ABSTRACT

SORROW, PATRICIA ANN. Epidemiology as a Hypothesis Generation Tool for Toxicology: A Case for Acetaminophen and Adverse Health Outcomes in Children (Under the direction of Dr. Cathrine Hoyo).

Recently, obesity and its comorbidities have reached epidemic proportions for children in the United States. Some children seem to be more susceptible to developing obesity and for these children lifestyle interventions are often ineffective. No more than 5% increased risk for developing obesity can be attributed to genetic predisposition, and developmental exposures are known to contribute to the incidence of metabolic disease later in life. Current blood tests for metabolic disease are not useful in children, and thus novel biomarkers are needed to detect, at birth, those with the greatest risk of developing obesity while prevention is still possible.

An untargeted metabolomics assay was used in a subset of 50 cord blood samples from the Newborn Epigenetics Cohort to compare the circulating small molecule profile in children who became obese (n=25) (weight for height z-score (WHz) >95%) compared with children who were not obese (n=25) (WHz <85%) by age 3-5 years. The assay identified 384 different metabolites using a combination of GC and LC-MS, and 70 metabolites were found at significantly different levels. The most significant of these included species of medium and long chain fatty acids and metabolites of the analgesic/antipyretic medication acetaminophen. These associations remained after the role of maternal pre-pregnancy obesity and ethnicity were adjusted for in regression models.

Prenatal acetaminophen exposure is related adverse child outcomes including asthma, attention deficit hyperactive disorder (ADHD), decreased anogenital distance and now obesity. This suggests some level of metabolic disruption maybe linked to acetaminophen use in pregnancy.

To strengthen these findings, targeted HPLC-MS assays were developed to determine the concentration of acetaminophen, acetaminophen sulfate, acetaminophen glucuronide, and acetaminophen glutathione in a larger population. Significant associations were found between detectable cord blood levels of acetaminophen, acetaminophen sulfate, and acetaminophen glucuronide with childhood obesity (WHz) in boys by 3 years of age after adjustment for maternal pre-pregnancy obesity. No associations were found in girls, and these associations did not persist in boys at ages 4 and 5 years, although sample size remains small. No associations were found between detectable cord blood concentrations of acetaminophen and its metabolites with systolic and diastolic blood pressure.

Because of the longitudinal cohort design, temporal sequence is retained between exposure and disease incidence granting considerable strength to the associations presented here, but even after increasing sample size from 50 to 120 individuals, the number of individuals with detectable exposure to acetaminophen remains small (21%) limiting the power of this finding. Although it is impossible to determine causality, due to the extreme heterogeneity within the human population, this work shows that epidemiology is an excellent tool for hypothesis generation for toxicologists.

Epidemiology as a Hypothesis Generation Tool for Toxicology: A Case for Acetaminophen and Adverse Health Outcomes in Children

by
Patricia Ann Sorrow

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Toxicology

Raleigh, North Carolina
2019

APPROVED BY:

_____	_____
Cathrine Hoyo Committee Chair	Scott Belcher
_____	_____
Michel Cowley	Cynthia Moylan

DEDICATION

I dedicate this work to my husband David. You are truly a jack of all trades: artist, attentive pseudo-student, game-show host, cook, and most recently father of “the cutest”.

BIOGRAPHY

Along with her identical twin sister, Patricia Ann Lawrence (Sorrow) was born to proud parents David and Laura. The ultrasound months before had determined, later assumed to be the unfortunate position of a tiny baby foot, the presence of fraternal boy-girl twins. However, after the first baby girl, Katherine, was born they were surprised to discover that the second baby was also a girl. Although he never said it, David was probably disappointed, but his disappointment would be short lived as the second girl, given the impromptu name "Patricia Ann" after her mother "Laura Patricia" and her maternal grandmother "Myrtis Ann," grew to be a nature loving, soccer playing, tomboy that couldn't seem to get dirty enough.

Her father took her hunting for the first time when she was ten years old. At the time, she found it mind numbingly boring, however over the years she grew to cherish every moment spent in the beauty of nature. While sitting in a tree or beside a rippling creek, she found herself pondering nature's many intricacies. Beginning with a Bachelor of Science in Environmental Science she started unearthing some of the inner workings of biology. As a senior in college, she attended the SC Water Resources Conference, and was intrigued with a talk discussing the presence of synthetic hormones and pharmaceuticals in the park's most isolated regions. This moment compelled her to learn more about topics such as these, and ultimately decide to pursue a career in toxicology.

