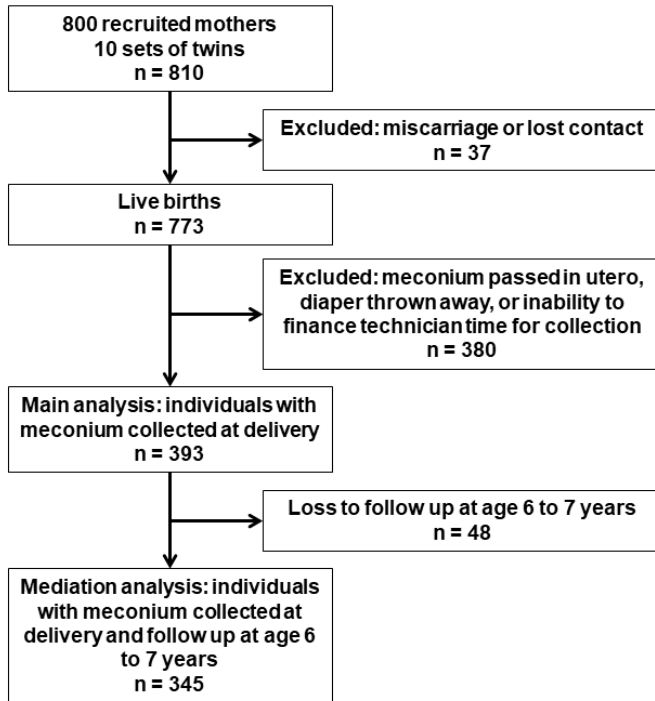
Table 4: Analysis of mediation of the association between prenatal acetaminophen and ADHD by birth outcomes (N = 345) ^a

Mediator	Total effect on ADHD		Direct effect of prenatal acetaminophen		Average causal mediation effect	
	Estimate	P value	Estimate	P value	Estimate	P value
Birthweight	0.07 [0.01, 0.14]	0.028	0.07 [0, 0.13]	0.036	0.007 [-0.003, 0.021]	0.188
Birthweight for gestational age z-score	0.07 [0.01, 0.13]	0.024	0.07 [0.01, 0.13]	0.032	0.004 [-0.003, 0.015]	0.314
Gestational age	0.07 [0.01, 0.13]	0.032	0.07 [0.01, 0.13]	0.036	0.002 [-0.005, 0.011]	0.612
Small for gestational age	0.07 [0.01, 0.13]	0.02	0.08 [0.01, 0.14]	0.018	-0.007 [-0.028, 0.009]	0.354
Large for gestational age	0.07 [0.01, 0.13]	0.028	0.07 [0.01, 0.13]	0.028	0 [-0.005, 0.006]	0.89
Preterm birth	0.07 [0.01, 0.13]	0.028	0.07 [0.01, 0.13]	0.032	0 [-0.015, 0.009]	0.818

^a Unadjusted models shown. See eTable 3 for adjusted models in 10 datasets imputed for missing covariates.

3.7 Figures

Figure 1: GESTation and the Environment (GESTE) cohort flowchart



3.8 Supplement

eTable 1: Characteristics of study sample stratified by meconium collection in the GESTation and the Environment (GESTE) cohort (n = 810)

	Meconium collected (N=393)	No meconium collected (N=417)	Total (N=810)	p value
Sex				0.429
N-Miss	0	37	37	
Female	188 (47.8%)	171 (45.0%)	359 (46.4%)	
Male	205 (52.2%)	209 (55.0%)	414 (53.6%)	
Maternal age at delivery				0.004
N-Miss	0	33	33	
Mean (SD)	28.91 (4.56)	27.97 (4.62)	28.45 (4.61)	
Range	18.00 - 43.00	18.00 - 41.00	18.00 - 43.00	
Maternal education				< 0.001
No College or University	156 (39.7%)	227 (54.4%)	383 (47.3%)	
College or University	237 (60.3%)	190 (45.6%)	427 (52.7%)	
Family income (Canadian dollars)				0.052
N-Miss	39	93	132	
Mean (SD)	68353.67 (46153.47)	62420.68 (30869.48)	65518.44 (39675.38)	
Range	2600.00 - 500000.00	7000.00 - 180000.00	2600.00 - 500000.00	
Maternal BMI				0.145
N-Miss	1	7	8	
Mean (SD)	25.67 (5.64)	25.06 (6.16)	25.35 (5.91)	

Range	17.71 - 49.08	14.99 - 55.60	14.99 - 55.60	
Smoked during pregnancy				0.163
N-Miss	13	73	86	
No	328 (86.3%)	284 (82.6%)	612 (84.5%)	
Yes	52 (13.7%)	60 (17.4%)	112 (15.5%)	
Alcohol during pregnancy				0.406
N-Miss	13	73	86	
No	293 (77.1%)	274 (79.7%)	567 (78.3%)	
Yes	87 (22.9%)	70 (20.3%)	157 (21.7%)	

eTable 2: E-value sensitivity analysis for unmeasured confounding

Outcome	Cohen's d cutoff for dichotomized continuous outcome ^a	Risk ratio conversion ^b	E-value ^c
Birthweight (continuous)	-0.29	0.77 (0.64, 0.92)	1.93
Birthweight z-score (continuous)	-0.21	0.83 (0.69, 0.99)	1.7
Gestational age (hazard ratio for delivery)	NA	1.12 (1.00, 1.28)	1.52
Large for gestational age (odds ratio)	NA	0.38 (0.20, 0.75)	4.64

^a Continuous outcomes are dichotomized based on the effect size (Cohen's d), computed as the linear regression estimate divided by the standard deviation of the outcome.

^b All estimates are converted to risk ratios for calculation of the E-value. See VanderWeele (2017) for details and conversion formulae.

^c E-values indicate the minimum strength of association, on the risk ratio scale, of an unmeasured confounder with both the exposure and outcome that would completely explain the observed association between the exposure and outcome.

eTable 3: Covariate adjusted analysis of mediation of the association between prenatal acetaminophen and ADHD by birth outcomes in 10 datasets imputed for missing covariates (N = 345)

Mediator	ACME estimate	ACME lower CI	ACME upper CI	ADE estimate	ADE lower CI	ADE upper CI	Total effect estimate	Total effect lower CI	Total effect upper CI
birthweight	0.004	-0.008	0.019	0.069	0.009	0.139	0.072	0.014	0.142
birthweight	0.005	-0.007	0.020	0.071	0.011	0.140	0.074	0.016	0.142
birthweight	0.005	-0.008	0.020	0.071	0.011	0.141	0.074	0.016	0.143
birthweight	0.005	-0.008	0.020	0.071	0.010	0.140	0.073	0.015	0.144
birthweight	0.005	-0.007	0.021	0.070	0.010	0.140	0.073	0.015	0.144
birthweight	0.004	-0.008	0.020	0.069	0.007	0.139	0.071	0.013	0.142
birthweight	0.005	-0.007	0.020	0.069	0.008	0.139	0.072	0.013	0.142
birthweight	0.005	-0.007	0.020	0.071	0.010	0.140	0.074	0.015	0.144
birthweight	0.005	-0.008	0.020	0.070	0.009	0.139	0.073	0.014	0.143
birthweight	0.005	-0.007	0.021	0.070	0.010	0.140	0.073	0.015	0.144
birthweight for gestational age zscore	0.002	-0.007	0.014	0.070	0.011	0.138	0.071	0.013	0.138
birthweight for gestational age zscore	0.003	-0.006	0.014	0.072	0.014	0.141	0.073	0.015	0.141
birthweight for gestational age zscore	0.002	-0.007	0.015	0.072	0.014	0.140	0.073	0.015	0.140
birthweight for gestational age zscore	0.003	-0.007	0.015	0.071	0.013	0.140	0.073	0.014	0.139

birthweight for gestational age zscore	0.003	-0.006	0.015	0.071	0.012	0.139	0.073	0.014	0.139
birthweight for gestational age zscore	0.002	-0.007	0.014	0.069	0.009	0.138	0.070	0.011	0.138
birthweight for gestational age zscore	0.002	-0.007	0.014	0.070	0.010	0.137	0.071	0.012	0.139
birthweight for gestational age zscore	0.002	-0.007	0.015	0.071	0.013	0.139	0.073	0.014	0.140
birthweight for gestational age zscore	0.002	-0.007	0.014	0.070	0.011	0.139	0.072	0.013	0.139
birthweight for gestational age zscore	0.003	-0.007	0.015	0.071	0.012	0.139	0.072	0.014	0.139
gestational age	0.001	-0.006	0.009	0.071	0.012	0.142	0.071	0.012	0.142
gestational age	0.001	-0.006	0.010	0.073	0.014	0.142	0.074	0.014	0.144
gestational age	0.001	-0.006	0.009	0.073	0.014	0.144	0.073	0.014	0.144
gestational age	0.001	-0.006	0.009	0.072	0.014	0.143	0.073	0.014	0.143
gestational age	0.001	-0.006	0.009	0.072	0.014	0.144	0.073	0.014	0.143
gestational age	0.001	-0.006	0.009	0.070	0.010	0.142	0.070	0.011	0.141
gestational age	0.001	-0.006	0.009	0.070	0.011	0.143	0.071	0.011	0.142
gestational age	0.001	-0.006	0.009	0.072	0.014	0.144	0.073	0.014	0.144
gestational age	0.001	-0.006	0.009	0.071	0.012	0.143	0.072	0.012	0.143
gestational age	0.001	-0.006	0.009	0.072	0.013	0.144	0.072	0.013	0.143

small for gestational age	0.000	-0.008	0.007	0.070	0.011	0.137	0.070	0.012	0.138
small for gestational age	0.000	-0.007	0.008	0.072	0.015	0.140	0.072	0.015	0.140
small for gestational age	0.000	-0.007	0.007	0.072	0.014	0.139	0.072	0.015	0.140
small for gestational age	0.000	-0.008	0.008	0.071	0.013	0.140	0.071	0.015	0.140
small for gestational age	0.000	-0.007	0.007	0.071	0.014	0.140	0.071	0.013	0.140
small for gestational age	0.000	-0.008	0.007	0.069	0.009	0.137	0.069	0.011	0.137
small for gestational age	0.000	-0.007	0.008	0.070	0.011	0.138	0.070	0.012	0.138
small for gestational age	0.000	-0.007	0.007	0.071	0.013	0.139	0.071	0.014	0.138
small for gestational age	0.000	-0.008	0.007	0.070	0.012	0.138	0.070	0.013	0.139
small for gestational age	0.000	-0.008	0.007	0.071	0.013	0.139	0.071	0.013	0.139
large for gestational age	-0.002	-0.019	0.008	0.072	0.010	0.146	0.071	0.011	0.144
large for gestational age	-0.001	-0.018	0.009	0.073	0.012	0.144	0.073	0.014	0.142
large for gestational age	-0.001	-0.019	0.008	0.074	0.013	0.147	0.073	0.013	0.145
large for gestational age	-0.001	-0.019	0.009	0.073	0.012	0.146	0.072	0.013	0.143

large for gestational age	0.000	-0.018	0.010	0.072	0.011	0.144	0.071	0.013	0.140
large for gestational age	-0.001	-0.019	0.008	0.071	0.008	0.146	0.070	0.010	0.144
large for gestational age	-0.001	-0.018	0.008	0.072	0.009	0.146	0.071	0.010	0.144
large for gestational age	-0.001	-0.019	0.009	0.072	0.012	0.145	0.071	0.012	0.144
large for gestational age	-0.001	-0.018	0.008	0.072	0.010	0.146	0.071	0.011	0.144
large for gestational age	-0.001	-0.018	0.009	0.072	0.010	0.145	0.071	0.012	0.142
preterm birth	-0.006	-0.026	0.008	0.077	0.017	0.143	0.073	0.013	0.141
preterm birth	-0.006	-0.027	0.010	0.079	0.020	0.146	0.075	0.016	0.143
preterm birth	-0.006	-0.027	0.009	0.079	0.020	0.145	0.075	0.015	0.143
preterm birth	-0.005	-0.025	0.009	0.078	0.019	0.144	0.074	0.015	0.142
preterm birth	-0.006	-0.026	0.010	0.078	0.019	0.145	0.074	0.015	0.143
preterm birth	-0.006	-0.026	0.008	0.076	0.015	0.143	0.072	0.012	0.140
preterm birth	-0.006	-0.026	0.009	0.077	0.017	0.144	0.073	0.013	0.141
preterm birth	-0.005	-0.026	0.010	0.078	0.019	0.144	0.075	0.016	0.142
preterm birth	-0.006	-0.026	0.008	0.077	0.018	0.143	0.073	0.013	0.141
preterm birth	-0.006	-0.026	0.009	0.078	0.019	0.144	0.074	0.015	0.142

Chapter 4: Sex-Specific Neurobehavioral and Frontal Cortex Gene Expression Alterations Following Developmental Acetaminophen Exposure in Mice

Brennan H. Baker,¹ Elizabeth E. Rafikian,² Paul B. Hamblin,² Madeleine D. Strait,³ Mu Yang,²
Brandon L. Pearson¹

¹Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, New York

²The Mouse Neurobehavior Core, Institute of Genomic Medicine, Columbia University College of Physicians and Surgeons, New York, New York

³Department of Biology, University of Washington, Seattle, Washington

4.1 Introduction

Acetaminophen (N-acetyl-p-aminophenol (APAP), also known as paracetamol) is the most common pharmaceutical ingredient in the United States: it is the active ingredient in over 600 prescription and over-the-counter drugs that treat pain, fever, cough, cold, and allergies.^{125,126} APAP-containing drugs are among the only analgesics not contraindicated during pregnancy.⁶⁵ Consequently, their use is reported by over half of pregnant women in many populations.¹²⁷⁻¹²⁹

Despite such widespread use, evidence from human observational studies suggests that prenatal APAP exposure may be associated with attention deficit hyperactivity disorder (ADHD),¹³⁰⁻¹⁴² autism spectrum disorders (ASD),^{130,135,143} and a multitude of other behavioral and neurodevelopmental abnormalities.¹²⁵ Although many of these observational studies accounted for potential confounding by indications, including fever and headaches, and maternal characteristics, including age at birth, race/ethnicity, body mass index, neuropsychiatric conditions, and tobacco and alcohol use, unobserved and unmeasured confounding remain a possibility. For instance, maternal polygenic risk scores for ADHD are associated with use of APAP during late pregnancy,¹⁴⁴ indicating a strong potential for genetic confounding, yet no observational studies of prenatal APAP and child ADHD have explicitly accounted for genetics. Furthermore, a sibling study to examine unmeasured familial confounding in the Norwegian national cohort found a substantial family effect, suggesting that unmeasured familial factors, including genetics, may partially explain the association of maternal APAP use with child ADHD.¹³⁴

In addition to human observational studies, animal models have been employed to investigate health risks associated with APAP exposure. Supporting the human epidemiological

evidence, various randomized experimental studies have found adverse effects of pre- and early postnatal APAP exposure on rodent behavior and cognition.¹⁴⁵⁻¹⁵³ Although translating complex neurodevelopmental disorders to animal behaviors can be challenging, and mechanisms may sometimes differ between humans and animals, animal models maintain several advantages over human studies. First, confounding is impossible in experimental studies that randomize animals to control or treatment groups. Additionally, mechanisms are easier to explore in animals. Treatments designed to explore specific mechanisms are more ethical and practical to implement in animals, and target tissues, such as those in the central nervous system, can be readily collected and studied. Finally, novel treatments to block the pathways between exposures and diseases cannot be ethically studied in humans without first being tested in animal models.

Despite these advantages, studies of neurodevelopmental effects of prenatal APAP exposure in animal models remain limited. Although many animal studies have uncovered behavior and brain abnormalities following prenatal acetaminophen exposure,¹⁴⁵⁻¹⁵³ the results of these studies are not always in agreement. For instance, one study found no behavioral effects of prenatal acetaminophen exposure in mice.¹⁵⁴ Second, while many observational studies have linked prenatal APAP with child ADHD in humans,¹³⁰⁻¹⁴² no mouse studies of prenatal APAP exposure have investigated offspring attention deficits in behavior tasks specifically designed to measure attention. Finally, the mechanisms linking APAP exposure to abnormal neurodevelopment are unclear. Rodent studies have explored APAP effects on the cannabinoid^{151,155} and prostaglandin¹⁵⁶ pathways, altered neurotransmission,^{152,157-160} brain-derived neurotrophic factor,^{146,150,153} neuronal number in the sexually dimorphic nucleus of the hypothalamus,¹⁴⁵ and oxidative stress.^{146,148} However, no studies have employed ‘omics’ technologies for an untargeted exploration of potential mechanisms. Here, we address these

limitations by 1) examining the effect of pre- and early postnatal APAP on offspring behavior, including attention deficits in the 5-choice serial reaction time task; and 2) exploring mechanistic pathways that may underly the effects of APAP exposure via unbiased RNA sequencing.

4.2 Methods

Mice

Timed pregnant C57BL/6J females were purchased from the Jackson Laboratory (Bar Harbor, ME). Half were randomized to control and half to APAP treatments. Mice were ordered and randomized at an early embryonic stage, so pregnancy was not guaranteed. We therefore anticipated that the number of control and treatment might be uneven.

The first cohort of 12 dams were ordered at embryonic day 3 (E3) and randomized into control and APAP treatment groups at E4. Only 1 APAP-treated and 3 control mice were pregnant among the first cohort. Owing to this low pregnancy rate (25%), we ordered the second cohort of 28 timed pregnant females at E7 and randomized them at E10. The second cohort had 23 (82%) pregnancies. One litter in the second cohort had only 3 pups. All other litters in the second cohort were reduced to 4 pups per dam, with remaining offspring euthanized for tissue collection.

Treatment

Pregnant mice were given 150 mg/kg/day APAP (rounded to the nearest gram of mouse weight) or 0 mg APAP (control) in gelatin tablets. Gelatin tablets consisted of 14% gelatin powder, 5% sucrose, and 0.5% fruit flavor (SodaStream™ drops) in water. Mice received one 0.5 mL tablet each day with the appropriate APAP dose or control. One day prior to the start of treatment, all mice received a control gelatin tablet in their home cage to become habituated.

During treatment, pregnant mice were weighed daily to determine dosage, and monitored to ensure gelatin tablets were completely consumed (typically in under 5 minutes). The dose of 150mg/kg/day was chosen because it has previously been shown to result in the highest serum concentrations of acetaminophen without inducing liver toxicity in mice.¹⁶¹⁻¹⁶⁵ We continued to dose mice through postnatal day 14 because the peak brain growth that occurs during gestation in humans does not occur until the postnatal period in mice.

Behavior Tasks

Mice behavioral analyses were conducted in the Columbia Mouse NeuroBehavior core. Offspring of control and APAP treated dams from the 1st cohort (born October 9, 2020) were tested in the open field test, elevated plus maze, and 5-choice serial reaction time task. Offspring in the 2nd cohort (born February 8, 2021) underwent the same tasks, and were additionally tested for pup ultrasonic vocalizations, CatWalk XT (Noldus), and pre-pulse inhibition. Behavior tasks tested 21 offspring from the 1st cohort and 90 offspring from the 2nd cohort (n = 111).

Pup Ultrasonic Vocalizations (USV)

Pup ultrasonic vocalizations were measured for 6 pups per dam among 4 control and 4 treatment dams (22 female and 26 male pups) in the second cohort. In a random order, pups were removed one at a time from the dam and placed into an insulated chamber where USVs were recorded for 3 min by an ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16; Avisoft Bioacoustics, Berlin, Germany). Calls were recorded in 16-bit resolution with a sampling rate of 250 kHz by Avisoft Recorder software. Calls were manually counted from USV spectrograms displayed in Avisoft SASLab Pro by a trained investigator blind to

treatment status. Repeated testing occurred on postnatal days 2, 5, 8, and 11, and pup paw tattoos were employed to track individual pups.

Open Field Test (OFT)

Mice (7 weeks old) were allowed to roam freely in a clear Plexiglas open field arena (27.31 × 27.31 × 20.32 cm, Med Associates ENV-510) for 60 minutes. Activity Monitor Version 7 tracking software (Med Associates Inc.) was used in conjunction with infrared beams along the x, y, and z planes of the arena to automatically measure total distance traveled (ambulatory movement), time spent in the center versus edge zones, and the total number of rearings which were defined as the number of times the mouse disrupted two infrared beams by standing on its hind limbs.