While at NCSU, Patricia joined a molecular epidemiology lab, in the Program of Molecular and Cellular Toxicology, studying the etiology of childhood obesity. There, she increased her knowledge of human metabolic disease, dug deeper into the fascinating world of molecular biology, and even fell in love with computer programming. Since joining the program,

Patricia and her husband welcomed the birth of their first child and has since decided to redirect her career path and return home to be closer to family.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The number of children suffering from type 2 diabetes and non-alcoholic fatty liver disease have reached unprecedented levels in recent years. Nearly 20% of American children are plagued with obesity, and these numbers are expected to rise significantly over the next decade (1–3). Because lifestyle interventions fail for approximately 40% of the obese population, there must be an underlying mechanism other than inequality in caloric energy balance (4). Furthermore, genome wide association studies (GWAS) have found that genetic markers obesity only account for approximately a 5% increase in risk of developing the condition (5).

Using Metabolomics to Inform Diagnosis of Childhood Obesity

Physicians use blood chemistry markers including circulating triglycerides, HDL/LDL, blood glucose and A1C to assess metabolic health, but these measures are not useful for diagnosis of metabolic disease in children. Non-targeted metabolomic profiling is a mass spectrometry technique that enables the simultaneous detection of hundreds of small molecules in biological matrices, and as such may be useful to uncover novel biomarkers for early detection of children at risk of becoming obese later in life (6–15).

Many groups have profiled the circulating small molecule profile or "metabolome" of obesity in adults and children. The majority of these studies published differences in acylcarnitines, branched-chain amino acids (BCAAs) non-esterified fatty acids (NEFAs), mono and poly-unsaturated fatty acids (MUFAs and PUFAs), phospholipids (PL), phosphatidylcholines (PC), and short, medium, and long-chain free fatty acids (FFAs) (6–11,15–17). Since these studies compare the metabolic profile of individuals with and without disease it is impossible to know whether metabolites found at significantly different levels are informative of the causal pathway or if they are simply biomarkers of the disease itself.

Few longitudinal cohort studies have investigated the cord blood metabolome in search of novel biomarkers of those with increased risk of becoming obese (13–15,18). Though the endpoints of these four studies vary, low birth weight, maternal gestational diabetes, and elevated postanal growth trajectory and obesity (weight for height z-score >95th percentile) by age 3-5 years are all risk factors for obesity that is persistent into adulthood (15). Each of these longitudinal cohort studies observed elevations in circulating lipids signatures (13–15,18). Unexpectedly though, the non-targeted metabolomics experiment revealed metabolites acetaminophen at significantly greater levels in children who became obese by age 3-5 years (15).

Acetaminophen Pharmacology and Use during Pregnancy

Acetaminophen is widely used for the treatment of non-specific pain and fever in the general population. It is used in 80 countries worldwide and is an active ingredient in more than 300 mixed formulation medications (19). The therapeutic range of acetaminophen is narrow with therapeutic effects beginning at 1-2grams/day and hepatotoxicity becoming apparent with as little as 4 grams/day for an average adult. More than 88% of the drug is absorbed into the bloodstream within 90-minutes, and at therapeutic doses it has a half-life of 1.5-2.5 hours (19). Acetaminophen elicits its pain-relieving effects by inhibition of eicosanoid, endocannabinoid, serotonergic, and nitric oxide pathways and many of these have not yet been fully described (20,21). The most well described pathway for acetaminophen analgesia is via inhibition of prostaglandin (PG) synthesis. Acetaminophen is known to readily cross the blood-brain barrier, and interfere with both prostaglandin-endoperoxide synthase enzymes, more commonly known as cyclooxygenase-1 and 2 (COX-1 and COX-2); this occurs when acetaminophen interacts with the peroxidase binding sites of these enzymes (20,21). Since PGs are proinflammatory cytokines,

inhibition of this pathway results in an anti-inflammatory action. This analgesic activity of acetaminophen is better suited for acute instances of inflammation in comparison to other non-steroidal anti-inflammatories (NSAIDs) because inhibition of PG synthesis by acetaminophen only occurs when the PG precursor arachidonic acid is low (22).

Metabolism of acetaminophen occurs through two major pathways: sulfation, and glucuronidation. A smaller portion is metabolized via CYP450 monooxygenation resulting in the formation of a highly reactive metabolite NAPQI (23). In the majority of the population, the amount of NAPQI generated by administration of a therapeutic dose of acetaminophen is sufficiently conjugated into acetaminophen glutathione by glutathione-s-transferase (GST) (19). In situations of overdose, glutathione stores become completely sequestered and can no longer neutralize NAPQI. Unconjugated, NAPQI is a potent hepatotoxin and binds various cellular proteins resulting in oxidative stress, mitochondrial dysfunction, and ultimately necrosis (24).

Sex, metabolic condition and alcohol consumption are known to alter the rate and ratio of hepatic metabolism (19). Sex differences in drug metabolism involve varied rates of both phase I and II metabolism which appear to be modulated by sex hormones (19,25). Moreover, the rise in progesterone during pregnancy alters hepatic metabolism, and pregnant mice have been shown to experience slower acetaminophen metabolism and increased instances of hepatotoxicity below the typical toxic doses (26). Alcohol consumption is known to upregulate CYP2E1 resulting in preferential metabolism of acetaminophen into NAPQI and increasing the risk of hepatoxicity at sub-toxic doses (19). Additionally, the abundance of CYP2E1 increases with the progression of non-alcoholic fatty liver disease (NAFLD) as well as in morbidly obese patients, in conjunction with decreases in sulfotransferase enzymes may also lead to increased likelihood for hepatoxicity from a subtoxic doses (27).

Acetaminophen is generally considered safe for use during pregnancy by the majority of the medical community, and this assertion of safety stems from early work which described the drug as a "safer" alternative to ibuprofen and aspirin which have been shown to be related to placental hemorrhaging and gastrointestinal distress (28). Obstetricians often recommend acetaminophen to combat non-specific pain or fever, and studies report 47-65% of mothers using acetaminophen at least once over the course of their pregnancy (29,30).

During pregnancy, changes in hormone regulation are known to alter hepatic metabolism, and it is important to note that pregnant mice were found to be more susceptible to hepatic acetaminophen toxicity than non-pregnant controls (31). Additionally, acetaminophen readily crosses the placenta by passive diffusion, and thus it can be assumed that the level of acetaminophen exposure for the mother and the fetus are similar (32). Metabolites containing glutathione, sulfate, and glucuronide conjugates are transferred from the mother via ABCC1 transporters at the basolateral membrane of the maternal compartment and transferred into the fetal capillaries through ABCC3 transporters (33,34). It is also widely accepted that acetaminophen metabolites can return from fetal to maternal circulation (35).

For most of gestation, the fetus is incapable of glucuronide conjugation because of its lack of the enzyme UGT1A6, so acetaminophen metabolism in the fetus is dominated by sulfation (36). The fetus is also capable of CYP450 formation of NAPQI by the 14th week, and, since the fetus lacks glucuronidation activity, it is thought that the increase in the ratio of CYP450 metabolism would result in higher levels of NAPQI fetus than in the adult (37).

Acetaminophen Toxicology

Since its first compounding in 1955, there has been a wealth of toxicology research regarding acetaminophen overdose including overdose during pregnancy (19). Studies have

found no evidence of miscarriage, pre-term birth, low birth weight, or gross fetal malformations attributable to exposure in pregnant women hospitalized and treated for acetaminophen overdose (38,39). Similar results derived using animal models further confirm little risk to the fetus from acetaminophen overdose (19).

Chronic therapeutic exposures are also known to elicit nomonotonic toxicity. In these instances, chronic "non-toxic" exposure can also result in toxic phenotypes, described by a "U" shaped dose response curve, and thus it is important to investigate these effects when probable cause exists.

Associations between Chronic Gestational Use and Adverse Child Outcomes.