Elevated Plus Maze (EPM)

Mice (6 weeks old) were evaluated in one trial in the elevated plus maze (EPM), an apparatus with two open arms (30 cm x 5 cm) and two closed arms (30 cm x 5 cm) extending from a central junction (5 cm x 5 cm). Mice were placed in the junction facing a closed arm and allowed to roam the maze for five-minutes, during which the time spent in the open arms, closed arms, and junction was recorded automatically by the MED-PC V 64 bit Software (Med Associates). Due to equipment malfunction, data were not recorded for 7 offspring in the first cohort. The task could not be re-run for these mice because their first trial partially habituated them to the maze.

CatWalk

Mice (8 weeks old) were allowed to ambulate freely across the CatWalk XT apparatus (Noldus Information Technology, Leesburg, VA), an illuminated walled glass walkway (130 cm x 10 cm) until they completed three full crossings without stopping/hesitation. During the task,

walking patterns were captured by a high-speed camera underneath the walkway, and CatWalk XT software was used to automatically measure 226 parameters related to mouse gait and locomotion.

Pre-pulse inhibition (PPI)

Each mouse (29-30 weeks old) was placed inside a plexiglass tube and subjected to a five-minute habituation period with ambient light and a background noise of 68 dB. Following habituation, each mouse was presented with seven trial types across six discrete blocks of trials for a total of 42 trials of noise stimuli separated by randomly generated inter-trial intervals ranging between 10-20 seconds. Trials were presented pseudo randomly such that each type occurred once within each block. Mouse startle response was measured as the pressure exerted against the tube, which was translated into a voltage. One trial measured the response to 100 ms 68 dB background noise (baseline), and another measured the response to a 40 ms 110 dB sound burst. The remaining five trial types consisted of a 20 ms pre-pulse, followed by 100 ms background, then a 40 ms, 110 burst. Pre-pulses were 74, 78, 82, 86, or 92 dB. PPI % inhibition was calculated separately for each pre-pulse dB level as: $(\text{response to 110 dB alone} - \text{response to 110 dB with pre-pulse}) / \text{response to 110 dB alone}$. The baseline response was defined as: $(\text{response to 110 dB alone} - \text{response to 68 dB alone}) / \text{response to 110 dB alone}$.

5-Choice Serial Reaction Time Task (5CSRTT)

From each cohort, 4 males and 4 females were randomly selected to participate in this task. Testing started at 14-16 weeks of age and continued through 26-27 weeks of age. Mice were tested in a chamber with five touch screens and a food reward tray (Campden Instruments Ltd, Loughborough, UK), which can deliver food reward of 1:1 dilution of water to strawberry flavored Ensure. The deployment of tasks and measurement of outcomes was managed with

Whisker Server and ABET II software (Layfette Instruments, Layfette, Indiana). Before beginning the pre-training, mice were restricted to 85% free-feeding weight and underwent one 20-minute session of habituation in the chamber with freely available reward (ABET II habituation 2a procedure).

Pre-training

Mice were trained to initiate trials by poking their heads into the food tray, which illuminates when the reward is delivered (initial touch procedure). Then mice were trained to nose-poke a white square stimulus displayed on one of the five touch screens (must touch procedure). In this task, poking the correct screen with the white square delivered 3-times the reward. In the final habituation task, the mice must enter and exit the illuminated food tray for a stimulus to appear on one of the five screens (must initiate procedure). The mouse must then touch the stimulus to elicit the reward paired with the tray light and tone. For the initial touch and must touch procedures, mice were required to complete 30 trials within 60 minutes on one day of testing before moving on. For the must initiate procedure, mice were required to complete 30 trials within 60 minutes during two consecutive days of testing to proceed to the 5CSRTT.

5CSRTT Training

All sessions begin with 7 μ L reward in the food tray and the light illuminated. When the mouse exits the reward tray the first trial is initiated. There is a delay interval of 5 seconds before a white square stimulus is presented on one of the five touch screens. A nose-poke of the screen with the white square (correct response) triggers a 7 μ L food reward paired with the tray light and tone. Upon exiting the food reward tray, the next trial begins. Selecting a touch screen during the 5 second delay before a stimulus is presented (premature response), selecting any of the four touchscreens that do not have the white square stimulus (incorrect response), or making

no response at all during the stimulus duration (omission) causes a five-second time-out paired with illumination of the entire chamber. On the first day of the 5CSRTT Procedure, the stimulus duration was set to 32 seconds. For each mouse, the stimulus duration was halved upon successfully completing the task. Task completion consisted of the mouse completing at least 50 trials in 60 minutes with >80% accuracy and <20% omissions on two consecutive testing days. Mice were tested daily until they successfully completed the task with a 2-second stimulus duration. Then mice were tested with the probes described below.

5CSRTT Probes

In the variable stimulus duration probe, mice were randomly presented stimulus durations of 1.5, 1, 0.8, and 0.6s (5 of each in 3 blocks of 20 trials) rather than the standard 2s duration. In the variable delay probe, mice were randomly presented delays (between initiating the trial and the stimulus appearing on screen) of 5, 6, 7 and 8s (5 of each in each block of 20 trials) rather than the standard 5s delay. In the distraction probe, mice experienced the standard 2s stimulus duration procedure with the addition of a 0.5s burst of noise sounded at a pseudorandom time during the 5s delay period.

Statistical Analysis

For all behavior tasks, summary outcome variables were modeled with fixed effects for treatment, sex, and cohort, and random intercepts for dam. Repeated measures models additionally included the repeated measures variable (e.g., time-bin for open field, pre-pulse dB level for PPI, and stimulus duration for 5CSRTT) and its interaction with treatment, and random intercepts for individual nested within dam. Treatment by sex interactions were retained in final models when significant. We performed Bonferroni-corrected post-hoc pairwise comparisons for treatment interactions with sex and repeated measures variables. CatWalk models additionally

controlled for mass and average speed during the CatWalk test, and only included mice with less than 60% speed variation. CatWalk results were similar in sensitivity analyses 1) excluding mass and speed, and 2) not filtering for speed variation.

RNA-sequencing

In the second cohort, 24 pups (equal number per sex and treatment) among those euthanized at birth were randomly selected. Frontal cortex was collected, and RNA extracted with the RNeasy Kit (Qiagen, Hilden, Germany). Library preparation and RNA-sequencing (TruSeq RNA Library Prep Kit v2 and HiSeq 4000, Illumina, San Diego, CA, USA) were performed by Genewiz (South Plainfield, NJ, USA).

FASTQ files were preprocessed with fastp using the default settings to filter bad reads, trim low quality bases, and cut adaptors.¹⁶⁶ Reads were mapped to the GRCM38(mm10) mouse genome using BWA-MEM¹⁶⁷ and assigned to exons using featureCounts¹⁶⁸ before performing differential expression analysis using DESeq2.¹⁶⁹ Gene expression fold-changes compared APAP treated to control offspring, accounting for sex. Normalized counts from DESeq2 were input into Ensemble of Gene Set Enrichment Analysis (EGSEA)¹⁷⁰ to determine enrichment for MSigDB hallmark gene sets^{171,172} and KEGG Pathways.¹⁷³ We examined the top 20 EGSEA enrichments sorted by median ranking score, and also present enriched pathway-level information using FDR-adjusted P-values. To investigate sex-specific effects, we repeated EGSEA analysis stratified by sex. RNA-seq data are deposited at Gene Expression Omnibus (GEO) accession number GSE198424.

4.3 Results

Mean [SD] mass (grams) did not differ between control (1.30 [0.095]) and APAP-treated (1.30 [0.104]) litters at birth, nor between control (22.6 [3.15]) and APAP-treated (22.9 [3.05])

adult mice at 8 weeks of age. Offspring sex ratios did not differ between control (37 females [46.3%]) and APAP-treated (46 females [49.5%]) groups.

Pup vocalizations upon separation from the mother peaked on postnatal day 8 for control and treated males and females (Figure 1). Treatment by sex by day contrasts revealed increased calls on postnatal day 8 among offspring of dams treated with APAP during pregnancy (Figure 1, $P = 0.0472$).

In the open field test, control and treated females shared similar total ambulation, while control males had higher total ambulation compared to the male offspring of APAP treated dams (Figure 2A, sex by treatment $P = 0.0281$). There was also a sex by treatment interaction in the repeated measures model ($P = 0.001$), and post hoc contrasts revealed elevated ambulatory movement among control males in the first 10-minute time-bin (Figure 2B, $P = 0.004$).

Following this pattern, control offspring had more total rearings than those exposed prenatally to APAP (Figure 2C, $P = 0.0325$), although there was no interaction with sex. Treatment by time-bin contrasts showed the largest difference in rearings during the third time bin (Figure 2D, $P = 0.0528$). While control offspring spent more time in the center of the open field chamber, the treatment term was non-significant in the overall and repeated measures models for the center duration outcome (Figure 2E, F).

Although a small number of CatWalk parameters appeared to differ between prenatal APAP and control mice, none survived adjustment for multiple comparisons (Figure 3, see eTable 1 for details).

There was no difference among groups in the time mice spent in the open arms, closed arms, or junction of the elevated plus maze, nor in the ratio of time spent in the open arms versus the closed arms (Figure 4).

Although the prenatal APAP group had reduced PPI at a pre-pulse of 86 dB, the main effect of prenatal APAP was non-significant in the repeated measures model ($P = 0.5247$), and no PPIs were significantly different between groups in post-hoc contrasts (Figure 5).

Control and treatment groups shared similar percent accuracy, omissions, and premature responses during 5CSRTT training (Figure 6 A,B,C), in the variable stimulus duration probe (Figure 6 D,E,F), the variable delay probe (Figure 6 G,H,I), and the distraction probe (Figure 6 J,K,L). Treatment and treatment by sex interactions were non-significant in all 5CSRTT models.

RNA-sequencing revealed ten differentially expressed genes in prefrontal cortex between control and prenatal APAP-exposed pups (Table 1). Enriched pathways from EGSEA (Figure 7, eTable 2) were related to the known metabolism of APAP by glutathione S-transferase and cytochrome p450 enzymes (HALLMARK_XENOBIOTIC_METABOLISM, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, Glutathione metabolism), DNA damage (HALLMARK_G2M_CHECKPOINT, HALLMARK_DNA_REPAIR, HALLMARK_E2F_TARGETS, HALLMARK_APOPTOSIS, Homologous recombination, Chemical carcinogenesis), the endocrine system (HALLMARK_ESTROGEN_RESPONSE_LATE, HALLMARK_ESTROGEN_RESPONSE_EARLY, Autoimmune thyroid disease, Steroid hormone biosynthesis, Steroid biosynthesis), and the immune system (HALLMARK_ALLOGRAFT_REJECTION, HALLMARK_INTERFERON_ALPHA_RESPONSE, HALLMARK_IL6_JAK_STAT3_SIGNALING, HALLMARK_INTERFERON_GAMMA_RESPONSE, HALLMARK_COMPLEMENT, HALLMARK_INFLAMMATORY_RESPONSE, HALLMARK_IL2_STAT5_SIGNALING,

Autoimmune thyroid disease, Antigen processing and presentation, Graft-versus-host disease, Systemic lupus erythematosus).

Sex-stratified EGSEA revealed which pathways were either consistently or differentially enriched depending on sex (Figure 7, eTable 2). Both sexes saw enrichment of pathways related to DNA damage (HALLMARK_G2M_CHECKPOINT, HALLMARK_DNA_REPAIR, HALLMARK_APOPTOSIS) and immune activation (HALLMARK_ALLOGRAFT_REJECTION, HALLMARK_INTERFERON_ALPHA_RESPONSE, HALLMARK_IL6_JAK_STAT3_SIGNALING, HALLMARK_INTERFERON_GAMMA_RESPONSE, HALLMARK_COMPLEMENT).

However, developmental APAP effects on hormone regulation and APAP metabolism were sex-specific. APAP treatment caused upregulation of the estrogen response in females but not males, and upregulation of autoimmune thyroid disease in males but not females. In females but not males, APAP treatment upregulated pathways related to APAP metabolism by glutathione S-transferase and cytochrome p450 enzymes (HALLMARK_XENOBIOTIC_METABOLISM, Drug metabolism – other enzymes, Metabolism of xenobiotics by cytochrome P450, Glutathione metabolism).

4.4 Discussion

In this study of more than 100 mouse offspring from 27 dams, prenatal APAP treatment was associated with increased pup vocalizations after separation from the litter, as well as decreased ambulation and rearings in the open field task among male offspring. Prenatal APAP treatment did not affect locomotion patterns in the CatWalk, time spent in the open versus closed arms in the elevated plus maze, precent inhibition in PPI, or accuracy, omissions, and premature

responses in the 5CSRTT. In addition to behavioral changes, prenatal APAP treatment was associated with altered prefrontal cortex gene expression relating to glutathione and cytochrome p450 metabolism, DNA damage, and the endocrine and immune systems.

Behavioral changes among male offspring in the treatment group may indicate elevated anxiety. First, treated male offspring exhibited decreased total movement and rearings during the open field task. Reduced exploratory behavior in the novel environment of the open field could indicate increased anxiety when confronted with a stressful situation. Although the open field task has certain shortcomings as a model for human anxiety disorders, the test is still sensitive to drugs used for the clinical treatment of anxiety including benzodiazepines and 5-HT_{1A} receptor agonists.¹⁷⁴ Given the null treatment effect on the CatWalk test, altered exploratory behavior in the open field may not be not attributable to motor deficits. Furthermore, treated male pups exhibited increased peak ultrasonic vocalization calls after being separated from the dam and litter. Vocalizations have commonly been used as a measure of anxiety in rodent adults and infants.¹⁷⁵⁻¹⁷⁷ Despite concordance of anxiety-like behaviors across two tasks, we still cannot rule out the possibility that changes in pup vocalizations were influenced by some effect of APAP exposure on maternal responsiveness.¹⁷⁸

Based on genetic animal models, our results demonstrating a lack of hyperactivity does not preclude ADHD relevance. Complex human neurodevelopmental conditions such as ADHD are difficult to replicate in animal models. In fact, some of the most common mouse models of ADHD do not exhibit hyperactivity.¹⁷⁹ Spontaneously hypertensive rats (SHR), which were developed by selective breeding of Wistar-Kyoto (WKY) rats,¹⁸⁰ are less active than WKY rats in running wheel and less active than Sprague Dawley rats in open field tests.¹⁸¹ Nevertheless, SHR rats have been used as a model for ADHD because they exhibit hyperactivity, impulsivity,

and inattentiveness in other tasks, and these ADHD-like symptoms can be attenuated with stimulant treatment.¹⁷⁹

In agreement with well-known sex differences across a wide range of neurodevelopmental conditions, we found that APAP treatment interacted with sex in its effect on mouse behavior. Regarding associations of prenatal and early life APAP exposure with neurodevelopmental outcomes in humans, there are several reports of sex interactions,^{130,141,182,183} although some studies explicitly testing sex interactions have found no evidence.¹⁸⁴⁻¹⁸⁶ These contrasting results could result from the use of different neurobehavioral outcome measures or the lack of generalizability across study populations. Investigators have also uncovered sex interactions with APAP exposure in animal models. One study found more vertical exploration in the open field in female but not male rats exposed to 350 mg/kg/day APAP during gestation compared to controls.¹⁴⁶ In another study, compared to control animals, male but not female rats exposed to 350 mg/kg/day APAP during pregnancy and lactation had increased apomorphine-induced stereotyped behavior, while males exposed to 35 mg/kg/day APAP displayed elevated ambulation in the open field.¹⁴⁸

RNA sequencing uncovered several candidate mechanisms of the prenatal toxicity of APAP. Many of the pathways significantly enriched in the prefrontal cortex of treated offspring have been previously studied in rodents following prenatal and early life APAP treatment. For instance, we found pathway enrichment for DNA damage and glutathione metabolism. In agreement with our findings, one study found that 350 mg/kg/day APAP during pregnancy and lactation resulted in decreased hippocampal glutathione levels and striatal superoxide dismutase activity.¹⁴⁸ However, another study found no changes in prefrontal cortex or hippocampus glutathione levels following 350 mg/kg/day APAP from gestational day 6 until delivery.¹⁴⁶ We

also found altered endocrine pathways between control and treated offspring, which may align with prior work showing APAP interference with sexual development in rodents. Prenatal APAP exposure in rodents may cause decreased anogenital distance,^{49,187-189} decreased pelvic floor muscle weights,¹⁹⁰ increased nipple retention,^{190,191} increased testosterone,¹⁹² and decreased male and female fertility¹⁹³⁻¹⁹⁷ (reviewed by¹²⁵).

In addition to pathways previously studied, we uncovered pathways that, to our knowledge, have not been studied as candidate mechanisms for the effects of prenatal APAP *in vivo*. RNA-sequencing revealed an upregulation of pathways related to thyroid disease in pups prenatally exposed to APAP. Although the effects of prenatal APAP exposure on thyroid hormone regulation has not been previously studied, there is evidence for APAP-associated thyroid dysregulation following adult exposure in humans¹⁹⁸ and rats.⁵¹ Furthermore, in humans, maternal thyroid hormone dysregulation is associated with a wide range of child neurodevelopmental disorders,¹⁹⁹ including ADHD.²⁰⁰⁻²⁰⁴ Thus, thyroid hormone changes might link APAP exposure with altered neurodevelopment. Similarly, transcriptomics revealed upregulation of immune system pathways in our treatment group compared to controls. The immune response remains understudied in relation to prenatal APAP exposure. Maternal immune adaptation to pregnancy involves suppressing the immune response to the fetus, which is allogeneic to the mother. Prior work indicates that non-hepatotoxic doses of APAP may interfere with fetal immune tolerance,²⁰⁵ and a recent synthesis of emerging evidence suggests a critical role of the immune system in normal neurodevelopment.²⁰⁶ Therefore, immune system alterations might link APAP exposure and neurodevelopment, and should be the subject of future studies.

Sex-stratified EGSEA revealed differential enrichment of hormone and APAP metabolic pathways, allowing us to generate hypotheses to explain the sex-specific effects of APAP on behavior observed here. One possibility is that developmental APAP exposure negatively impacted thyroid hormone signaling in males but not females. In humans, thyroid dysfunction during pregnancy is associated with child intellectual disability⁵³ and ADHD.⁵⁴⁻⁵⁶ Sex-specific effects on behavior could also be explained by differential APAP metabolism by males and females. Females may have been better able to upregulate glutathione and cytochrome p450 metabolism and thereby efficiently detoxify APAP and avoid adverse effects on the developing brain.

While being the first assessment of the effects of prenatal APAP exposure on gene expression in the brain and attention in the 5CSRTT is a considerable strength of this study, the results should be considered in the context of several limitations. First, despite strong evidence for associations of prenatal APAP with ADHD in humans,¹³⁰⁻¹⁴² prenatal APAP was not associated with mouse attention deficits in the 5CSRTT. In humans, not all individuals exposed prenatally to APAP develop ADHD. It is possible that only sub-groups with genetic predispositions or concurrent additional exposures develop ADHD following prenatal APAP exposure. Such interactions could be tested in future mouse work by using genetic models with altered neurodevelopment, or by exposing mice to multiple chemicals in addition to APAP. It is also possible that ADHD is too complex a human disorder to be translated into mouse behavior. Second, although the open field and pup ultrasonic vocalizations tests indicated elevated anxiety in male offspring exposed developmentally to APAP, we saw no effect of APAP treatment on another common assay for anxiety-related behavior, the elevated plus maze.^{207,208} Third, gene expression was measured in the brains of offspring euthanized at delivery, and not in the animals

that underwent behavior testing. While this design reduced the time-lag between prenatal APAP exposure and RNA-sequencing, it also prevented us from conducting mediation analyses to assess whether specific pathways linked APAP exposure to altered mouse behavior.

In conclusion, 150 mg/kg/day APAP exposure during gestation and lactation was associated with increased pup vocalizations after being separated from the dam and reduced exploratory behavior in the open field. Exposure was also associated with gene expression changes in offspring prefrontal cortex indicative of increased DNA damage and altered endocrine and immune system activity. Future studies are needed to explore whether the potential mechanisms revealed by RNA-sequencing directly link perinatal APAP exposure with behavior changes.