In utero exposure to acetaminophen has been associated with immunological, neurodevelopmental, and endocrine disorders in children between 6 months and 10 years of age (38–52) Immunological assessment of clinically diagnosed asthma, wheeze, rhinitis, and blood IgE found a higher incidence in children prenatally exposed to acetaminophen (40,41,47–50,53). Animal studies in mice found prenatal exposure to acetaminophen resulted in decreased fetal liver stem cells, which is thought to contribute to altered immune function/allergic response later in life (31). Neurodevelopmental phenotypes include clinically diagnosed ADHD, increased hyperactivity, decreased IQ, and motor milestone delay (42–46,52,54). Mouse and zebrafish studies, however, have found no instance of hyperactivity or neurodevelopmental delay after perinatal acetaminophen exposure (55,56). Gervin et al in 2017 found significant changes in CpG methylation associated with acetaminophen exposure and the outcome of ADHD. Methylome changes were specific to individuals who had both the exposure and the outcome but were not found in acetaminophen exposed children who did not develop ADHD nor in children with ADHD who were not gestationally exposed to acetaminophen supporting the existence of a

correlation (57). Finally, the Danish cohort found evidence of endocrine disruption in the form of reduced ano-genital distance in children whose mothers reported taking acetaminophen during pregnancy (58). Similar results have been found in animal studies postulating an insult to maternal immune adaptation, placental morphology, and fetal hormone regulation (59).

To date, no mouse studies have been conducted linking in utero exposure to acetaminophen and obesity but results from the animal work, previously described, support the hypothesis that prenatal acetaminophen may result in maternal/fetal liver insult and subsequent alteration to metabolic programming. The causes of obesity have yet to be fully described, and although liver disease and obesity are often found concurrent, it is not known whether liver dysfunction leads to obesity or if instead, the converse is true. It is plausible, though, that in-utero exposure to acetaminophen could result in altered liver function at birth and that dysfunction in the primary organ of lipid metabolism may lead to obesity later in childhood.

REFERENCES

1. 1. Ogden CL, Carroll MD, Lawman HG, Fryar CD, Kruszon-Moran D, Kit BK, et al. Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988-1994 Through 2013-2014. JAMA [Internet]. 2016 Jun 7 [cited 2017 Jul 20];315(21):2292. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2016.6361
2. Temple JL, Cordero P, Li J, Nguyen V, Oben JA. A Guide to Non-Alcoholic Fatty Liver Disease in Childhood and Adolescence. Int J Mol Sci [Internet]. 2016 Jun 15 [cited 2018 Sep 28];17(6). Available from: http://www.ncbi.nlm.nih.gov/pubmed/27314342
3. Arslanian S. Type 2 Diabetes in Children: Clinical Aspects and Risk Factors. Horm Res Paediatr [Internet]. 2002 [cited 2018 Sep 28];57(1):19–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11979018
4. Burguera B, Jesús Tur J, Escudero AJ, Alos M, Pagán A, Cortés B, et al. An Intensive Lifestyle Intervention Is an Effective Treatment of Morbid Obesity: The TRAMOMTANA Study-A Two-Year Randomized Controlled Clinical Trial. Int J Endocrinol [Internet]. 2015 [cited 2018 Sep 28];2015:194696. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26257780
5. Bouchard C. Childhood obesity: are genetic differences involved? Am J Clin Nutr [Internet]. 2009 May [cited 2018 Mar 20];89(5):1494S–1501S. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19261728
6. Rauschert S, Uhl O, Koletzko B, Kirchberg F, Mori TA, Huang R-C, et al. Lipidomics Reveals Associations of Phospholipids With Obesity and Insulin Resistance in Young Adults. J Clin Endocrinol Metab [Internet]. 2016 Mar 1 [cited 2018 Apr 11];101(3):871–9. Available from: https://academic.oup.com/jcem/article-lookup/doi/10.1210/jc.2015-3525

7. Puri P, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min H-K, et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. Hepatology [Internet]. 2009 Dec [cited 2017 Apr 17];50(6):1827–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19937697

8. Fiehn O, Timothy Garvey W, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS One. 2010;

9. Yi L-Z, He J, Liang Y-Z, Yuan D-L, Chau F-T. Plasma fatty acid metabolic profiling and biomarkers of type 2 diabetes mellitus based on GC/MS and PLS-LDA [Internet]. Vol. 580, FEBS Letters. 2006 [cited 2017 Apr 17]. Available from: http://www.sciencedirect.com/science/article/pii/S001457930601372X

10. Liu L, Wang M, Yang X, Bi M, Na L, Niu Y, et al. Fasting Serum Lipid and Dehydroepiandrosterone Sulfate as Important Metabolites for Detecting Isolated Postchallenge Diabetes: Serum Metabolomics via Ultra-High-Performance LC-MS. Clin Chem [Internet]. 2013 [cited 2017 Apr 17];59(9). Available from: http://clinchem.aaccjnls.org/content/59/9/1338.long

11. Tulipani S, Palau-Rodriguez M, Miñarro Alonso A, Cardona F, Marco-Ramell A, Zonja B, et al. Biomarkers of Morbid Obesity and Prediabetes by Metabolomic Profiling of Human Discordant Phenotypes. Clin Chim Acta [Internet]. 2016 [cited 2017 Apr 13];463:53–61. Available from: http://www.sciencedirect.com/science/article/pii/S0009898116304028

12. Costa SMR, Isganaitis E, Matthews TJ, Hughes K, Daher G, Dreyfuss JM, et al. Maternal obesity programs mitochondrial and lipid metabolism gene expression in infant umbilical

vein endothelial cells. Int J Obes [Internet]. 2016 Nov 17 [cited 2017 Aug 1];40(11):1627–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27531045

13. Ivorra C, García-Vicent C, Chaves FJ, Monleón D, Morales JM, Lurbe E. Metabolomic profiling in blood from umbilical cords of low birth weight newborns. J Transl Med [Internet]. 2012 Jul 9 [cited 2017 Sep 21];10(1):142. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22776444
14. Yee JK, Mao CS, Ross MG, Lee WNP, Desai M, Toda A, et al. High oleic/stearic fatty-acid desaturation index in cord plasma from infants of mothers with gestational diabetes. J Perinatol [Internet]. 2014 May [cited 2018 Mar 8];34(5):357–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24577432
15. Sorrow P, Maguire R, Murphy SK, Belcher SM, Hoyo C. Elevated metabolites of acetaminophen in cord blood of children with obesity. Pediatr Obes [Internet]. 2018 Sep 25 [cited 2018 Sep 28]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/30253079
16. Rauschert S, Uhl O, Koletzko B, Hellmuth C. Metabolomic Biomarkers for Obesity in Humans: A Short Review. Ann Nutr Metab [Internet]. 2014 [cited 2017 Sep 28];64(3–4):314–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25300275
17. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic Signature Shift in Type 2 Diabetes Mellitus Revealed by Mass Spectrometry-based Metabolomics. J Clin Endocrinol Metab [Internet]. 2013 Jun [cited 2017 Apr 17];98(6):E1060–5. Available from: https://academic.oup.com/jcem/article-lookup/doi/10.1210/jc.2012-4132
18. Isganaitis E, Rifas-Shiman SL, Oken E, Dreyfuss JM, Gall W, Gillman MW, et al. Associations of cord blood metabolites with early childhood obesity risk. Int J Obes

[Internet]. 2015 Jul 25 [cited 2017 May 10];39(7):1041–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25804930

19. Mazaleuskaya LL, Sangkuhl K, Thorn CF, FitzGerald GA, Altman RB, Klein TE. PharmGKB summary: pathways of acetaminophen metabolism at the therapeutic versus toxic doses. Pharmacogenet Genomics [Internet]. 2015 Aug [cited 2017 Mar 28];25(8):416–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26049587
20. Toussaint K, Yang XC, Zielinski MA, Reigle KL, Sacavage SD, Nagar S, et al. What do we (not) know about how paracetamol (acetaminophen) works? J Clin Pharm Ther [Internet]. 2010 Dec [cited 2018 Sep 28];35(6):617–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21054454
21. Smith HS. Potential analgesic mechanisms of acetaminophen. Pain Physician [Internet]. [cited 2018 Sep 28];12(1):269–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19165309
22. Graham GG, Davies MJ, Day RO, Mohamudally A, Scott KF. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. Inflammopharmacology [Internet]. 2013 Jun 30 [cited 2017 Mar 27];21(3):201–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23719833
23. Tabrizchi R. Essential Pain Pharmacology (The Prescriber's Guide). Can J Anesth Can d'anesthésie [Internet]. 2013 Aug 18 [cited 2017 Mar 18];60(8):828–9. Available from: http://link.springer.com/10.1007/s12630-013-9939-1
24. Waring R. Molecules of Death. 2nd ed. London: Imperial College Press; 2007. 253-263 p.