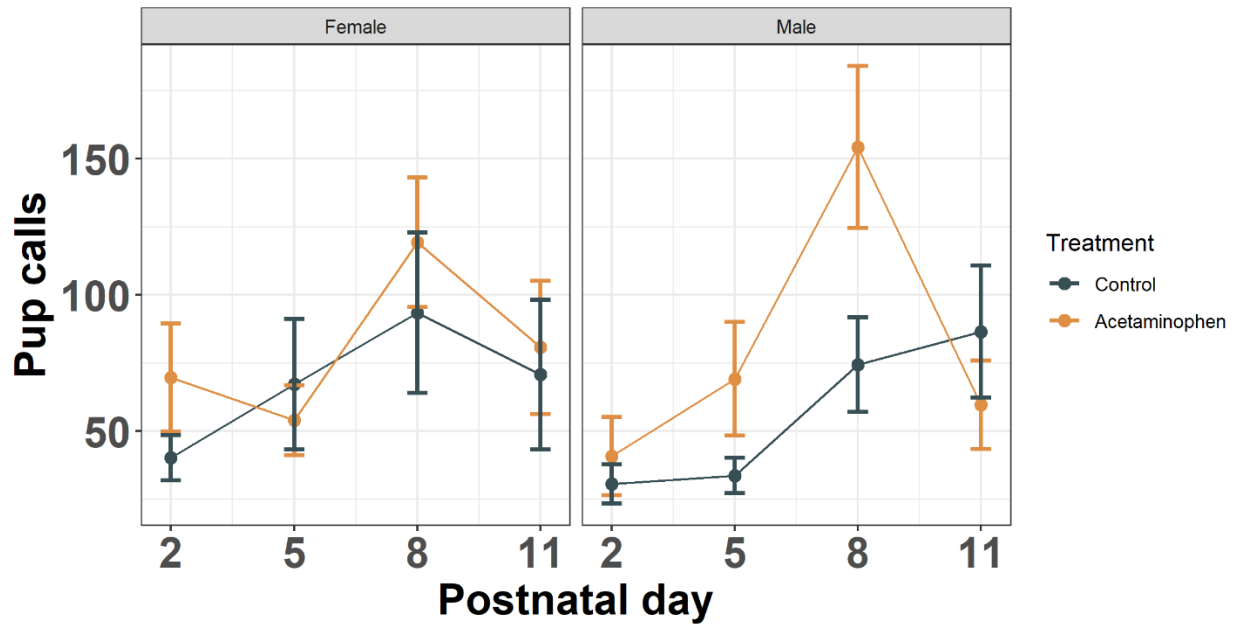
4.5 Tables

Table 1: Acetaminophen treatment over control fold change for significantly differentially expressed genes

Gene Symbol	log ₂ (Fold Change)	Standard Error	P-adj
Rad54l	0.575	0.091	0
Car3	0.373	0.077	0.009
Gm7285	0.453	0.093	0.009
Gm20762	0.442	0.093	0.009
Derl3	0.404	0.087	0.013
Pax6os1	0.415	0.091	0.013
Plbd1	0.43	0.093	0.013
H2-Ea	0.414	0.091	0.016
H4c8	0.417	0.093	0.017
Myc	0.368	0.082	0.017

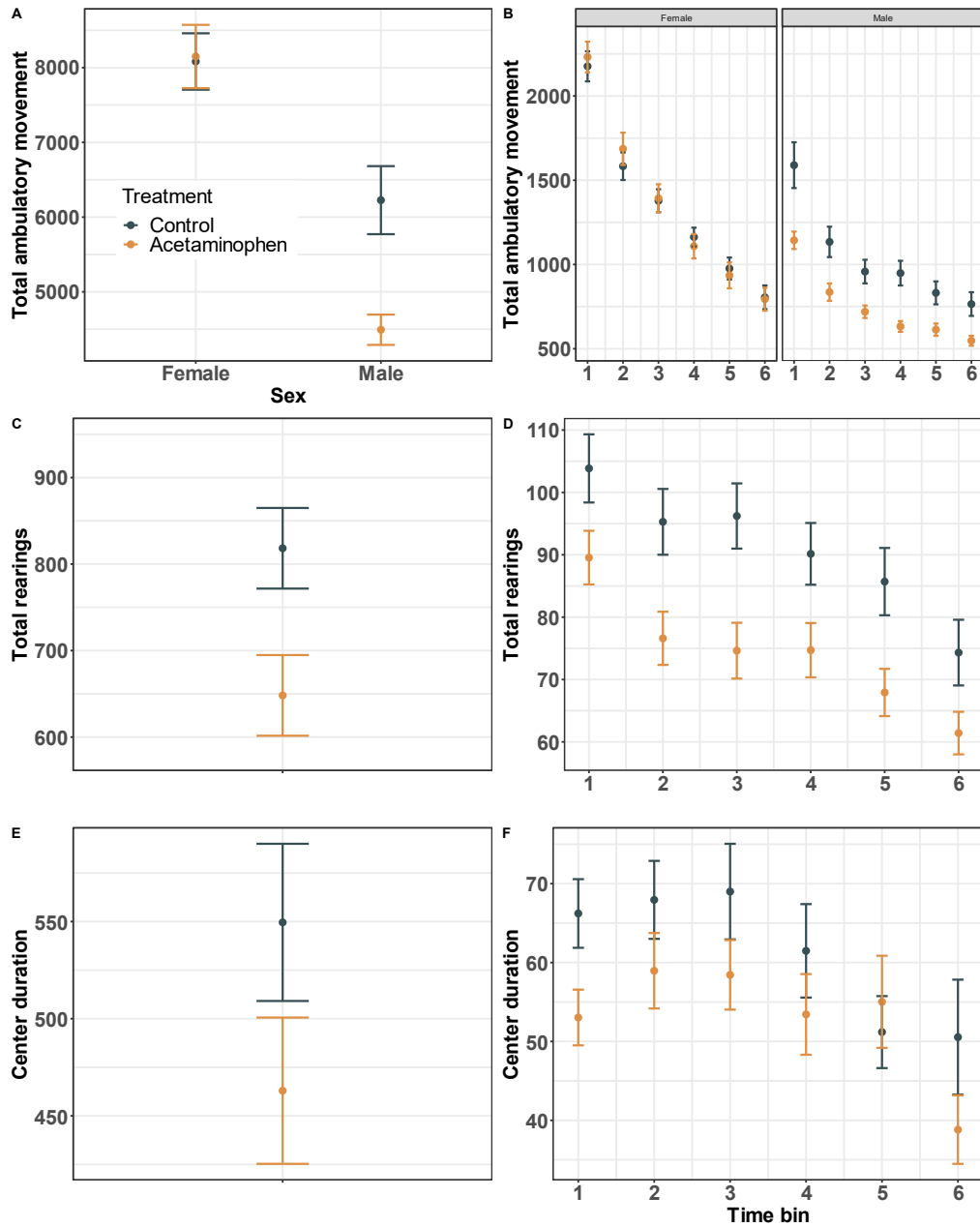
4.6 Figures

Figure 1: Pup Ultrasonic Vocalizations (USV)



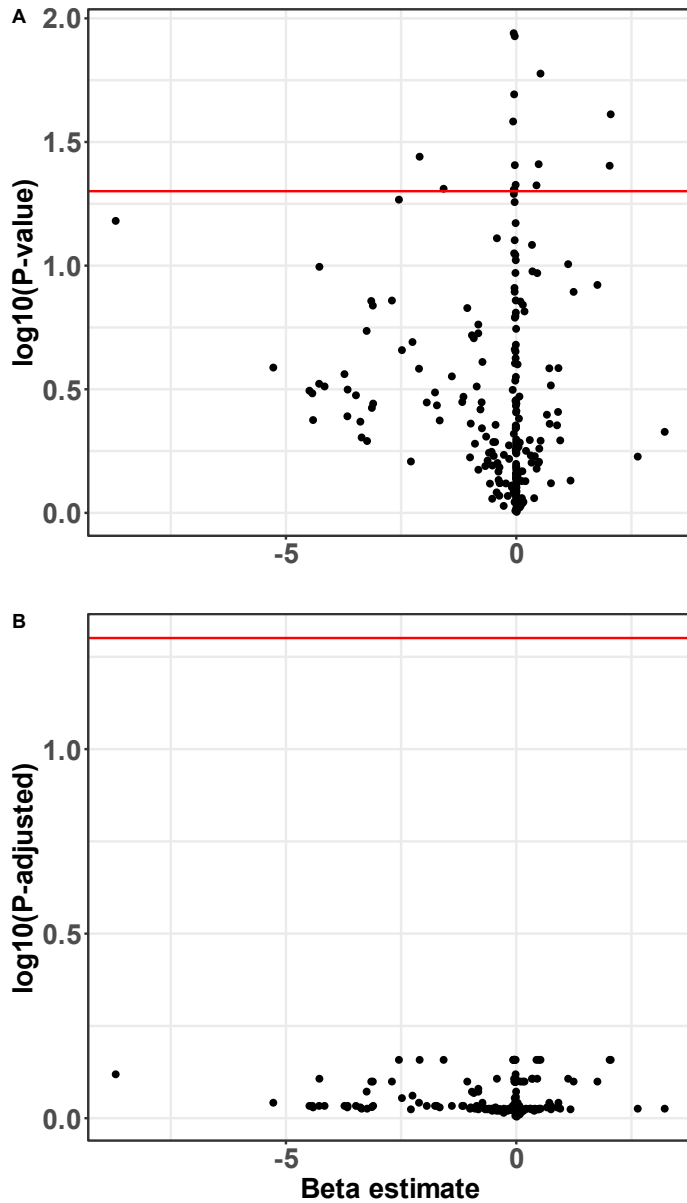
Legend: Among 4 control and 4 treatment dams, 6 pups per dam were removed one at a time from the dam and placed into an insulated chamber where USVs were recorded for 3 min by an ultrasonic microphone. Mean \pm standard error shown for number of USVs recorded across 4 days of testing, stratified by sex owing to significant treatment by sex interaction ($n = 48$).

Figure 2: Open Field Test (OFT)



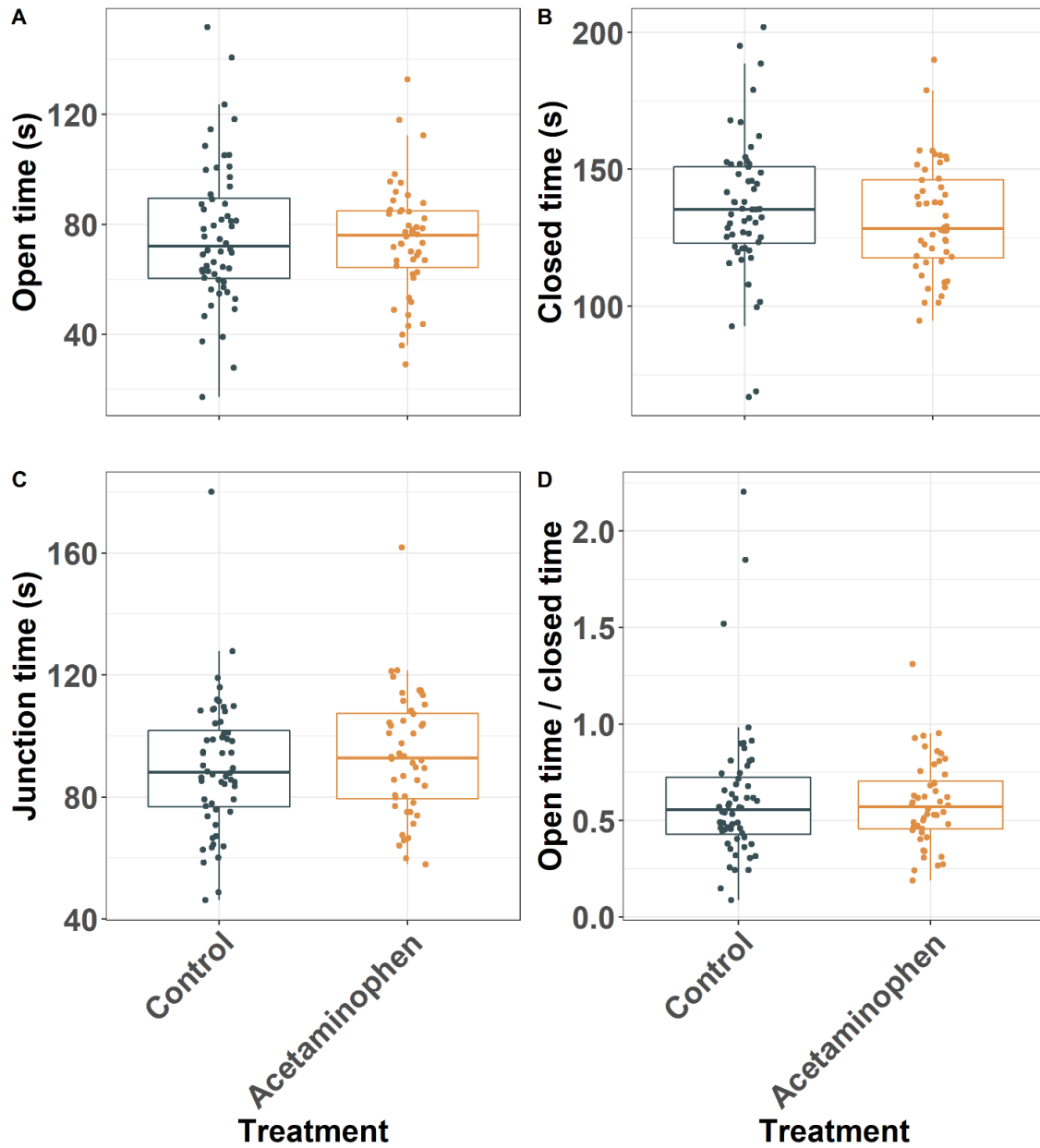
Legend: Mice were allowed to roam freely in a clear Plexiglas open field arena for 60 minutes. Mean \pm standard error shown for (A, B) total distance traveled (ambulatory movement, stratified by sex owing to significant treatment by sex interaction), (C, D) total number of rearings defined as the number of times the mouse disrupted two infrared beams by standing on its hind limbs, and (E, F) time spent in the center zone of the arena (n = 111).

Figure 3: CatWalk



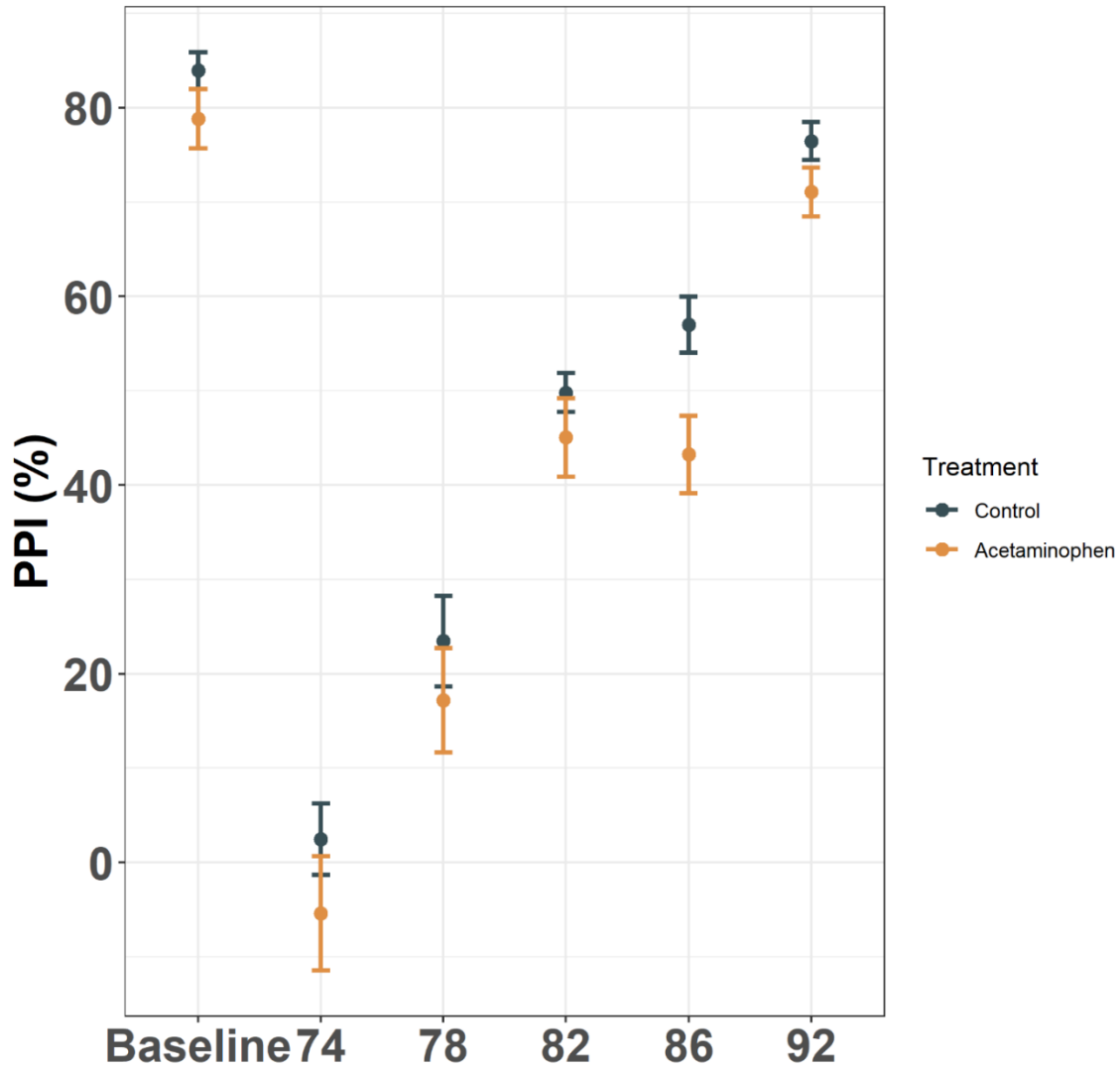
Legend: Mice completed three full crossings over an illuminated walled glass walkway. CatWalk XT software automatically measured 226 parameters related to mouse gait and locomotion via a high-speed camera underneath the walkway. Raw (A) and FDR-adjusted (B) P-values and beta estimates from separate linear regressions of treatment on each parameter shown. Red horizontal line at significance threshold of $P=0.05$ ($n = 90$).

Figure 4: Elevated plus maze (EPM)



Legend: Mice were placed in the central junction facing a closed arm and allowed to roam the maze for five-minutes. Individual data points and box and whiskers shown for the time spent in (A) open arms, (B) closed arms, (C) the central junction, and (D) the ratio of time spent in the open versus closed arms (n = 104).

Figure 5: Pre-pulse inhibition (PPI)



Legend: Mean \pm standard error shown for PPI % inhibition, calculated separately for each pre-pulse dB level as: (response to 110 dB alone - response to 110 dB with pre-pulse) / response to 110 dB alone. The baseline response was defined as: (response to 110 dB alone - response to 68 dB alone) / response to 110 dB alone (n = 90).