25. Waxman DJ, Holloway MG. Sex differences in the expression of hepatic drug metabolizing enzymes. Mol Pharmacol [Internet]. 2009 Aug [cited 2017 Mar 29];76(2):215–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19483103

26. Wen X, Donepudi AC, Thomas PE, Slitt AL, King RS, Aleksunes LM. Regulation of hepatic phase II metabolism in pregnant mice. J Pharmacol Exp Ther [Internet]. 2013 Jan [cited 2017 Mar 29];344(1):244–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23055538

27. Merrell MD, Cherrington NJ. Drug metabolism alterations in nonalcoholic fatty liver disease. Drug Metab Rev [Internet]. 2011 Aug [cited 2018 Dec 26];43(3):317–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21612324

28. Schoenfeld A, Bar Y, Merlob P, Ovadia Y. NSAIDs: maternal and fetal considerations. Am J Reprod Immunol [Internet]. [cited 2017 Dec 4];28(3–4):141–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1285865

29. Glover DD, Amonkar M, Rybeck BF, Tracy TS. Prescription, over-the-counter, and herbal medicine use in a rural, obstetric population. Am J Obstet Gynecol [Internet]. 2003 Apr [cited 2018 Sep 28];188(4):1039–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12712107

30. Ystrom E, Gustavson K, Brandlistuen RE, Knudsen GP, Magnus P, Susser E, et al. Prenatal Exposure to Acetaminophen and Risk of ADHD. Pediatrics [Internet]. 2017 Nov 30 [cited 2018 Sep 28];140(5):e20163840. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29084830

31. Karimi K, Keßler T, Thiele K, Ramisch K, Erhardt A, Huebener P, et al. Prenatal acetaminophen induces liver toxicity in dams, reduces fetal liver stem cells, and increases

airway inflammation in adult offspring. J Hepatol [Internet]. 2015 May [cited 2017 Mar 29];62(5):1085–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25529619

32. Griffiths SK, Campbell JP. Placental structure, function and drug transfer. Contin Educ Anaesthesia, Crit Care Pain [Internet]. 2014 May 30 [cited 2017 Mar 18];103(2):mku013. Available from: https://academic.oup.com/bjaed/article-lookup/doi/10.1093/bjaceaccp/mku013

33. Ni Z, Mao Q. ATP-binding cassette efflux transporters in human placenta. Curr Pharm Biotechnol [Internet]. 2011 Apr [cited 2017 Mar 29];12(4):674–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21118087

34. Bloise E, Ortiga-Carvalho TM, Reis FM, Lye SJ, Gibb W, Matthews SG. ATP-binding cassette transporters in reproduction: a new frontier. [cited 2017 Mar 29]; Available from: https://oup.silverchair-cdn.com/oup/backfile/Content_public/Journal/humupd/22/2/10.1093_humupd_dmv049/2/dmv049.pdf?Expires=1491153198&Signature=dimySfVQ4RKUR1VuZcw8bk-LD2Svs1T5XuIWeKjpQWN55CXB49hCHMcXCuFXhQU7xIKn1wT7CpprRSvKP1vySPPqjkPbtvPwziixsBLogl-hVWmd0qrEh6sRVNLlufqbFQ7ZNU6bJ8BIcItBJzG8Ni0arbqOvgY80EJ7buUTR2JDTYGGdPPwmYmRS5XxKLUmqd-PPzXNWDIG20de90RIVCOKb~5RSlyq3~01z~L1UzgvvwLiovRQbC0xdndtf4His1VNV5CPiY5ywCuQSFaHJf6KrLYNpzPd0bUY4mDHmrrDAmLIDUvXY8e3sbDehdzXVOGGcDXQsT-3OvWmSsH-MQ__&Key-Pair-Id=APKAIUCZBIA4LVPAVW3Q

35. Conings TP, Qi A, Annaert VC. TRANSPLACENTAL TRANSPORT OF PARACETAMOL AND ITS METABOLITES USING THE EX-VIVO HUMAN

PLACENTA PERFUSION MODEL — KU Leuven congressen. In: 16 European Society for Developmental Perinatal and Paediatric Pharmacology Congress [Internet]. Leuven, Belgium; 2017 [cited 2018 Sep 24]. Available from: https://kuleuvencongres.be/ESDPPP2017/articles/abstracts/Conings-Sigrid