Figure 6: 5-Choice Serial Reaction Time Task (5CSRTT)

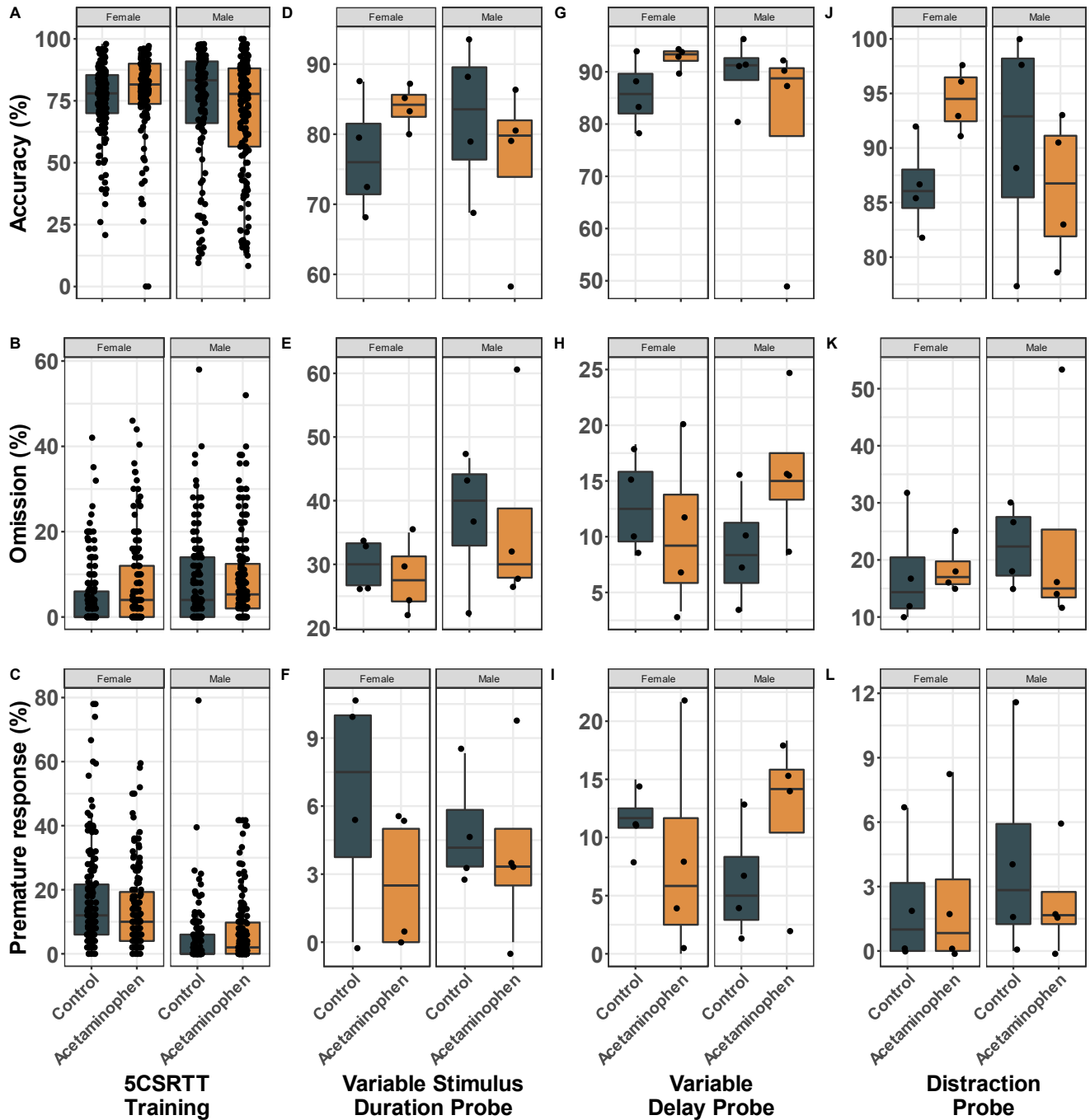
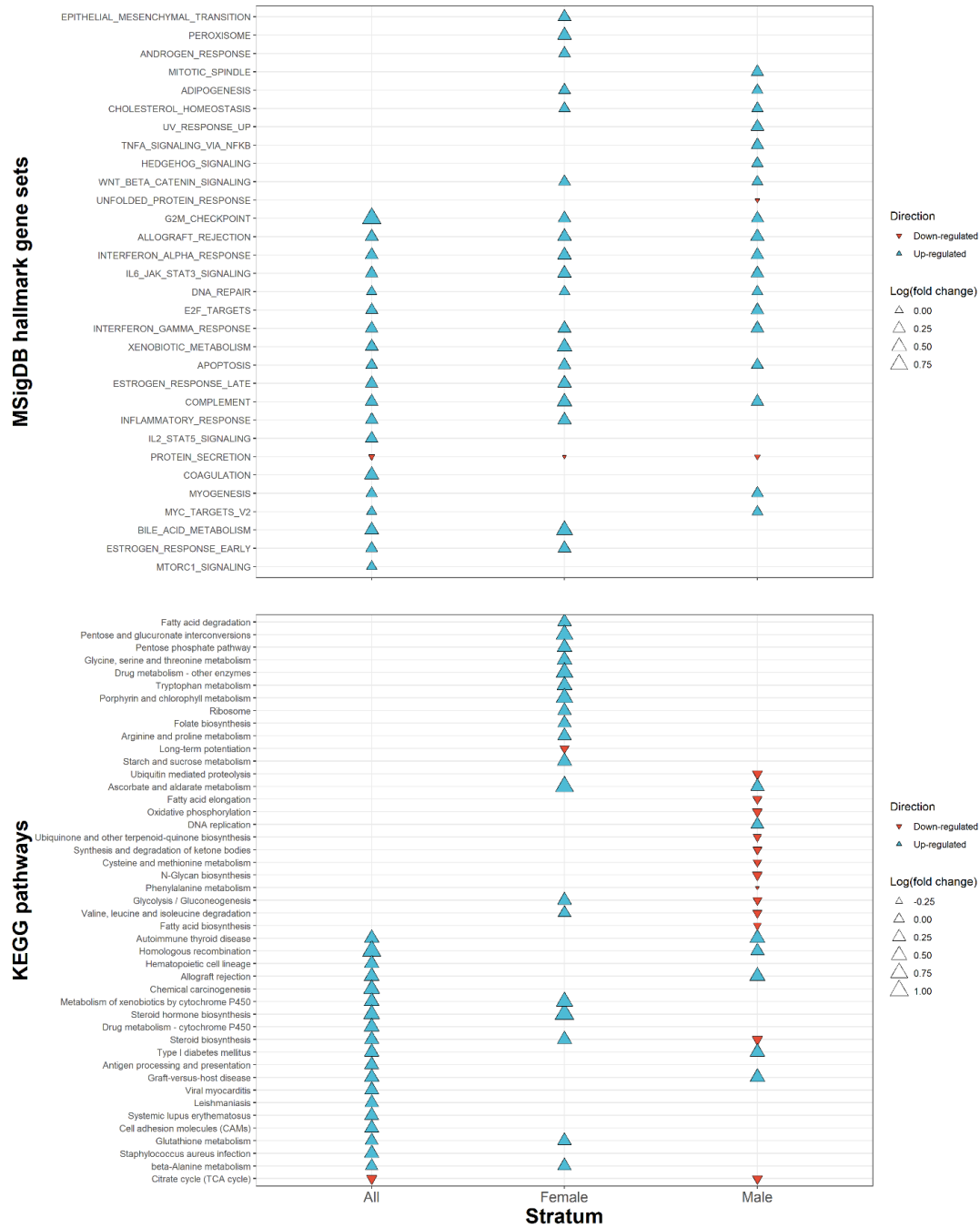


Figure 7: Ensemble of Gene Set Enrichment Analysis (EGSEA)



Legend: APAP treatment over control fold changes in top 20 enriched terms for (A) MSigDB hallmark gene sets and (B) KEGG pathways sorted from top to bottom by EGSEA median ranking score. Analysis repeated for all mice and stratified by sex (n = 24).

4.7 Supplement

eTable 1: Beta estimates from linear regression of APAP versus control treatment on individual CatWalk parameters

Estimate	Standard error	Statistic	Outcome	P value	P adj
-0.056	0.022	-2.584	rh_print_width_c m_mean	0.011	0.695
-0.033	0.013	-2.573	couplings_lh_rf_c _stat_r	0.012	0.695
0.525	0.215	2.441	hp_min_intensity _mean	0.017	0.695
-0.046	0.020	-2.365	hp_print_width_c m_mean	0.020	0.695
2.050	0.895	2.291	couplings_lh_rh_ mean	0.024	0.695
-0.068	0.030	-2.264	couplings_rh_lh_c _stat_r	0.026	0.695
-2.098	0.986	-2.128	couplings_rh_lf_ mean	0.036	0.695
0.486	0.232	2.098	lh_min_intensity_ mean	0.039	0.695
-0.032	0.015	-2.094	couplings_rh_lf_c _stat_r	0.039	0.695

2.027	0.969	2.092	rf_max_contact_a t_percent_mean	0.039	0.695
-0.014	0.007	-2.014	lf_max_contact_a rea_cm2_mean	0.047	0.695
0.439	0.218	2.012	support_girdle_pe rcent	0.047	0.695
-1.578	0.790	-1.998	step_sequence_re gularity_index_pe rcent	0.049	0.695
-0.053	0.027	-1.995	couplings_rh_rf_c _stat_r	0.049	0.695
-0.039	0.020	-1.993	phase_dispersions _rf_lh_c_stat_r	0.050	0.695
-0.053	0.027	-1.977	phase_dispersions _lh_rh_c_stat_r	0.051	0.695
-2.548	1.304	-1.953	support_diagonal_ percent	0.054	0.695
-0.036	0.018	-1.943	couplings_rf_lh_c _stat_r	0.055	0.695
-8.696	4.668	-1.863	couplings_lf_rh_ mean	0.066	0.760

-0.014	0.008	-1.853	lf_print_length_c m_mean	0.067	0.760
-0.421	0.235	-1.787	rf_stand_index_m ean	0.078	0.782
-0.035	0.020	-1.778	phase_dispersions _lf_lh_c_stat_r	0.079	0.782
0.341	0.194	1.757	support_lateral_pe rcent	0.082	0.782
-0.046	0.027	-1.719	couplings_lh_rh_c _stat_r	0.089	0.782
-0.020	0.012	-1.712	lh_max_contact_a rea_cm2_mean	0.091	0.782
-0.012	0.007	-1.688	fp_max_contact_a rea_cm2_mean	0.095	0.782
1.126	0.674	1.670	step_sequence_rb _percent	0.099	0.782
-4.274	2.579	-1.657	rf_max_intensity_ mean	0.101	0.782
0.351	0.214	1.636	lf_stand_index_m ean	0.105	0.782
-0.018	0.011	-1.629	hp_max_contact_ area_cm2_mean	0.107	0.782

0.455	0.280	1.628	rh_min_intensity_ mean	0.107	0.782
1.761	1.120	1.572	support_three_per cent	0.120	0.796
-0.039	0.025	-1.557	phase_dispersions _rf_rh_c_stat_r	0.123	0.796
-0.036	0.023	-1.539	couplings_lf_rh_c _stat_r	0.128	0.796
1.245	0.809	1.538	fp_max_contact_a t_percent_mean	0.128	0.796
-0.012	0.008	-1.496	fp_print_length_c m_mean	0.138	0.796
-2.700	1.805	-1.496	fp_mean_intensity _of_the15most_in tense_pixels_me an	0.138	0.796
-3.148	2.108	-1.494	fp_max_intensity _mean	0.139	0.796
0.091	0.061	1.491	print_positions_le ft_paws_mean_c m	0.140	0.796

0.143	0.097	1.475	fp_min_intensity_ mean	0.144	0.796
-3.114	2.118	-1.471	rf_mean_intensity _of_the15most_in tense_pixels_me an	0.145	0.796
-1.064	0.729	-1.458	lf_mean_intensity _mean	0.148	0.796
0.178	0.124	1.441	rf_min_intensity_ mean	0.153	0.796
-0.010	0.007	-1.435	lf_print_area_cm2 _mean	0.155	0.796
-0.019	0.013	-1.416	lh_print_area_cm 2_mean	0.160	0.798
-0.031	0.022	-1.409	lh_print_width_c m_mean	0.162	0.798
-0.824	0.600	-1.374	couplings_rf_lf_c _stat_mean	0.173	0.832
-0.004	0.003	-1.351	lh_single_stance_ s_mean	0.180	0.847

-3.248	2.423	-1.340	rf_max_contact_ max_intensity_me an	0.184	0.847
-0.825	0.621	-1.328	fp_max_contact_ mean_intensity_m ean	0.188	0.848
-0.963	0.731	-1.317	fp_mean_intensity _mean	0.191	0.848
-0.922	0.709	-1.301	rf_max_contact_ mean_intensity_m ean	0.197	0.855
-2.253	1.759	-1.281	lf_mean_intensity _of_the15most_in tense_pixels_me an	0.204	0.869
-0.009	0.007	-1.266	fp_print_area_cm 2_mean	0.209	0.874
-0.033	0.027	-1.241	couplings_rf_rh_c _stat_r	0.218	0.882
-2.482	2.007	-1.237	fp_max_contact_ max_intensity_me an	0.220	0.882

-0.016	0.013	-1.229	hp_print_area_cm 2_mean	0.223	0.882
-0.015	0.012	-1.191	rh_max_contact_a rea_cm2_mean	0.237	0.908
-0.734	0.628	-1.170	lf_max_contact_ mean_intensity_m ean	0.245	0.908
-0.028	0.024	-1.162	phase_dispersions _lf_rh_c_stat_r	0.248	0.908
0.029	0.025	1.156	bos_hind_paws_ mean_cm	0.251	0.908
-5.274	4.635	-1.138	phase_dispersions _lf_rh_c_stat_mea n	0.258	0.908
0.915	0.806	1.135	phase_dispersions _lf_rf_mean	0.260	0.908
0.717	0.633	1.134	other_statistics_n umber_of_steps	0.260	0.908
-2.112	1.867	-1.131	step_sequence_aa _percent	0.261	0.908
-3.730	3.392	-1.099	hp_mean_intensit y_of_the15most_i	0.275	0.926

			ntense_pixels_me an		
-1.398	1.288	-1.086	couplings_rh_rf_ mean	0.280	0.926
-0.009	0.008	-1.083	rf_max_contact_a rea_cm2_mean	0.282	0.926
-0.022	0.021	-1.061	couplings_lf_lh_c _stat_r	0.292	0.926
-4.280	4.109	-1.042	hp_max_contact_ max_intensity_me an	0.301	0.926
0.751	0.727	1.032	phase_dispersions _lh_rh_c_stat_me an	0.305	0.926
-4.162	4.058	-1.025	hp_max_intensity _mean	0.308	0.926
-0.860	0.838	-1.025	rf_mean_intensity _mean	0.308	0.926
-3.661	3.639	-1.006	lh_mean_intensity _of_the15most_in tense_pixels_me an	0.317	0.926

-0.078	0.077	-1.004	lh_stride_length_c m_mean	0.318	0.926
-4.490	4.494	-0.999	lh_max_intensity_ mean	0.321	0.926
-1.764	1.785	-0.988	rh_max_intensity _at_percent_mean	0.326	0.926
-4.427	4.505	-0.983	lh_max_contact_ max_intensity_me an	0.329	0.926
-3.480	3.586	-0.971	rh_mean_intensity _of_the15most_in tense_pixels_mea n	0.335	0.926
0.069	0.071	0.963	support_zero_perc ent	0.338	0.926
-1.147	1.193	-0.961	phase_dispersions _lh_rh_mean	0.339	0.926
-0.002	0.002	-0.941	hp_single_stance_ s_mean	0.350	0.926
-0.018	0.019	-0.936	couplings_lh_lf_c _stat_r	0.352	0.926

-1.173	1.264	-0.928	hp_max_intensity_ at_percent_mean	0.356	0.926
-0.752	0.812	-0.926	lh_duty_cycle_percent_mean	0.357	0.926
-1.943	2.101	-0.925	lf_max_intensity_mean	0.358	0.926
-3.107	3.385	-0.918	other_statistics_maximum_variation_percent	0.361	0.926
0.002	0.002	0.918	fp_swing_s_mean	0.361	0.926
-1.723	1.901	-0.906	rh_body_speed_variation_percent_mean	0.367	0.926
-0.008	0.009	-0.903	rf_print_area_cm2_mean	0.369	0.926
-3.135	3.524	-0.890	run_maximum_variation_percent	0.376	0.933
-0.778	0.885	-0.879	couplings_rh_lh_c_stat_mean	0.382	0.933
0.002	0.003	0.869	lf_swing_s_mean	0.387	0.933
0.906	1.050	0.863	lf_swing_speed_cm_s_mean	0.391	0.933

-0.004	0.004	-0.860	lf_initial_dual_sta nce_s_mean	0.392	0.933
0.665	0.787	0.844	couplings_lh_rh_c _stat_mean	0.401	0.935
-3.666	4.398	-0.834	rh_max_contact_ max_intensity_me an	0.407	0.935
0.057	0.070	0.818	print_positions_ri ght_paws_mean_c m	0.416	0.935
-4.411	5.458	-0.808	couplings_rf_lh_ mean	0.421	0.935
-1.663	2.064	-0.805	lf_max_contact_ max_intensity_me an	0.423	0.935
-3.385	4.247	-0.797	rh_max_intensity _mean	0.428	0.935
-0.989	1.262	-0.784	couplings_lh_rf_ mean	0.435	0.935
0.725	0.927	0.782	couplings_lh_lf_ mean	0.436	0.935

-0.450	0.580	-0.775	lf_duty_cycle_per cent_mean	0.441	0.935
0.886	1.147	0.772	run_average_spee d_cm_s	0.442	0.935
-0.011	0.014	-0.771	rh_print_area_cm 2_mean	0.443	0.935
-0.008	0.011	-0.765	rf_print_length_c m_mean	0.446	0.935
-0.005	0.007	-0.756	lh_stand_s_mean	0.452	0.935
-0.746	0.993	-0.751	rh_mean_intensity _mean	0.455	0.935
-0.013	0.017	-0.751	hp_print_length_c m_mean	0.455	0.935
3.221	4.440	0.726	couplings_lh_rf_c _stat_mean	0.470	0.942
-0.054	0.076	-0.711	hp_stride_length_ cm_mean	0.479	0.942
-0.654	0.947	-0.691	hp_mean_intensit y_mean	0.492	0.942
-3.357	4.904	-0.685	couplings_lf_rh_c _stat_mean	0.495	0.942

-0.002	0.003	-0.671	fp_initial_dual_st ance_s_mean	0.504	0.942
0.293	0.440	0.666	support_single_pe rcent	0.507	0.942
0.954	1.439	0.663	rh_max_contact_a t_percent_mean	0.509	0.942
0.528	0.800	0.660	couplings_rf_rh_c _stat_mean	0.511	0.942
-3.239	4.919	-0.658	couplings_rh_lf_c _stat_mean	0.512	0.942
-0.500	0.768	-0.651	couplings_lh_lf_c _stat_mean	0.517	0.942
-0.459	0.705	-0.651	hp_duty_cycle_pe rcent_mean	0.517	0.942
-0.004	0.006	-0.648	hp_stand_s_mean	0.519	0.942
0.076	0.117	0.647	lf_min_intensity_ mean	0.519	0.942
-0.897	1.405	-0.638	lh_max_contact_a t_percent_mean	0.525	0.942
-0.159	0.254	-0.626	other_statistics_av erage_speed_cm_ s	0.533	0.942

0.002	0.004	0.619	rf_swing_s_mean	0.538	0.942
0.048	0.078	0.614	lf_stride_length_c m_mean	0.541	0.942
0.499	0.831	0.600	couplings_rf_lf_m ean	0.550	0.942
-0.011	0.018	-0.598	lh_print_length_c m_mean	0.551	0.942
0.003	0.006	0.588	rh_terminal_dual_ stance_s_mean	0.558	0.942
-0.011	0.018	-0.588	rh_print_length_c m_mean	0.558	0.942
0.212	0.363	0.583	lh_stand_index_m ean	0.561	0.942
-0.536	0.931	-0.576	phase_dispersions _lf_lh_mean	0.566	0.942
-0.005	0.008	-0.575	lf_step_cycle_s_ mean	0.567	0.942
-0.592	1.042	-0.568	lh_mean_intensity _mean	0.571	0.942
-0.003	0.005	-0.568	fp_stand_s_mean	0.572	0.942
0.002	0.003	0.563	hp_initial_dual_st ance_s_mean	0.575	0.942

-0.269	0.488	-0.552	fp_duty_cycle_percent_mean	0.583	0.942
0.314	0.574	0.547	phase_dispersions_lf_rf_c_stat_mean	0.586	0.942
-0.489	0.901	-0.543	rh_max_contact_mean_intensity_mean	0.588	0.942
0.402	0.744	0.541	phase_dispersions_rf_rh_c_stat_mean	0.590	0.942
2.636	4.902	0.538	phase_dispersions_rf_lh_c_stat_mean	0.592	0.942
-1.004	1.889	-0.532	hp_body_speed_variation_percent_mean	0.596	0.943
-0.155	0.298	-0.520	lh_body_speed_cm_s_mean	0.605	0.946
-0.623	1.231	-0.506	lh_swing_speed_cm_s_mean	0.614	0.946

-2.288	4.593	-0.498	couplings_rf_lh_c _stat_mean	0.620	0.946
0.494	0.996	0.496	couplings_rh_rf_c _stat_mean	0.622	0.946
0.332	0.679	0.488	phase_dispersions _lf_lh_c_stat_mean n	0.626	0.946
-0.409	0.842	-0.486	hp_max_contact_ mean_intensity_m ean	0.628	0.946
0.477	0.983	0.485	fp_swing_speed_c m_s_mean	0.629	0.946
-0.004	0.008	-0.478	lf_print_width_c m_mean	0.634	0.946
-0.519	1.117	-0.465	phase_dispersions _rf_rh_mean	0.643	0.946
-0.672	1.461	-0.460	lh_max_intensity_ at_percent_mean	0.647	0.946
-0.003	0.007	-0.457	lh_step_cycle_s_ mean	0.649	0.946
0.002	0.005	0.450	rh_initial_dual_st ance_s_mean	0.654	0.946

-0.365	0.813	-0.449	fp_max_intensity _at_percent_mean	0.654	0.946
0.441	1.009	0.437	lf_max_contact_at _percent_mean	0.663	0.946
-0.003	0.006	-0.433	hp_step_cycle_s_ mean	0.666	0.946
-0.823	1.919	-0.429	lf_body_speed_va riation_percent_m ean	0.669	0.946
0.132	0.317	0.416	mass	0.678	0.946
-0.387	0.938	-0.413	lh_max_contact_ mean_intensity_m ean	0.681	0.946
-0.007	0.019	-0.397	phase_dispersions _lf_rf_c_stat_r	0.692	0.946
-0.002	0.006	-0.395	rf_stand_s_mean	0.694	0.946
-0.003	0.006	-0.392	lf_stand_s_mean	0.696	0.946
0.027	0.073	0.373	fp_stride_length_ cm_mean	0.710	0.946
0.001	0.003	0.370	rh_single_stance_ s_mean	0.712	0.946

-0.002	0.005	-0.365	lh_terminal_dual_ stance_s_mean	0.716	0.946
0.003	0.008	0.361	rf_step_cycle_s_ mean	0.719	0.946
0.001	0.003	0.354	rf_single_stance_s_ _mean	0.724	0.946
-0.007	0.020	-0.347	couplings_lf_rf_c_ _stat_r	0.729	0.946
0.026	0.077	0.339	other_statistics_d uration_s	0.735	0.946
-0.385	1.137	-0.338	hp_swing_speed_ cm_s_mean	0.736	0.946
1.177	3.538	0.333	phase_dispersions_ _rf_lh_mean	0.740	0.946
0.118	0.359	0.328	step_sequence_ra_ _percent	0.743	0.946
0.198	0.606	0.327	couplings_lf_rf_c_ _stat_mean	0.744	0.946
-0.363	1.170	-0.310	rf_max_intensity_ at_percent_mean	0.757	0.946
-0.001	0.004	-0.310	rf_terminal_dual_ stance_s_mean	0.758	0.946

-0.354	1.146	-0.309	support_four_percent	0.758	0.946
0.753	2.445	0.308	step_sequence_callback_percent	0.759	0.946
-0.226	0.736	-0.306	couplings_lf_lh_mean	0.760	0.946
-0.572	1.878	-0.305	fp_body_speed_variation_percent_mean	0.761	0.946
0.001	0.002	0.304	fp_single_stance_s_mean	0.762	0.946
-0.090	0.313	-0.288	rf_body_speed_cm_s_mean	0.774	0.946
-0.006	0.020	-0.288	couplings_rf_lf_callback_stat_r	0.774	0.946
-0.001	0.004	-0.287	rf_initial_dual_stance_s_mean	0.775	0.946
-0.001	0.003	-0.272	fp_terminal_dual_stance_s_mean	0.787	0.951
-0.096	0.358	-0.270	rh_stand_index_mean	0.788	0.951

0.001	0.005	0.265	lh_initial_dual_sta nce_s_mean	0.791	0.951
-0.022	0.089	-0.253	run_duration_s	0.801	0.955
-0.002	0.007	-0.236	rh_stand_s_mean	0.814	0.955
0.002	0.011	0.234	rf_print_width_c m_mean	0.816	0.955
-0.426	1.937	-0.220	rf_body_speed_va riation_percent_m ean	0.826	0.955
-0.002	0.007	-0.219	fp_step_cycle_s_ mean	0.827	0.955
-0.001	0.003	-0.219	rh_swing_s_mean	0.827	0.955
-0.061	0.281	-0.216	hp_body_speed_c m_s_mean	0.830	0.955
-0.037	0.176	-0.209	fp_stand_index_m ean	0.835	0.955
-0.002	0.008	-0.190	fp_print_width_c m_mean	0.850	0.955
0.001	0.004	0.187	hp_terminal_dual _stance_s_mean	0.852	0.955

-0.368	1.971	-0.187	lh_body_speed_v ariation_percent_ mean	0.852	0.955
-0.185	1.004	-0.184	lf_max_intensity_ at_percent_mean	0.854	0.955
-0.004	0.020	-0.183	bos_front_paws_ mean_cm	0.856	0.955
0.000	0.002	-0.177	hp_swing_s_mean	0.860	0.955
0.121	0.743	0.162	couplings_lf_lh_c _stat_mean	0.871	0.955
0.013	0.080	0.162	rf_stride_length_c m_mean	0.872	0.955
0.389	2.403	0.162	step_sequence_ca _percent	0.872	0.955
0.045	0.285	0.158	rh_body_speed_c m_s_mean	0.875	0.955
-0.522	3.358	-0.156	phase_dispersions _lf_rh_mean	0.877	0.955
0.000	0.003	0.139	lh_swing_s_mean	0.889	0.955
0.127	0.916	0.139	couplings_rf_rh_ mean	0.890	0.955

-0.019	0.143	-0.133	step_sequence_number_of_patterns	0.895	0.955
0.155	1.264	0.123	rh_swing_speed_cm_mean	0.903	0.955
-0.037	0.304	-0.122	fp_body_speed_cm_mean	0.903	0.955
-0.024	0.212	-0.115	other_statistics_candence	0.909	0.955
0.011	0.095	0.113	rh_stride_length_cm_mean	0.910	0.955
0.140	1.247	0.113	rf_swing_speed_cm_mean	0.911	0.955
0.034	0.312	0.110	hp_stand_index_mean	0.912	0.955
0.061	0.596	0.103	couplings_lf_rf_mean	0.919	0.957
0.096	1.208	0.080	couplings_rh_lh_mean	0.937	0.967
-0.273	3.448	-0.079	step_sequence_ab_percent	0.937	0.967
0.072	1.179	0.061	hp_max_contact_at_percent_mean	0.952	0.977

0.000	0.004	0.054	lf_terminal_dual_ stance_s_mean	0.957	0.979
0.000	0.007	0.032	rh_step_cycle_s_ mean	0.975	0.985
0.000	0.003	-0.030	lf_single_stance_s_ _mean	0.976	0.985
-0.021	0.825	-0.025	rh_duty_cycle_pe rcent_mean	0.980	0.985
0.007	0.311	0.024	lf_body_speed_c m_s_mean	0.981	0.985
0.008	0.707	0.012	rf_duty_cycle_per cent_mean	0.990	0.990

eTable 2: Top 20 APAP over control enrichments from EGSEA for all mice and stratified by sex

Gene Set	Description	Number of Genes	P adj	Medium Rank Score	Average log(Fold Change)	Pathway	Stratum
G2M_CHECKPOINT	Genes involved in the G2/M checkpoint, as in progression through the cell division cycle.	311/320	0.026	3	0.960	MutSig DB Hallmark	All
ALLOGRAFT_REJECTION	Genes up-regulated during transplant rejection.	319/333	0.002	3	0.326	MutSig DB Hallmark	All
INTERFERON_ALPHA_RESPONSE	Genes up-regulated in response to alpha interferon proteins.	177/187	0.000	7	0.314	MutSig DB Hallmark	All
IL6_JAK_STAT3_SIGNALING	Genes up-regulated by IL6 [GeneID=3569]	122/124	0.019	8	0.280	MutSig DB Hallmark	All

	via STAT3 [GeneID=6774] , e.g., during acute phase response.						
DNA_REPAIR	Genes involved in DNA repair.	206/218	0.177	9	0.113	MutSig DB Hallmark	All
E2F_TARGETS	Genes encoding cell cycle related targets of E2F transcription factors.	304/312	0.020	9	0.246	MutSig DB Hallmark	All
INTERFERON_GAMMA_RESPONSE	Genes up- regulated in response to IFNG [GeneID=3458] .	317/327	0.000	10	0.273	MutSig DB Hallmark	All

XENOBIOTIC _METABOLI SM	Genes encoding proteins involved in processing of drugs and other xenobiotics.	342/349	0.050	12	0.329	MutSig DB Hallmar k	All
APOPTOSIS	Genes mediating programmed cell death (apoptosis) by activation of caspases.	221/224	0.180	13	0.217	MutSig DB Hallmar k	All
ESTROGEN_ RESPONSE_ L ATE	Genes defining late response to estrogen.	323/327	0.180	14	0.279	MutSig DB Hallmar k	All
COMPLEME NT	Genes encoding components of the complement system, which is part of the	362/367	0.060	15	0.323	MutSig DB Hallmar k	All

	innate immune system.						
INFLAMMATORY_RESPONSE	Genes defining inflammatory response.	301/303	0.180	16	0.288	MutSig DB Hallmark	All
IL2_STAT5_SIGNALING	Genes up-regulated by STAT5 in response to IL2 stimulation.	337/343	0.094	16	0.270	MutSig DB Hallmark	All
PROTEIN_SECRETION	Genes involved in protein secretion pathway.	146/146	0.083	17	-0.068	MutSig DB Hallmark	All
COAGULATION	Genes encoding components of blood coagulation system; also up-regulated in platelets.	222/225	0.012	18	0.446	MutSig DB Hallmark	All

MYOGENESIS	Genes involved in development of skeletal muscle (myogenesis).	333/335	0.472	19	0.198	MutSig DB Hallmark	All
MYC_TARGETS_V2	A subgroup of genes regulated by MYC - version 2 (v2).	69/71	0.180	21	0.111	MutSig DB Hallmark	All
BILE_ACID_METABOLISM	Genes involved in metabolism of bile acids and salts.	176/176	0.214	21	0.384	MutSig DB Hallmark	All
ESTROGEN_RESPONSE_EARLY	Genes defining early response to estrogen.	307/310	0.214	22	0.219	MutSig DB Hallmark	All
MTORC1_SIGNALING	Genes up-regulated through activation of	297/310	0.180	23	0.136	MutSig DB Hallmark	All

	mTORC1 complex.						
G2M_CHECKPOINT	Genes involved in the G2/M checkpoint, as in progression through the cell division cycle.	311/320	0.004	2	0.271	MutSig DB Hallmark	Male
E2F_TARGETS	Genes encoding cell cycle related targets of E2F transcription factors.	304/312	0.040	3	0.290	MutSig DB Hallmark	Male
MITOTIC_SPINDLE	Genes important for mitotic spindle assembly.	357/358	0.064	5	0.252	MutSig DB Hallmark	Male
APOPTOSIS	Genes mediating programmed cell death	221/224	0.316	10	0.235	MutSig DB Hallmark	Male

	(apoptosis) by activation of caspases.						
ALLOGRAFT _REJECTION	Genes up-regulated during transplant rejection.	319/333	0.316	10	0.372	MutSig DB Hallmar k	Male
ADIPOGENE SIS	Genes up-regulated during adipocyte differentiation (adipogenesis).	300/303	0.314	12	0.163	MutSig DB Hallmar k	Male
CHOLESTER OL_HOMEOS TASIS	Genes involved in cholesterol homeostasis.	110/110	0.443	13	0.187	MutSig DB Hallmar k	Male
MYOGENESI S	Genes involved in development of skeletal muscle (myogenesis).	333/335	0.414	16	0.224	MutSig DB Hallmar k	Male

IL6_JAK_STA T3_SIGNALI NG	Genes up- regulated by IL6 [GeneID=3569] via STAT3 [GeneID=6774] , e.g., during acute phase response.	122/124	0.544	17	0.297	MutSig DB Hallmar k	Male
PROTEIN_SE CRETION	Genes involved in protein secretion pathway.	146/146	0.064	17	-0.078	MutSig DB Hallmar k	Male
INTERFERO N_ALPHA_R ESPONSE	Genes up- regulated in response to alpha interferon proteins.	177/187	0.331	17	0.298	MutSig DB Hallmar k	Male
DNA_REPAI R	Genes involved in DNA repair.	206/218	0.519	18	0.168	MutSig DB Hallmar k	Male

UV_RESPON SE_UP	Genes up- regulated in response to ultraviolet (UV) radiation.	315/322	0.331	18	0.302	MutSig DB Hallmar k	Male
INTERFERO N_GAMMA_ RESPONSE	Genes up- regulated in response to IFNG [GeneID=3458] .	317/327	0.331	19	0.290	MutSig DB Hallmar k	Male
MYC_TARGE TS_V2	A subgroup of genes regulated by MYC - version 2 (v2).	69/71	0.240	19	0.150	MutSig DB Hallmar k	Male
TNFA_SIGNA LING_VIA_N FKB	Genes regulated by NF-kB in response to TNF [GeneID=7124] .	321/327	0.583	22	0.268	MutSig DB Hallmar k	Male

HEDGEHOG_ SIGNALING	Genes up-regulated by activation of hedgehog signaling.	69/73	0.414	22	0.198	MutSig DB Hallmar k	Male
WNT_BETA_ CATENIN_ SIGNALING	Genes up-regulated by activation of WNT signaling through accumulation of beta catenin CTNNB1 [GeneID=1499].	68/69	0.600	23	0.161	MutSig DB Hallmar k	Male
COMPLEMENT	Genes encoding components of the complement system, which is part of the innate immune system.	362/367	0.536	23	0.299	MutSig DB Hallmar k	Male

UNFOLDED_ PROTEIN_RE SPONSE	Genes up- regulated during unfolded protein response, a cellular stress response related to the endoplasmic reticulum.	174/176	0.259	24	-0.091	MutSig DB Hallmar k	Male
WNT_BETA_ CATENIN_SI GNALING	Genes up- regulated by activation of WNT signaling through accumulation of beta catenin CTNNB1 [GeneID=1499] .	68/69	0.029	5	0.215	MutSig DB Hallmar k	Fem ale

BILE_ACID_ METABOLIS M	Genes involve in metabolism of bile acids and salts.	176/176	0.000	5	0.604	MutSig DB Hallmar k	Fem ale
IL6_JAK_STA T3_SIGNALI NG	Genes up- regulated by IL6 [GeneID=3569] via STAT3 [GeneID=6774] , e.g., during acute phase response.	122/124	0.005	7	0.372	MutSig DB Hallmar k	Fem ale
COMPLEME NT	Genes encoding components of the complement system, which is part of the innate immune system.	362/367	0.024	11	0.447	MutSig DB Hallmar k	Fem ale
ADIPOGENE SIS	Genes up- regulated during	300/303	0.020	12	0.220	MutSig DB	Fem ale

	adipocyte differentiation (adipogenesis).					Hallmark	
EPITHELIAL_MESENCHYMAL_TRANSITION	Genes defining epithelial-mesenchymal transition, as in wound healing, fibrosis and metastasis.	302/304	0.044	12	0.305	MutSigDB Hallmark	Female
G2M_CHECKPOINT	Genes involved in the G2/M checkpoint, as in progression through the cell division cycle.	311/320	0.506	13	0.286	MutSigDB Hallmark	Female
PEROXISOME	Genes encoding components of peroxisome.	179/180	0.065	13	0.407	MutSigDB Hallmark	Female
DNA_REPAIR	Genes involved in DNA repair.	206/218	0.506	14	0.141	MutSigDB	Female

						Hallmar k	
ESTROGEN_ RESPONSE_L ATE	Genes defining late response to estrogen.	323/327	0.087	14	0.386	MutSig DB Hallmar k	Fem ale
APOPTOSIS	Genes mediating programmed cell death (apoptosis) by activation of caspases.	221/224	0.107	15	0.288	MutSig DB Hallmar k	Fem ale
ANDROGEN_ RESPONSE	Genes defining response to androgens.	182/186	0.366	15	0.233	MutSig DB Hallmar k	Fem ale
INTERFERO N_ALPHA_R ESPONSE	Genes up- regulated in response to alpha interferon proteins.	177/187	0.000	15	0.374	MutSig DB Hallmar k	Fem ale

ALLOGRAFT _REJECTION	Genes up-regulated during transplant rejection.	319/333	0.000	16	0.392	MutSig DB Hallmark	Female
CHOLESTEROL_HOMEOSTASIS	Genes involved in cholesterol homeostasis.	110/110	0.146	17	0.201	MutSig DB Hallmark	Female
ESTROGEN_RESPONSE_EARLY	Genes defining early response to estrogen.	307/310	0.506	17	0.305	MutSig DB Hallmark	Female
PROTEIN_SECRETION	Genes involved in protein secretion pathway.	146/146	0.464	17	-0.096	MutSig DB Hallmark	Female
INFLAMMATORY_RESPONSE	Genes defining inflammatory response.	301/303	0.151	18	0.342	MutSig DB Hallmark	Female

INTERFERON_GAMMA_RESPONSE	Genes up-regulated in response to IFNG [GeneID=3458]	317/327	0.000	19	0.333	MutSigDB Hallmark	Female
XENOBIOTIC_METABOLISM	Genes encoding proteins involved in processing of drugs and other xenobiotics.	342/349	0.000	19	0.509	MutSigDB Hallmark	Female
Autoimmune thyroid disease	Disease	73/79	0.000	2	0.426	KEGG	All
Homologous recombination	Signaling	41/41	0.023	3	0.960	KEGG	All
Hematopoietic cell lineage	Signaling	94/94	0.003	11	0.375	KEGG	All
Allograft rejection	Disease	57/63	0.001	12	0.456	KEGG	All
Chemical carcinogenesis	Disease	83/84	0.001	14	0.587	KEGG	All

Metabolism of xenobiotics by cytochrome P450	Metabolism	72/73	0.003	15	0.473	KEGG	All
Steroid hormone biosynthesis	Metabolism	90/92	0.003	16	0.624	KEGG	All
Drug metabolism - cytochrome P450	Metabolism	70/71	0.002	17	0.462	KEGG	All
Steroid biosynthesis	Metabolism	20/20	0.091	18	0.359	KEGG	All
Type I diabetes mellitus	Disease	64/70	0.005	23	0.390	KEGG	All
Antigen processing and presentation	Signaling	83/90	0.003	24	0.338	KEGG	All
Graft-versus-host disease	Disease	57/63	0.000	28	0.421	KEGG	All
Viral myocarditis	Disease	82/88	0.035	33	0.360	KEGG	All

Leishmaniasis	Disease	70/70	0.049	35	0.290	KEGG	All
Systemic lupus erythematosus	Disease	144/148	0.041	35	0.323	KEGG	All
Cell adhesion molecules (CAMs)	Signaling	166/174	0.046	37	0.355	KEGG	All
Glutathione metabolism	Metabolism	71/71	0.022	39	0.228	KEGG	All
Staphylococcus aureus infection	Disease	116/124	0.000	41	0.379	KEGG	All
beta-Alanine metabolism	Metabolism	32/32	0.091	42	0.169	KEGG	All
Citrate cycle (TCA cycle)	Metabolism	32/32	0.091	43	-0.051	KEGG	All
Citrate cycle (TCA cycle)	Metabolism	32/32	0.000	2	-0.077	KEGG	Male
Ubiquitin mediated proteolysis	Signaling	139/140	0.078	9	-0.067	KEGG	Male

Ascorbate and aldarate metabolism	Metabolism	27/27	0.299	12	0.272	KEGG	Male
Graft-versus-host disease	Disease	58/65	0.084	12	0.456	KEGG	Male
Fatty acid elongation	Metabolism	27/27	0.319	13	-0.132	KEGG	Male
Oxidative phosphorylation	Metabolism	134/134	0.078	16	-0.085	KEGG	Male
DNA replication	Signaling	35/35	0.016	16	0.186	KEGG	Male
Homologous recombination	Signaling	41/41	0.094	16	0.259	KEGG	Male
Ubiquinone and other terpenoid-quinone biosynthesis	Metabolism	11-Nov	0.387	18	-0.182	KEGG	Male
Autoimmune thyroid disease	Disease	71/79	0.142	19	0.476	KEGG	Male

Allograft rejection	Disease	56/64	0.156	21	0.478	KEGG	Male
Synthesis and degradation of ketone bodies	Metabolism	11-Nov	0.160	26	-0.139	KEGG	Male
Cysteine and methionine metabolism	Metabolism	47/48	0.174	27	-0.194	KEGG	Male
N-Glycan biosynthesis	Metabolism	49/49	0.156	27	-0.087	KEGG	Male
Phenylalanine metabolism	Metabolism	23/23	0.447	30	-0.310	KEGG	Male
Steroid biosynthesis	Metabolism	19/19	0.545	32	-0.052	KEGG	Male
Glycolysis / Gluconeogenesis	Metabolism	66/66	0.270	34	-0.143	KEGG	Male
Valine, leucine and isoleucine degradation	Metabolism	56/56	0.278	35	-0.134	KEGG	Male
Fatty acid biosynthesis	Metabolism	14/14	0.362	40	-0.206	KEGG	Male

Type I diabetes mellitus	Disease	63/70	0.319	41	0.403	KEGG	Male
Ascorbate and aldarate metabolism	Metabolism	27/27	0.000	7	0.905	KEGG	Female
Fatty acid degradation	Metabolism	49/49	0.000	10	0.316	KEGG	Female
Steroid biosynthesis	Metabolism	19/19	0.145	12	0.407	KEGG	Female
Pentose and glucuronate interconversions	Metabolism	34/34	0.002	15	0.722	KEGG	Female
Steroid hormone biosynthesis	Metabolism	86/87	0.000	15	1.041	KEGG	Female
Glycolysis / Gluconeogenesis	Metabolism	66/66	0.004	23	0.332	KEGG	Female
Pentose phosphate pathway	Metabolism	32/32	0.167	24	0.433	KEGG	Female

Glycine, serine and threonine metabolism	Metabolism	40/40	0.012	25	0.428	KEGG	Female
Drug metabolism - other enzymes	Metabolism	51/51	0.004	25	0.779	KEGG	Female
Tryptophan metabolism	Metabolism	46/46	0.041	31	0.458	KEGG	Female
Porphyrin and chlorophyll metabolism	Metabolism	41/41	0.012	32	0.713	KEGG	Female
Ribosome	Signaling	169/177	0.000	34	0.276	KEGG	Female
Valine, leucine and isoleucine degradation	Metabolism	56/56	0.018	35	0.189	KEGG	Female
Folate biosynthesis	Metabolism	14/14	0.019	36	0.269	KEGG	Female
Arginine and proline metabolism	Metabolism	50/50	0.020	37	0.266	KEGG	Female

Glutathione metabolism	Metabolism	59/59	0.000	38	0.289	KEGG	Female
Long-term potentiation	Signaling	67/67	0.020	38	-0.152	KEGG	Female
Starch and sucrose metabolism	Metabolism	33/33	0.010	39	0.373	KEGG	Female
Metabolism of xenobiotics by cytochrome P450	Metabolism	65/65	0.000	41	0.681	KEGG	Female
beta-Alanine metabolism	Metabolism	33/33	0.058	42	0.290	KEGG	Female

Conclusion

Summary of findings

Despite dozens of observational studies over the past few decades showing consistent associations of maternal acetaminophen use during pregnancy with child ADHD, major gaps in the literature remain. The first gap addressed in this dissertation (Chapter 2) was the reliance on maternal self-reported acetaminophen intake in nearly all prior studies. Here, we have shown an association of acetaminophen detected in meconium with child ADHD, a result that is not prone to recall bias. Only one other study, the Boston Birth Cohort, measured acetaminophen in a biological substrate rather than relying on self-report.²¹ A meta-analysis of prior studies relying on self-report found a pooled risk ratio of 1.34 for child ADHD following prenatal acetaminophen exposure.¹⁹ Both our study and the Boston Birth Cohort found stronger associations: odds ratios of 2.43 and 2.86 respectively. These stronger associations from studies that directly measured acetaminophen could indicate that the effect size from prior literature is smaller than the true effect owing to recall bias toward the null.

Another major gap in the literature is the lack of studies investigating the mechanisms that may link prenatal acetaminophen exposure with child ADHD in humans. We found that detection of acetaminophen in meconium was associated with altered child brain connectivity measured via fMRI (Chapter 2) and adverse birth outcomes (Chapter 3), including reduced birthweight and gestational age. We explored these potential mechanisms in mediation analyses, and found that the association of prenatal acetaminophen exposure with child ADHD was mediated by brain connectivity but not birth outcomes.

Although observational research has many strengths, it is difficult to infer causality in observational studies where unmeasured confounding is unavoidable. Accordingly, we employed a randomized experiment in mice to assess the causal role of prenatal acetaminophen exposure in neurodevelopment (Chapter 4). Developmental acetaminophen exposure resulted in sex-specific changes in mouse behavior, but the neurodevelopmental changes were consistent with elevated anxiety and not ADHD-like behaviors. While some mechanisms can be explored in human studies, it is difficult to analyze molecular mechanisms in the human brain without relying on circulating biomarkers or postmortem tissues. In our mouse study, on the other hand, we had the opportunity to measure frontal cortex gene expression. RNA sequencing of the mouse frontal cortex revealed many pathways that could be targeted for future interventions, including immune activation, metabolic pathways of oxidative stress and DNA damage, and endocrine disruption.

Limitations

A major limitation of the human observational studies presented here (Chapters 2 and 3) was the lack of data on indications for acetaminophen use. Confounding by indication is a critical factor preventing causal inference in the association of prenatal acetaminophen exposure with adverse child health outcomes. Confounding occurs when a common cause of the predictor (prenatal acetaminophen) and outcome (adverse child health outcomes) induces a non-causal association between them. For instance, if older age: 1) causes pregnant women to take more acetaminophen and 2) increases the risk for child ADHD, then maternal age could confound the association of prenatal acetaminophen exposure with child ADHD. Many confounders, including maternal characteristics like age and socioeconomic status, and maternal exposures such as alcohol and tobacco smoke, are easily adjusted for in statistical models. Indications for a drug's use, on the other hand, are inexorably tied to exposure to that drug, and cannot be easily included

in statistical models. Controlling for the indications for use can partially or completely explain away the effect of a drug. For instance, if someone takes a drug every time they experience a particular symptom, we cannot distinguish from the downstream effects of the drug and the downstream effects of the symptom. Acetaminophen, on the other hand, has several indications including pain, headaches, and fever, so indications for acetaminophen use can be included as a model covariate. Although the human studies presented here lack these data, many prior studies have adjusted for indications and reported minimal confounding.^{21,85-88,90}

An inherent limitation of all observational epidemiology studies is confounding by unknown factors. Despite several decades of extensive observational research on prenatal acetaminophen and child ADHD, the possibility always remains that certain confounding factors have not been discovered and accounted for. Concerns about unknown confounding can be addressed in several ways. First, the best way to account for confounding is with randomized experiments. In a randomized clinical trial, confounding bias is impossible, and the only source of bias is from random chance. Similarly, the mouse study presented here (Chapter 4) was randomized, so confounding by unknown factors is impossible. Furthermore, experimental mice are genetically homogeneous and experience uniform laboratory environmental conditions, reducing the impact of random chance compared to randomized clinical trials. However, results from mouse studies may not always be easily translated to benefit humans. Translatability is particularly relevant for complex neurodevelopmental diseases like ADHD, which may not have a clear analogue in mice.

Aside from alternative study designs such as randomized clinical trials, unmeasured confounding can also be partially ameliorated within observational studies. One hypothesis is that unmeasured familial confounding, whether genetic or environmental, may explain

associations of prenatal acetaminophen exposure with adverse health outcomes. Such familial confounding can be accounted for with negative control exposure (NCE) methods. An NCE is an exposure that shares similar confounding structure with the exposure of interest but cannot have a causal effect on the outcome. Prior studies have analyzed maternal acetaminophen use after birth and partner's acetaminophen use as NCEs.^{90,209} These variables likely share familial confounders with maternal acetaminophen use during pregnancy, yet they cannot causally affect the developing fetus. Both studies found that only maternal acetaminophen use during pregnancy was associated with child neurodevelopment, and that these associations were not likely explained by unmeasured familial factors, since child neurodevelopment was not linked to postnatal or partner's acetaminophen use.

In addition to accounting for unmeasured confounding, there are methods for understanding and quantifying levels of unmeasured confounding. One such method, the E-value,¹²⁰ was employed here. E-values indicate the minimum strength of association of an unmeasured confounder with both the exposure and outcome that would confound a null effect to the observed effect estimate (i.e., completely explain the observed association between the exposure and outcome). In our study, the E-value revealed that to completely explain away the association of prenatal acetaminophen exposure and reduced birthweight, an unmeasured confounder would need to be associated with 93% increased risk of prenatal acetaminophen exposure and 93% increased risk for low birthweight (Chapter 3). While such strong confounding is unlikely, a smaller E-value would signal increased probability that the association was artificially induced by unobserved confounding, which could be addressed by gathering data on known confounders that were never measured, or discovering new confounders.

Future directions

In the face of new evidence for adverse neurodevelopmental outcomes associated with prenatal acetaminophen exposure, the United States Food and Drug Administration and the Society for Maternal-Fetal Medicine maintained that acetaminophen should be considered safe for the treatment of pain and/or fever during pregnancy. In the years since the release of those statements, in 2015 by the FDA²¹⁰ and 2017 by the SMFM,⁶⁴ overwhelming evidence has continued to accumulate suggesting that maternal use of acetaminophen during pregnancy puts children at increased risk of developing ADHD, Autism Spectrum Disorders (ASD), asthma, and reproductive disorders. A recent consensus statement supported by 91 scientists, clinicians and public health professionals from across the globe comprehensively reviewed the literature and summarized these clear and prevalent associations.¹⁰¹ The consensus statement authors did not recommend complete contraindication of acetaminophen use during pregnancy, but rather a more cautious approach to “minimize exposure by using the lowest effective dose for the shortest possible time.”

One common position is that current recommendations should not change, because pregnant women are already urged to “discuss all medicines with their health care professionals before using them”²¹⁰ (also see statements by the American College of Obstetricians and Gynecologists (ACOG)²¹¹ and the Society of Obstetricians and Gynecologists of Canada (SOGC)²¹² for similar recommendations). Yet, this expectation that all pregnant women consult with their health care professional before taking over-the-counter drugs is far from reality. Although pregnant women may be generally cautious when contemplating medication use during pregnancy, pregnant women and mothers perceive minimal risk conferred by acetaminophen.²¹³ Consequently, many women routinely use acetaminophen during pregnancy without informing their physician.²¹⁴ This status quo is consistent with minimal precaution regarding

acetaminophen exposure during pregnancy, and may in part explain such astoundingly high current levels of use, by upwards of 70% of pregnant women in many populations.^{10,210,213}

Ultimately, the position that current clinical recommendations are adequate for the safe use of acetaminophen amounts to an affirmation that the drugs continued, unbridled use during pregnancy is favorable for public health.

Unfortunately, much of the contemporary debate^{211,212} considers in relative absolutes if acetaminophen is or is not safe for use during pregnancy. A far more constructive debate and research agenda explores not just whether acetaminophen is safe, but when during pregnancy and for what indications the drug is recommended for use. Surveying the literature, several testable hypotheses related to the timing and indications of use can be easily generated.

One hypothesis is that acetaminophen during pregnancy can benefit the developing fetus when used to treat maternal fever but is harmful when used for all other indications.

Acetaminophen has several indications for use. The drug is primarily used to treat pain, headaches, and fever. Because there is not a 100% correlation of acetaminophen use with any singular indication, these indications can be included in statistical models, allowing us to distinguish from the effects of the symptoms and the effects of the drug itself on child health outcomes. While maternal pain and headaches during pregnancy are unlikely to increase the risk for child ADHD, many studies have shown that maternal fever during pregnancy may be associated with increased risk for child ASD and ADHD.²¹⁵⁻²²¹ One study even found that the increased ASD risk associated with maternal fever was attenuated among mothers taking antipyretic medications (any antipyretic, including acetaminophen).²¹⁶ Moving forward, it will be critical for researchers to consider that if left untreated, certain indications for acetaminophen use like maternal fevers may cause adverse child development and should be treated with the drug.

For other indications like maternal headache, however, acetaminophen might cause more harm than good. Headaches and pain account for upwards of 70% of the indications for acetaminophen use during pregnancy, while only 8% of women report using acetaminophen to treat fever.¹²⁹ Therefore, recommendations based on this hypothesis would drastically reduce the prevalence of prenatal acetaminophen exposure. Less than 3% of pregnant women report migraines as the reason for acetaminophen use,¹²⁹ and untreated migraines in pregnancy may be associated with pregnancy complications and adverse birth outcomes.²²² However, triptans may be a better option for migraine relief, as they are associated with equal or better outcomes compared with acetaminophen in the individual using them,²²³ and their use during pregnancy is not associated with child ADHD.²²⁴

Another hypothesis is that compared with earlier gestational exposure, maternal acetaminophen use late in pregnancy is more harmful to the developing fetus. During gestation, brain development is most active during the 3rd trimester of pregnancy. Consequently, several studies have found stronger neurodevelopmental effects of prenatal acetaminophen exposure in the 3rd trimester compared to earlier gestational exposure.^{85,86,90} Studies have similarly shown that associations of prenatal acetaminophen with symptoms related to asthma are stronger for exposure later in gestation.²²⁵⁻²²⁷ Finally, clinical case-studies suggest that prenatal acetaminophen exposure in the 3rd trimester may lead to premature ductus arteriosus constriction or closure,²²⁸ which can result in congestive heart failure or even intrauterine death.

Several cohort studies have already refuted the hypotheses that confounding by indication and/or recall bias are responsible for associations of prenatal acetaminophen with child neurodevelopment. Many more hypotheses can be generated and explored in future observational research. Ultimately, however, medical and regulatory organizations are unlikely to change their

position in the absence of a conclusive randomized clinical trial. Responding to the recent consensus statement on the harms of acetaminophen use during pregnancy,¹⁰¹ ACOG argued that neurodevelopmental disorders “are multifactorial and very difficult to associate with a singular cause. The brain does not stop developing until at least 15 months of age, which leaves room for children to be exposed to a number of factors that could potentially lead to these issues.”²¹¹ In fact, this is the case for almost any exposure-health outcome relationship, and the reason for the development of epidemiological methods to control for sources of bias such as confounding. For example, many experts agree that there is no safe level of lead exposure,²²⁹ yet the time between birth and the end of brain development leaves room for children to be exposed to many factors other than prenatal lead. Arguments such as this one point to the requirement imposed by many health professionals for a randomized clinical trial to constitute definitive evidence, and a reluctance to give any weight to observational research. Given their importance for informing clinical practice, we should expect that a previously published randomized clinical trial has shown definitive evidence that the most common drug used during pregnancy, acetaminophen, is safe for fetal development. Yet prior experimental studies have only shown the safety of developmental acetaminophen exposure for the pediatric liver, while neurodevelopment has never been assessed.²³⁰ Furthermore, if the drug were developed under today’s regulatory standards, acetaminophen would likely not meet safety criteria in preclinical animal studies, precluding a phase I clinical trial.

The accumulating evidence of adverse health outcomes resulting from prenatal acetaminophen exposure combined with continued high levels of use, by well over 50% of pregnant women in many populations, points to the possibility of adequate clinical equipoise for a randomized trial to occur. Designing the clinical trial will undoubtedly prove difficult. Clinical

trial controls typically receive the current standard of care, which is acetaminophen for the treatment of maternal pain and fevers during pregnancy. An experimental group assigned to take placebo control to treat maternal pain and/or fever during pregnancy might be considered unethical, although one recent trial randomly assigned preterm infants (n = 48) to either intravenous acetaminophen or saline control groups. Advancements in future observational studies will likely inform alternative clinical trial designs for achieving more favorable health outcomes. For example, the control groups could be advised to take acetaminophen for pain, fever, and headaches following the current standard of care, while the treatment group could be advised to only take acetaminophen to treat elevated fevers.

Concluding remarks

This dissertation addressed several known gaps in prenatal acetaminophen and neurodevelopment research. However, as discussed in this chapter, many gaps and challenges for future research remain. Although mediating mechanisms were explored in every chapter of this dissertation, more work is needed. The mediating brain connectivity changes uncovered in Chapter 2 are not easily intervened upon, while the potential molecular mechanisms uncovered through mouse frontal cortex RNA sequencing relating to DNA damage, hormone disruption, metabolic changes, and immune activation (Chapter 4) need to be further investigated in humans. If similar mechanisms play a role in the fetal toxicity of acetaminophen in humans, then interventions can target those pathways in mouse studies. Ultimately, definitive evidence of developmental harm caused by prenatal acetaminophen exposure may be lacking until a randomized clinical trial is performed. Until such a trial is completed, however, failure to change recommendations regarding the safety of acetaminophen use during pregnancy risks further harm

to public health. Therefore, recommendations to minimize prenatal acetaminophen exposure by using the lowest effect dose, and for the shortest duration possible, may be warranted.

References

1. Polanczyk G, De Lima MS, Horta BL, Biederman J, Rohde LA. The worldwide prevalence of ADHD: a systematic review and metaregression analysis. *American journal of psychiatry*. 2007;164(6):942-948.
2. Simon V, Czobor P, Bálint S, Mészáros A, Bitter I. Prevalence and correlates of adult attention-deficit hyperactivity disorder: meta-analysis. *The British Journal of Psychiatry*. 2009;194(3):204-211.
3. Pliszka SR. Comorbidity of attention-deficit/hyperactivity disorder with psychiatric disorder: an overview. *The Journal of clinical psychiatry*. 1998;59:50-58.
4. Kessler RC, Adler L, Barkley R, et al. The prevalence and correlates of adult ADHD in the United States: results from the National Comorbidity Survey Replication. *American Journal of psychiatry*. 2006;163(4):716-723.
5. Shaw M, Hodgkins P, Caci H, et al. A systematic review and analysis of long-term outcomes in attention deficit hyperactivity disorder: effects of treatment and non-treatment. *BMC medicine*. 2012;10(1):99.
6. Posner J, Polanczyk GV, Sonuga-Barke E. Attention-deficit hyperactivity disorder. *Lancet (London, England)*. 2020;395(10222):450-462.
7. Polanczyk GV, Willcutt EG, Salum GA, Kieling C, Rohde LA, Joe. ADHD prevalence estimates across three decades: an updated systematic review and meta-regression analysis. 2014;43(2):434-442.
8. Faraone SV, Perlis RH, Doyle AE, et al. Molecular genetics of attention-deficit/hyperactivity disorder. 2005;57(11):1313-1323.

9. Faraone SV, Banaschewski T, Coghill D, et al. The world federation of ADHD international consensus statement: 208 evidence-based conclusions about the disorder. 2021.
10. Werler MM, Mitchell AA, Hernandez-Diaz S, Honein MA. Use of over-the-counter medications during pregnancy. *American journal of obstetrics and gynecology*. 2005;193(3):771-777.
11. Rebordosa C, Kogevinas M, Bech BH, Sørensen HT, Olsen J. Use of acetaminophen during pregnancy and risk of adverse pregnancy outcomes. *International journal of epidemiology*. 2009;38(3):706-714.
12. Li D-K, Liu L, Odouli R. Exposure to non-steroidal anti-inflammatory drugs during pregnancy and risk of miscarriage: population based cohort study. *Bmj*. 2003;327(7411):368.
13. Nielsen GL, Sørensen HT, Larsen H, Pedersen L. Risk of adverse birth outcome and miscarriage in pregnant users of non-steroidal anti-inflammatory drugs: population based observational study and case-control study. *BMJ: British Medical Journal*. 2001;322(7281):266.
14. Ofori B, Oraichi D, Blais L, Rey E, Bérard A. Risk of congenital anomalies in pregnant users of non-steroidal anti-inflammatory drugs: A nested case-control study. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*. 2006;77(4):268-279.
15. Ericson A, Källén BA. Nonsteroidal anti-inflammatory drugs in early pregnancy. *Reproductive Toxicology*. 2001;15(4):371-375.

16. Gyllenberg D, Marttila M, Sund R, et al. Temporal changes in the incidence of treated psychiatric and neurodevelopmental disorders during adolescence: an analysis of two national Finnish birth cohorts. *The Lancet Psychiatry*. 2018;5(3):227-236.
17. Etminan M, Sadatsafavi M, Jafari S, Doyle-Waters M, Aminzadeh K, FitzGerald JM. Acetaminophen use and the risk of asthma in children and adults: a systematic review and metaanalysis. *Chest*. 2009;136(5):1316-1323.
18. Aminoshariae A, Khan A. Acetaminophen: old drug, new issues. *Journal of endodontics*. 2015;41(5):588-593.
19. Masarwa R, Levine H, Gorelik E, Reif S, Perlman A, Matok I. Prenatal exposure to acetaminophen and risk for attention deficit hyperactivity disorder and autistic spectrum disorder: a systematic review, meta-analysis, and meta-regression analysis of cohort studies. *American journal of epidemiology*. 2018;187(8):1817-1827.
20. Gou X, Wang Y, Tang Y, et al. Association of maternal prenatal acetaminophen use with the risk of attention deficit/hyperactivity disorder in offspring: A meta-analysis. *Australian & New Zealand Journal of Psychiatry*. 2019;53(3):195-206.
21. Ji Y, Azuine RE, Zhang Y, et al. Association of Cord Plasma Biomarkers of In Utero Acetaminophen Exposure With Risk of Attention-Deficit/Hyperactivity Disorder and Autism Spectrum Disorder in Childhood. *JAMA psychiatry*. 2019:1-11.
22. Braun JM, Daniels JL, Poole C, et al. A prospective cohort study of biomarkers of prenatal tobacco smoke exposure: the correlation between serum and meconium and their association with infant birth weight. *Environmental Health*. 2010;9(1):53.
23. Bearer CF. Meconium as a biological marker of prenatal exposure. *Ambulatory Pediatrics*. 2003;3(1):40-43.

24. Moore C, Negrusz A, Lewis D. Determination of drugs of abuse in meconium. *Journal of Chromatography B: Biomedical Sciences and Applications*. 1998;713(1):137-146.
25. Harries J. Meconium in health and disease. *British medical bulletin*. 1978;34(1):75-78.
26. Ostrea EM, Brady M, Gause S, Raymundo AL, Stevens M. Drug screening of newborns by meconium analysis: a large-scale, prospective, epidemiologic study. *Pediatrics*. 1992;89(1):107-113.
27. Ghanem CI, Pérez MJ, Manautou JE, Mottino AD. Acetaminophen from liver to brain: new insights into drug pharmacological action and toxicity. *Pharmacological research*. 2016;109:119-131.
28. Posadas I, Santos P, Blanco A, Muñoz-Fernández M, Ceña V. Acetaminophen induces apoptosis in rat cortical neurons. *PloS one*. 2010;5(12):e15360.
29. da Silva MH, da Rosa EJJ, de Carvalho NR, et al. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. *Neurotoxicity research*. 2012;21(3):334-344.
30. Patten CJ, Thomas PE, Guy RL, et al. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chemical research in toxicology*. 1993;6(4):511-518.
31. Slattery JT, Wilson JM, Kalthorn TF, Nelson SDJCP, Therapeutics. Dose-dependent pharmacokinetics of acetaminophen: evidence of glutathione depletion in humans. 1987;41(4):413-418.
32. Dimova S, Hoet PH, Dinsdale D, Nemery BJTijob, biology c. Acetaminophen decreases intracellular glutathione levels and modulates cytokine production in human alveolar macrophages and type II pneumocytes in vitro. 2005;37(8):1727-1737.

33. Nuttall S, Khan J, Thorpe G, Langford N, Kendall MJ. J. Therapeutics. The impact of therapeutic doses of paracetamol on serum total antioxidant capacity. 2003;28(4):289-294.
34. Bajt ML, Knight TR, Lemasters JJ, Jaeschke H. Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine. *Toxicological sciences*. 2004;80(2):343-349.
35. McGill MR, Williams CD, Xie Y, Ramachandran A, Jaeschke H. Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. *Toxicology and applied pharmacology*. 2012;264(3):387-394.
36. McGill MR, Jaeschke H. Metabolism and disposition of acetaminophen: recent advances in relation to hepatotoxicity and diagnosis. *Pharmaceutical research*. 2013;30(9):2174-2187.
37. Du K, Ramachandran A, Jaeschke H. Oxidative stress during acetaminophen hepatotoxicity: Sources, pathophysiological role and therapeutic potential. *Redox biology*. 2016;10:148-156.
38. Jaeschke H, McGill MR, Williams CD, Ramachandran A. Current issues with acetaminophen hepatotoxicity—a clinically relevant model to test the efficacy of natural products. *Life sciences*. 2011;88(17-18):737-745.
39. Yan M, Huo Y, Yin S, Hu H. Mechanisms of acetaminophen-induced liver injury and its implications for therapeutic interventions. *Redox biology*. 2018.
40. Nassini R, Materazzi S, André E, et al. Acetaminophen, via its reactive metabolite N-acetyl-p-benzo-quinoneimine and transient receptor potential ankyrin-1 stimulation,

- causes neurogenic inflammation in the airways and other tissues in rodents. *The FASEB Journal*. 2010;24(12):4904-4916.
41. Chen T, Richie J, Lang C. Life span profiles of glutathione and acetaminophen detoxification. *Drug metabolism and disposition*. 1990;18(6):882-887.
 42. Micheli L, Cerretani D, Fiaschi A, Giorgi G, Romeo M, Runci F. Effect of acetaminophen on glutathione levels in rat testis and lung. *Environmental health perspectives*. 1994;102(Suppl 9):63.
 43. Slattery JT, Wilson JM, Kalthorn TF, Nelson SD. Dose-dependent pharmacokinetics of acetaminophen: evidence of glutathione depletion in humans. *Clinical Pharmacology & Therapeutics*. 1987;41(4):413-418.
 44. Dimova S, Hoet PH, Dinsdale D, Nemery B. Acetaminophen decreases intracellular glutathione levels and modulates cytokine production in human alveolar macrophages and type II pneumocytes in vitro. *The international journal of biochemistry & cell biology*. 2005;37(8):1727-1737.
 45. Nuttall S, Khan J, Thorpe G, Langford N, Kendall M. The impact of therapeutic doses of paracetamol on serum total antioxidant capacity. *Journal of clinical pharmacy and therapeutics*. 2003;28(4):289-294.
 46. Kristensen DM, Mazaud-Guittot S, Gaudriault P, et al. Analgesic use—prevalence, biomonitoring and endocrine and reproductive effects. *Nature Reviews Endocrinology*. 2016;12(7):381-393.
 47. Cohen IV, Cirulli ET, Mitchell MW, et al. Acetaminophen (paracetamol) use modifies the sulfation of sex hormones. 2018;28:316-323.

48. Klopčič I, Markovič T, Mlinarič-Raščan I, Dolenc MSJTl. Endocrine disrupting activities and immunomodulatory effects in lymphoblastoid cell lines of diclofenac, 4-hydroxydiclofenac and paracetamol. 2018;294:95-104.
49. Holm JB, Chalmey C, Modick H, et al. Aniline is rapidly converted into paracetamol impairing male reproductive development. 2015;148(1):288-298.
50. Addo KA, Palakodety N, Fry RCJTS. Acetaminophen modulates the expression of steroidogenesis-associated genes and estradiol levels in human placental JEG-3 cells. 2021;179(1):44-52.
51. Motawi TK, Ahmed SA, El-Boghdady NA, Metwally NS, Nasr NNJB. Protective effects of betanin against paracetamol and diclofenac induced neurotoxicity and endocrine disruption in rats. 2019;24(7):645-651.
52. Aminoshariae A, Khan AJJoe. Acetaminophen: old drug, new issues. 2015;41(5):588-593.
53. Thompson W, Russell G, Baragwanath G, Matthews J, Vaidya B, Thompson-Coon J. Maternal thyroid hormone insufficiency during pregnancy and risk of neurodevelopmental disorders in offspring: A systematic review and meta-analysis. *Clinical endocrinology*. 2018;88(4):575-584.
54. Ghassabian A, Bongers-Schokking JJ, Henrichs J, et al. Maternal thyroid function during pregnancy and behavioral problems in the offspring: the generation R study. *Pediatric research*. 2011;69(5, Part 1 of 2):454.
55. Ghassabian A, Bongers-Schokking JJ, De Rijke YB, et al. Maternal thyroid autoimmunity during pregnancy and the risk of attention deficit/hyperactivity problems in children: the Generation R Study. *Thyroid*. 2012;22(2):178-186.

56. Pääkkilä F, Männistö T, Pouta A, et al. The impact of gestational thyroid hormone concentrations on ADHD symptoms of the child. *The Journal of Clinical Endocrinology & Metabolism*. 2014;99(1):E1-E8.
57. Hoover RM, Hayes VAG, Erramouspe J. Association between prenatal acetaminophen exposure and future risk of attention deficit/hyperactivity disorder in children. *Annals of Pharmacotherapy*. 2015;49(12):1357-1361.
58. Olsen J, Liew Z. Fetal programming of mental health by acetaminophen? Response to the SMFM statement: prenatal acetaminophen use and ADHD. *Expert opinion on drug safety*. 2017;16(12):1395-1398.
59. Smilkstein MJ, Knapp GL, Kulig KW, Rumack BH. Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. *New England Journal of Medicine*. 1988;319(24):1557-1562.
60. McGill MR, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *The Journal of clinical investigation*. 2012;122(4):1574-1583.
61. Zhu J, Lee KP, Spencer TJ, Biederman J, Bhide PG. Transgenerational transmission of hyperactivity in a mouse model of ADHD. *Journal of Neuroscience*. 2014;34(8):2768-2773.
62. Albert O, Desdoits-Lethimonier C, Lesné L, et al. Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. *Human reproduction*. 2013;28(7):1890-1898.

63. Jégou B. Reproductive endocrinology: Paracetamol-induced endocrine disruption in human fetal testes. *Nature Reviews Endocrinology*. 2015;11(8):453.
64. Committee SfM-FMP. Prenatal acetaminophen use and outcomes in children. *Am J Obstet Gynecol*. 2017;216(3):B14-B15.
65. FDA has reviewed possible risks of pain medicine use during pregnancy. <https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-fda-has-reviewed-possible-risks-pain-medicine-use-during-pregnancy>. Accessed October 31, 2019.
66. Prescott L. Kinetics and metabolism of paracetamol and phenacetin. *British journal of clinical pharmacology*. 1980;10(S2):291S-298S.
67. Hill AB. The environment and disease: association or causation? In: Sage Publications; 1965.
68. Castellanos FX, Aoki Y. Intrinsic Functional Connectivity in Attention-Deficit/Hyperactivity Disorder: A Science in Development. *Biol Psychiatry Cogn Neurosci Neuroimaging*. 2016;1(3):253-261.
69. Gallo EF, Posner J. Moving towards causality in attention-deficit hyperactivity disorder: overview of neural and genetic mechanisms. *The Lancet Psychiatry*. 2016;3(6):555-567.
70. Cassoulet R, Haroune L, Abdelouahab N, et al. Monitoring of prenatal exposure to organic and inorganic contaminants using meconium from an Eastern Canada cohort. *Environmental research*. 2019;171:44-51.
71. Merenda PF. BASC: Behavior Assessment System for Children. *Measurement and Evaluation in Counseling and Development*. 1996.

72. Whitfield-Gabrieli S, Nieto-Castanon A. Conn: a functional connectivity toolbox for correlated and anticorrelated brain networks. *Brain connectivity*. 2012;2(3):125-141.
73. Austin PC. An introduction to propensity score methods for reducing the effects of confounding in observational studies. *Multivariate behavioral research*. 2011;46(3):399-424.
74. Rosenbaum PR, Rubin DB. The central role of the propensity score in observational studies for causal effects. *Biometrika*. 1983;70(1):41-55.
75. Austin PC, Stuart EA. Moving towards best practice when using inverse probability of treatment weighting (IPTW) using the propensity score to estimate causal treatment effects in observational studies. *Statistics in medicine*. 2015;34(28):3661-3679.
76. Imai K, Ratkovic M. Covariate balancing propensity score. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)*. 2014;76(1):243-263.
77. Fong C, Ratkovic M, Imai K. CBPS: R package for covariate balancing propensity score. *Comprehensive R Archive Network (CRAN)*. 2014.
78. MacKinnon DP, Lockwood CM, Hoffman JM, West SG, Sheets V. A comparison of methods to test mediation and other intervening variable effects. *Psychological methods*. 2002;7(1):83.
79. Tingley D, Yamamoto T, Hirose K, Keele L, Imai K. Mediation: R package for causal mediation analysis. 2014.
80. Team RC. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2018. In: ISBN3-900051-07-0 <https://www.R-project.org>; 2018.

81. Fatoumata Binta Diallo LR, Éric Pelletier, Alain Lesage, Annick Vincent, Helen-Maria Vasiliadis, Sylvain Palardy. *Surveillance of attention deficit disorder with or without hyperactivity (ADHD) in Quebec*. Montreal: National Institute of Public Health of Quebec;2019.
82. Austin PC. Using the standardized difference to compare the prevalence of a binary variable between two groups in observational research. *Communications in statistics-simulation and computation*. 2009;38(6):1228-1234.
83. Laue HE, Cassoulet R, Abdelouahab N, et al. Association between meconium acetaminophen and childhood neurocognitive development in GESTE, a Canadian cohort study. *Toxicological Sciences*. 2018;167(1):138-144.
84. Streissguth AP, Treder RP, Barr HM, et al. Aspirin and acetaminophen use by pregnant women and subsequent child IQ and attention decrements. *Teratology*. 1987;35(2):211-219.
85. Brandlistuen RE, Ystrom E, Nulman I, Koren G, Nordeng H. Prenatal paracetamol exposure and child neurodevelopment: a sibling-controlled cohort study. *International journal of epidemiology*. 2013;42(6):1702-1713.
86. Liew Z, Ritz B, Rebordosa C, Lee P-C, Olsen J. Acetaminophen use during pregnancy, behavioral problems, and hyperkinetic disorders. *JAMA pediatrics*. 2014;168(4):313-320.
87. Thompson JM, Waldie KE, Wall CR, Murphy R, Mitchell EA, Group AS. Associations between acetaminophen use during pregnancy and ADHD symptoms measured at ages 7 and 11 years. *PloS one*. 2014;9(9):e108210.

88. Avella-Garcia CB, Julvez J, Fortuny J, et al. Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. *International journal of epidemiology*. 2016;45(6):1987-1996.
89. Liew Z, Ritz B, Virk J, Olsen J. Maternal use of acetaminophen during pregnancy and risk of autism spectrum disorders in childhood: AD anish national birth cohort study. *Autism Research*. 2016;9(9):951-958.
90. Stergiakouli E, Thapar A, Smith GD. Association of acetaminophen use during pregnancy with behavioral problems in childhood: evidence against confounding. *JAMA pediatrics*. 2016;170(10):964-970.
91. Serme-Gbedo YK, Abdelouahab N, Pasquier J-C, Cohen AA, Takser L. Maternal levels of endocrine disruptors, polybrominated diphenyl ethers, in early pregnancy are not associated with lower birth weight in the Canadian birth cohort GESTE. *Environmental Health*. 2016;15(1):49.
92. Elton A, Alcauter S, Gao W. Network connectivity abnormality profile supports a categorical-dimensional hybrid model of ADHD. *Human brain mapping*. 2014;35(9):4531-4543.
93. Vatansever D, Bozhilova NS, Asherson P, Smallwood J. The devil is in the detail: exploring the intrinsic neural mechanisms that link attention-deficit/hyperactivity disorder symptomatology to ongoing cognition. *Psychological medicine*. 2019;49(7):1185-1194.
94. Pujol J, Martínez-Vilavella G, Macià D, et al. Traffic pollution exposure is associated with altered brain connectivity in school children. *Neuroimage*. 2016;129:175-184.

95. Tost H, Champagne FA, Meyer-Lindenberg A. Environmental influence in the brain, human welfare and mental health. *Nature Neuroscience*. 2015;18(10):1421.
96. Lugo-Candelas C, Cha J, Hong S, et al. Associations between brain structure and connectivity in infants and exposure to selective serotonin reuptake inhibitors during pregnancy. *JAMA pediatrics*. 2018;172(6):525-533.
97. SCHOENFELD A, BAR Y, MERLOB P, OVADIA Y. NSAIDs: maternal and fetal considerations. *American Journal of Reproductive Immunology*. 1992;28(3-4):141-147.
98. Nezvalová-Henriksen K, Spigset O, Nordeng H. Effects of ibuprofen, diclofenac, naproxen, and piroxicam on the course of pregnancy and pregnancy outcome: a prospective cohort study. *BJOG: An International Journal of Obstetrics & Gynaecology*. 2013;120(8):948-959.
99. Hernandez RK, Werler MM, Romitti P, Sun L, Anderka M, Study NBDP. Nonsteroidal antiinflammatory drug use among women and the risk of birth defects. *American journal of obstetrics and gynecology*. 2012;206(3):228. e221-228. e228.
100. Vermillion ST, Scardo JA, Lashus AG, Wiles HB. The effect of indomethacin tocolysis on fetal ductus arteriosus constriction with advancing gestational age. *American journal of obstetrics and gynecology*. 1997;177(2):256-261.
101. Bauer AZ, Swan SH, Kriebel D, et al. Paracetamol use during pregnancy—a call for precautionary action. 2021:1-10.
102. Arneja J, Hung RJ, Seeto RA, et al. Association between maternal acetaminophen use and adverse birth outcomes in a pregnancy and birth cohort. *Pediatric Research*. 2019.

103. Sonnenschein-Van Der Voort AM, Arends LR, de Jongste JC, et al. Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. *Journal of allergy and clinical immunology*. 2014;133(5):1317-1329.
104. Xu X-F, Li Y-J, Sheng Y-J, Liu J-L, Tang L-F, Chen Z-M. Effect of low birth weight on childhood asthma: a meta-analysis. *BMC pediatrics*. 2014;14(1):275.
105. Momany AM, Kamradt JM, Nikolas MA. A meta-analysis of the association between birth weight and attention deficit hyperactivity disorder. *Journal of abnormal child psychology*. 2018;46(7):1409-1426.
106. Sucksdorff M, Lehtonen L, Chudal R, et al. Preterm birth and poor fetal growth as risk factors of attention-deficit/hyperactivity disorder. *Pediatrics*. 2015;136(3):e599-e608.
107. Agrawal S, Rao SC, Bulsara MK, Patole SK. Prevalence of autism spectrum disorder in preterm infants: a meta-analysis. *Pediatrics*. 2018;142(3):e20180134.
108. Gardener H, Spiegelman D, Buka SL. Perinatal and neonatal risk factors for autism: a comprehensive meta-analysis. *Pediatrics*. 2011;128(2):344-355.
109. Baker BH, Lugo-Candelas C, Wu H, et al. Association of Prenatal Acetaminophen Exposure Measured in Meconium With Risk of Attention-Deficit/Hyperactivity Disorder Mediated by Frontoparietal Network Brain Connectivity. *JAMA Pediatrics*. 2020.
110. Coughlin SS. Recall bias in epidemiologic studies. *Journal of clinical epidemiology*. 1990;43(1):87-91.
111. WERLER MM, POBER BR, NELSON K, HOLMES LB. Reporting accuracy among mothers of malformed and nonmalformed infants. *American Journal of Epidemiology*. 1989;129(2):415-421.

112. Rockenbauer M, Olsen J, Czeizel AE, Pedersen L, Sørensen HT, Group TE. Recall bias in a case-control surveillance system on the use of medicine during pregnancy. *Epidemiology*. 2001;461-466.
113. Baker BH, Wu H, Laue HE, et al. Methylparaben in meconium and risk of maternal thyroid dysfunction, adverse birth outcomes, and Attention-Deficit Hyperactivity Disorder (ADHD). *Environment International*. 2020;139:105716.
114. Katz VL, Bowes Jr WA. Meconium aspiration syndrome: reflections on a murky subject. *American journal of obstetrics and gynecology*. 1992;166(1):171-183.
115. Alexander GR, Hulsey TC, Robillard P-Y, De Caunes F, Papiernik E. Determinants of meconium-stained amniotic fluid in term pregnancies. *Journal of Perinatology*. 1994;14(4):259-263.
116. Fenton TR, Kim JH. A systematic review and meta-analysis to revise the Fenton growth chart for preterm infants. *BMC pediatrics*. 2013;13(1):59.
117. Buuren Sv, Groothuis-Oudshoorn K. mice: Multivariate imputation by chained equations in R. *Journal of statistical software*. 2010:1-68.
118. Marshall A, Altman DG, Holder RL, Royston P. Combining estimates of interest in prognostic modelling studies after multiple imputation: current practice and guidelines. *BMC Medical Research Methodology*. 2009;9(1):57.
119. Rubin DB. *Multiple imputation for nonresponse in surveys*. Vol 81: John Wiley & Sons; 2004.
120. VanderWeele TJ, Ding PJAoim. Sensitivity analysis in observational research: introducing the E-value. 2017;167(4):268-274.

121. Nitsche JF, Patil AS, Langman LJ, et al. Transplacental passage of acetaminophen in term pregnancy. *Am J Perinatol*. 2017;34(6):541-543.
122. O'Brien WF, Krammer J, O'Leary TD, Mastrogiannis DS. The effect of acetaminophen on prostacyclin production in pregnant women. *American journal of obstetrics and gynecology*. 1993;168(4):1164-1169.
123. Gruslin A, Lemyre B. Pre-eclampsia: fetal assessment and neonatal outcomes. *Best practice & research Clinical obstetrics & gynaecology*. 2011;25(4):491-507.
124. Jetten MJ, Gaj S, Ruiz-Aracama A, et al. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. *Toxicology and applied pharmacology*. 2012;259(3):320-328.
125. Bauer AZ, Swan SH, Kriebel D, et al. Paracetamol use during pregnancy—a call for precautionary action. 2021;17(12):757-766.
126. Consumer Healthcare Products Association. <https://www.chpa.org/our-issues/otc-medicines/acetaminophen>. Accessed January 14, 2022.
127. Werler MM, Mitchell AA, Hernandez-Diaz S, Honein MAJAJoo, gynecology. Use of over-the-counter medications during pregnancy. 2005;193(3):771-777.
128. Rebordosa C, Kogevinas M, Bech BH, Sørensen HT, Olsen JJJjoe. Use of acetaminophen during pregnancy and risk of adverse pregnancy outcomes. 2009;38(3):706-714.
129. Bandoli G, Palmsten K, Chambers CJP, epidemiology p. Acetaminophen use in pregnancy: Examining prevalence, timing, and indication of use in a prospective birth cohort. 2020;34(3):237-246.

130. Avella-Garcia CB, Julvez J, Fortuny J, et al. Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. 2016;45(6):1987-1996.
131. Baker BH, Lugo-Candelas C, Wu H, et al. Association of prenatal acetaminophen exposure measured in meconium with risk of attention-deficit/hyperactivity disorder mediated by frontoparietal network brain connectivity. 2020;174(11):1073-1081.
132. Brandlistuen RE, Ystrom E, Nulman I, Koren G, Nordeng HJJioe. Prenatal paracetamol exposure and child neurodevelopment: a sibling-controlled cohort study. 2013;42(6):1702-1713.
133. Chen M-H, Pan T-L, Wang P-W, et al. Prenatal exposure to acetaminophen and the risk of attention-deficit/hyperactivity disorder: a nationwide study in Taiwan. 2019;80(5):15264.
134. Gustavson K, Ystrom E, Ask H, et al. Acetaminophen use during pregnancy and offspring attention deficit hyperactivity disorder—a longitudinal sibling control study. 2021;1(2):e12020.
135. Ji Y, Azuine RE, Zhang Y, et al. Association of cord plasma biomarkers of in utero acetaminophen exposure with risk of attention-deficit/hyperactivity disorder and autism spectrum disorder in childhood. 2020;77(2):180-189.
136. Liew Z, Kioumourtzoglou M-A, Roberts AL, O'Reilly EJ, Ascherio A, Weisskopf MGJAJoe. Use of negative control exposure analysis to evaluate confounding: an example of acetaminophen exposure and attention-deficit/hyperactivity disorder in Nurses' Health Study II. 2019;188(4):768-775.

137. Liew Z, Ritz B, Rebordosa C, Lee P-C, Olsen JJJp. Acetaminophen use during pregnancy, behavioral problems, and hyperkinetic disorders. 2014;168(4):313-320.
138. Stergiakouli E, Thapar A, Smith GDJJp. Association of acetaminophen use during pregnancy with behavioral problems in childhood: evidence against confounding. 2016;170(10):964-970.
139. Streissguth AP, Treder RP, Barr HM, et al. Aspirin and acetaminophen use by pregnant women and subsequent child IQ and attention decrements. 1987;35(2):211-219.
140. Thompson JM, Waldie KE, Wall CR, Murphy R, Mitchell EA, one ASGJP. Associations between acetaminophen use during pregnancy and ADHD symptoms measured at ages 7 and 11 years. 2014;9(9):e108210.
141. Tovo-Rodrigues L, Schneider BC, Martins-Silva T, et al. Is intrauterine exposure to acetaminophen associated with emotional and hyperactivity problems during childhood? Findings from the 2004 Pelotas birth cohort. 2018;18(1):1-11.
142. Ystrom E, Gustavson K, Brandlistuen RE, et al. Prenatal exposure to acetaminophen and risk of ADHD. 2017;140(5).
143. Liew Z, Ritz B, Virk J, Olsen JJAR. Maternal use of acetaminophen during pregnancy and risk of autism spectrum disorders in childhood: AD anish national birth cohort study. 2016;9(9):951-958.
144. Leppert B, Havdahl A, Riglin L, et al. Association of maternal neurodevelopmental risk alleles with early-life exposures. 2019;76(8):834-842.
145. Hay-Schmidt A, Finkielman OTE, Jensen BA, et al. Prenatal exposure to paracetamol/acetaminophen and precursor aniline impairs masculinisation of male brain and behaviour. 2017;154(2):145-152.

146. Klein RM, Rigobello C, Vidigal CB, et al. Gestational exposure to paracetamol in rats induces neurofunctional alterations in the progeny. 2020;77:106838.
147. Philippot G, Gordh T, Fredriksson A, Viberg HJJJoAT. Adult neurobehavioral alterations in male and female mice following developmental exposure to paracetamol (acetaminophen): characterization of a critical period. 2017;37(10):1174-1181.
148. Rigobello C, Klein RM, Debiassi JD, et al. Perinatal exposure to paracetamol: Dose and sex-dependent effects in behaviour and brain's oxidative stress markers in progeny. 2021;408:113294.
149. Suda N, Cendejas Hernandez J, Poulton J, et al. Therapeutic doses of acetaminophen with co-administration of cysteine and mannitol during early development result in long term behavioral changes in laboratory rats. 2021;16(6):e0253543.
150. Viberg H, Eriksson P, Gordh T, Fredriksson AJts. Paracetamol (acetaminophen) administration during neonatal brain development affects cognitive function and alters its analgesic and anxiolytic response in adult male mice. 2014;138(1):139-147.
151. Philippot G, Hallgren S, Gordh T, Fredriksson A, Fredriksson R, Viberg HJTS. A cannabinoid receptor type 1 (CB1R) agonist enhances the developmental neurotoxicity of acetaminophen (paracetamol). 2018;166(1):203-212.
152. Blecharz-Klin K, Piechal A, Jawna-Zboińska K, et al. Paracetamol– Effect of early exposure on neurotransmission, spatial memory and motor performance in rats. 2017;323:162-171.
153. Blecharz-Klin K, Wawer A, Jawna-Zboińska K, et al. Early paracetamol exposure decreases brain-derived neurotrophic factor (BDNF) in striatum and affects social behaviour and exploration in rats. 2018;168:25-32.

154. Saad A, Hegde S, Kechichian T, et al. Is there a causal relation between maternal acetaminophen administration and ADHD? 2016;11(6):e0157380.
155. Gould GG, Seillier A, Weiss G, et al. Acetaminophen differentially enhances social behavior and cortical cannabinoid levels in inbred mice. 2012;38(2):260-269.
156. Dean SL, Knutson JF, Krebs-Kraft DL, McCarthy MMJEJoN. Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. 2012;35(8):1218-1229.
157. Blecharz-Klin K, Joniec-Maciejak I, Jawna K, et al. Developmental exposure to paracetamol causes biochemical alterations in medulla oblongata. 2015;40(2):369-374.
158. Blecharz-Klin K, Joniec-Maciejak I, Jawna K, et al. Effect of prenatal and early life paracetamol exposure on the level of neurotransmitters in rats—Focus on the spinal cord. 2015;47:133-139.
159. Blecharz-Klin K, Joniec-Maciejak I, Jawna-Zbońska K, et al. Cerebellar level of neurotransmitters in rats exposed to paracetamol during development. 2016;68(6):1159-1164.
160. Blecharz-Klin K, Wawer A, Pyrzanowska J, Piechal A, Jawna-Zbońska K, Widy-Tyszkiewicz EJIJoDN. Hypothalamus—Response to early paracetamol exposure in male rats offspring. 2019;76:1-5.
161. Yang X, Greenhaw J, Shi Q, et al. Mouse liver protein sulfhydryl depletion after acetaminophen exposure. *Journal of Pharmacology and Experimental Therapeutics*. 2013;344(1):286-294.

162. Reel JR, Lawton AD, LAMB IV JC. Reproductive toxicity evaluation of acetaminophen in Swiss CD-1 mice using a continuous breeding protocol. *Toxicological Sciences*. 1992;18(2):233-239.
163. Larrey D, Letteron P, Foliot A, et al. Effects of pregnancy on the toxicity and metabolism of acetaminophen in mice. *Journal of Pharmacology and Experimental Therapeutics*. 1986;237(1):283-291.
164. Whitehouse L, Paul C, Wong L, Thomas B. Effect of aspirin on a subtoxic dose of ¹⁴C-acetaminophen in mice. *Journal of pharmaceutical sciences*. 1977;66(10):1399-1403.
165. Boyd E-M, Berezky G. Liver necrosis from paracetamol. *British journal of pharmacology and chemotherapy*. 1966;26(3):606-614.
166. Chen S, Zhou Y, Chen Y, Gu JJB. fastp: an ultra-fast all-in-one FASTQ preprocessor. 2018;34(17):i884-i890.
167. Li HJapa. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. 2013.
168. Liao Y, Smyth GK, Shi WJB. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. 2014;30(7):923-930.
169. Love MI, Huber W, Anders SJGb. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. 2014;15(12):1-21.
170. Alhamdoosh M, Ng M, Wilson NJ, et al. Combining multiple tools outperforms individual methods in gene set enrichment analyses. 2017;33(3):414-424.
171. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. 2005;102(43):15545-15550.

172. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The molecular signatures database hallmark gene set collection. 2015;1(6):417-425.
173. Kanehisa M, Furumichi M, Sato Y, Ishiguro-Watanabe M, Tanabe M. KEGG: integrating viruses and cellular organisms. 2021;49(D1):D545-D551.
174. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. 2003;463(1-3):3-33.
175. Branchi I, Santucci D, Alleva E. Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. 2001;125(1-2):49-56.
176. Hofer M. Multiple regulators of ultrasonic vocalization in the infant rat. 1996;21(2):203-217.
177. Sánchez C. Stress-induced vocalisation in adult animals. A valid model of anxiety? 2003;463(1-3):133-143.
178. D'Amato FR, Scalera E, Sarli C, Moles A. Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? 2005;35(1):103-112.
179. Regan SL, Williams MT, Vorhees CV. Review of rodent models of attention deficit hyperactivity disorder. 2022;132:621-637.
180. Okamoto K, Aoki K. Development of a strain of spontaneously hypertensive rats. 1963;27(3):282-293.
181. Ferguson SA, Cada AM. A longitudinal study of short- and long-term activity levels in male and female spontaneously hypertensive, Wistar-Kyoto, and Sprague-Dawley rats. 2003;117(2):271.

182. Bertoldi AD, Rifas-Shiman SL, Boing AC, et al. Associations of acetaminophen use during pregnancy and the first year of life with neurodevelopment in early childhood. 2020;34(3):267-277.
183. Laue HE, Cassoulet R, Abdelouahab N, et al. Association between meconium acetaminophen and childhood neurocognitive development in GESTE, a Canadian cohort study. 2019;167(1):138-144.
184. Tovo-Rodrigues L, Carpena MX, Martins-Silva T, et al. Low neurodevelopmental performance and behavioural/emotional problems at 24 and 48 months in Brazilian children exposed to acetaminophen during foetal development. 2020;34(3):278-286.
185. Rifas-Shiman SL, Cardenas A, Hivert MF, et al. Associations of prenatal or infant exposure to acetaminophen or ibuprofen with mid-childhood executive function and behaviour. 2020;34(3):287-298.
186. Liew Z, Ritz B, Virk J, Arah OA, Olsen JJE. Prenatal use of acetaminophen and child IQ: a Danish cohort study. 2016;27(6):912-918.
187. Kristensen DM, Hass U, Lesné L, et al. Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. 2011;26(1):235-244.
188. van Den Driesche S, Macdonald J, Anderson RA, et al. Prolonged exposure to acetaminophen reduces testosterone production by the human fetal testis in a xenograft model. 2015;7(288):288ra280-288ra280.
189. Holm JB, Mazaud-Guittot S, Danneskiold-Samsøe NB, et al. Intrauterine exposure to paracetamol and aniline impairs female reproductive development by reducing follicle reserves and fertility. 2016;150(1):178-189.

190. Axelstad M, Christiansen S, Boberg J, et al. Mixtures of endocrine-disrupting contaminants induce adverse developmental effects in preweaning rats. 2014;147(4):489-501.
191. Mandrup KR, Johansson HKL, Boberg J, et al. Mixtures of environmentally relevant endocrine disrupting chemicals affect mammary gland development in female and male rats. 2015;54:47-57.
192. Pereira MRF, Aleixo JF, de Freitas Cavalcanti L, et al. Can maternal exposure to paracetamol impair reproductive parameters of male rat offspring? 2020;93:68-74.
193. Dean A, Van Den Driesche S, Wang Y, et al. Analgesic exposure in pregnant rats affects fetal germ cell development with inter-generational reproductive consequences. 2016;6(1):1-12.
194. Johansson HKL, Jacobsen PR, Hass U, et al. Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging. 2016;61:186-194.
195. Axelstad M, Hass U, Scholze M, Christiansen S, Kortenkamp A, Boberg J. EDC IMPACT: Reduced sperm counts in rats exposed to human relevant mixtures of endocrine disrupters. 2018;7(1):139-148.
196. Rossitto M, Ollivier M, Déjardin S, et al. In utero exposure to acetaminophen and ibuprofen leads to intergenerational accelerated reproductive aging in female mice. 2019;2(1):1-13.
197. Rossitto M, Marchive C, Pruvost A, et al. Intergenerational effects on mouse sperm quality after in utero exposure to acetaminophen and ibuprofen. 2019;33(1):339-357.

198. Albert O, Desdoits-Lethimonier C, Lesné L, et al. Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. 2013;28(7):1890-1898.
199. Thompson W, Russell G, Baragwanath G, Matthews J, Vaidya B, Thompson-Coon J. Maternal thyroid hormone insufficiency during pregnancy and risk of neurodevelopmental disorders in offspring: A systematic review and meta-analysis. 2018;88(4):575-584.
200. Ghassabian A, Bongers-Schokking JJ, De Rijke YB, et al. Maternal thyroid autoimmunity during pregnancy and the risk of attention deficit/hyperactivity problems in children: the Generation R Study. 2012;22(2):178-186.
201. Pääkkilä F, Männistö T, Pouta A, et al. The impact of gestational thyroid hormone concentrations on ADHD symptoms of the child. 2014;99(1):E1-E8.
202. Modesto T, Tiemeier H, Peeters RP, et al. Maternal mild thyroid hormone insufficiency in early pregnancy and attention-deficit/hyperactivity disorder symptoms in children. 2015;169(9):838-845.
203. Ghassabian A, Bongers-Schokking JJ, Henrichs J, et al. Maternal thyroid function during pregnancy and behavioral problems in the offspring: the generation R study. 2011;69(7):454-459.
204. Baker BH, Wu H, Laue HE, et al. Methylparaben in meconium and risk of maternal thyroid dysfunction, adverse birth outcomes, and Attention-Deficit Hyperactivity Disorder (ADHD). 2020;139:105716.
205. Tiegs G, Karimi K, Brune K, Arck PJERoCP. New problems arising from old drugs: second-generation effects of acetaminophen. 2014;7(5):655-662.

206. Pronovost GN, Hsiao EYJI. Perinatal interactions between the microbiome, immunity, and neurodevelopment. 2019;50(1):18-36.
207. Walf AA, Frye CAJNp. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. 2007;2(2):322-328.
208. Pellow S, Chopin P, File SE, Briley MJJonm. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. 1985;14(3):149-167.
209. Liew Z, Kioumourtzoglou M-A, Roberts AL, O'Reilly ÉJ, Ascherio A, Weisskopf MG. Use of Negative Control Exposure Analysis to Evaluate Confounding: An Example of Acetaminophen Exposure and Attention-Deficit/Hyperactivity Disorder in Nurses' Health Study II. *American journal of epidemiology*. 2019;188(4):768-775.
210. FDA has reviewed possible risks of pain medicine use during pregnancy. <https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-fda-has-reviewed-possible-risks-pain-medicine-use-during-pregnancy>. Accessed June 18, 2022.
211. The American College of Obstetricians and Gynecologists (ACOG). ACOG Response to Consensus Statement on Paracetamol Use During Pregnancy. 2021.
212. Hutson JR, Smith GN, Codsí E, Garcia-Bournissen F. Statement on the use of acetaminophen for analgesia and fever in pregnancy. 2021.
213. Nordeng H, Ystrøm E, Einarson AJEjocp. Perception of risk regarding the use of medications and other exposures during pregnancy. 2010;66(2):207-214.

214. Van Calsteren K, Gersak K, Sundseth H, et al. Position statement from the European Board and College of Obstetrics & Gynaecology (EBCOG): The use of medicines during pregnancy—call for action. 2016;201:189-191.
215. Croen LA, Qian Y, Ashwood P, et al. Infection and fever in pregnancy and autism spectrum disorders: findings from the study to explore early development. 2019;12(10):1551-1561.
216. Zerbo O, Iosif A-M, Walker C, et al. Is maternal influenza or fever during pregnancy associated with autism or developmental delays? Results from the CHARGE (Childhood Autism Risks from Genetics and Environment) study. 2013;43(1):25-33.
217. Hornig M, Bresnahan M, Che X, et al. Prenatal fever and autism risk. 2018;23(3):759-766.
218. Brucato M, Ladd-Acosta C, Li M, et al. Prenatal exposure to fever is associated with autism spectrum disorder in the boston birth cohort. 2017;10(11):1878-1890.
219. Bilenberg N, Hougaard D, Norgaard-Pedersen B, Nordenbæk CM, Olsen J. Twin study on transplacental-acquired antibodies and attention deficit/hyperactivity disorder—A pilot study. 2011;236(1-2):72-75.
220. Mann JR, McDermott S. Are maternal genitourinary infection and pre-eclampsia associated with ADHD in school-aged children? 2011;15(8):667-673.
221. Antoun S, Ellul P, Peyre H, et al. Fever during pregnancy as a risk factor for neurodevelopmental disorders: results from a systematic review and meta-analysis. 2021;12(1):1-13.
222. Skajaa N, Szépligeti SK, Xue F, et al. Pregnancy, birth, neonatal, and postnatal neurological outcomes after pregnancy with migraine. 2019;59(6):869-879.

223. Cameron C, Kelly S, Hsieh SC, et al. Triptans in the acute treatment of migraine: A systematic review and network meta-analysis. 2015;55:221-235.
224. Harris GM, Wood M, Ystrom E, Nordeng HJJNO. Association of Maternal Use of Triptans During Pregnancy With Risk of Attention-Deficit/Hyperactivity Disorder in Offspring. 2022;5(6):e2215333-e2215333.
225. Shaheen SO, Newson RB, Ring SM, et al. Prenatal and infant acetaminophen exposure, antioxidant gene polymorphisms, and childhood asthma. 2010;126(6):1141-1148. e1147.
226. Liew Z, Yuan Y, Meng Q, et al. Prenatal Exposure to Acetaminophen and Childhood Asthmatic Symptoms in a Population-Based Cohort in Los Angeles, California. 2021;18(19):10107.
227. Perzanowski MS, Miller RL, Tang D, et al. Prenatal acetaminophen exposure and risk of wheeze at age 5 years in an urban low-income cohort. 2010;65(2):118-123.
228. Allegaert K, Mian P, Lapillonne A, van den Anker JNJBjocp. Maternal paracetamol intake and fetal ductus arteriosus constriction or closure: a case series analysis. 2019;85(1):245-251.
229. Dignam T, Kaufmann RB, LeStourgeon L, Brown MJJJophm, JPHMP p. Control of lead sources in the United States, 1970-2017: public health progress and current challenges to eliminating lead exposure. 2019;25(Suppl 1 LEAD POISONING PREVENTION):S13.
230. Cendejas-Hernandez J, Sarafian JT, Lawton VG, et al. Paracetamol (acetaminophen) use in infants and children was never shown to be safe for neurodevelopment: a systematic review with citation tracking. 2022:1-23.