36. Adjei AA, Gaedigk A, Simon SD, Weinshilboum RM, Leeder JS. Interindividual variability in acetaminophen sulfation by human fetal liver: Implications for pharmacogenetic investigations of drug-induced birth defects. Birth Defects Res Part A Clin Mol Teratol [Internet]. 2008 Mar [cited 2017 Mar 28];82(3):155–65. Available from: http://doi.wiley.com/10.1002/bdra.20535
37. D. E. Rollins, C. von Bahr, H. Glaumann PM and AR. Acetaminophen: Potentially Toxic Metabolite Formed by Human Fetal and Adult Liver Microsomes and Isolated Fetal Liver Cells on JSTOR. Science (80-) [Internet]. 1979 [cited 2017 Mar 18];205:1414–6. Available from: http://www.jstor.org.prox.lib.ncsu.edu/stable/1748904?pq-origsite=summon&seq=1#page_scan_tab_contents
38. McElhatton PR, Sullivan FM, Volans GN. Paracetamol overdose in pregnancy analysis of the outcomes of 300 cases referred to the Teratology Information Service. Reprod Toxicol [Internet]. [cited 2018 Sep 24];11(1):85–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9138637
39. Rebordosa C, Kogevinas M, Sorensen HT, Olsen J. Pre-natal exposure to paracetamol and risk of wheezing and asthma in children: A birth cohort study. Int J Epidemiol [Internet]. 2008 Jun 1 [cited 2017 Apr 12];37(3):583–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18400839

40. Hoeke H, Roeder S, Mueller A, Bertsche T, Borte M, Rolle-Kampczyk U, et al. Biomonitoring of prenatal analgesic intake and correlation with infantile anti-aeroallergens IgE. Allergy [Internet]. 2016 Jun [cited 2017 Apr 12];71(6):901–6. Available from: http://doi.wiley.com/10.1111/all.12897

41. Sordillo JE, Scirica C V, Rifas-Shiman SL, Gillman MW, Bunyavanich S, Camargo CA, et al. Prenatal and infant exposure to acetaminophen and ibuprofen and the risk for wheeze and asthma in children. J Allergy Clin Immunol [Internet]. 2015 Feb [cited 2017 Apr 12];135(2):441–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25441647

42. Weatherall M, Ioannides S, Braithwaite I, Beasley R. The association between paracetamol use and asthma: causation or coincidence? Clin Exp Allergy [Internet]. 2015 Jan [cited 2017 Apr 12];45(1):108–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25220564

43. Liew Z, Ritz B, Virk J, Arah OA, Olsen J. Prenatal Use of Acetaminophen and Child IQ. Epidemiology [Internet]. 2016 Nov [cited 2017 Apr 12];27(6):912–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27479646

44. Brandlistuen RE, Ystrom E, Nulman I, Koren G, Nordeng H. Prenatal paracetamol exposure and child neurodevelopment: a sibling-controlled cohort study. Int J Epidemiol [Internet]. 2013 Dec 1 [cited 2017 Apr 12];42(6):1702–13. Available from: https://academic.oup.com/ije/article-lookup/doi/10.1093/ije/dyt183

45. Vlenterie R, Wood ME, Brandlistuen RE, Roeleveld N, van Gelder MMHJ, Nordeng H. Neurodevelopmental problems at 18 months among children exposed to paracetamol in utero : a propensity score matched cohort study. Int J Epidemiol [Internet]. 2016 Aug 31

[cited 2017 Apr 12];dyw192. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27585674

46. Liew Z, Ritz B, Rebordosa C, Lee P-C, Olsen J. Acetaminophen Use During Pregnancy, Behavioral Problems, and Hyperkinetic Disorders. JAMA Pediatr [Internet]. 2014 Apr 1 [cited 2017 Apr 12];168(4):313. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24566677

47. Goksör E, Thengilsdottir H, Alm B, Norvenius G, Wennergren G. Prenatal paracetamol exposure and risk of wheeze at preschool age*. Acta Paediatr [Internet]. 2011 Dec [cited 2017 Apr 12];100(12):1567–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21767300

48. Bakkeheim E, Mowinckel P, Carlsen KH, Håland G, Carlsen KCL. Paracetamol in early infancy: the risk of childhood allergy and asthma. Acta Paediatr [Internet]. 2011 Jan [cited 2017 Apr 12];100(1):90–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21143295

49. Shaheen SO, Newson RB, Ring SM, Rose-Zerilli MJ, Holloway JW, Henderson AJ. Prenatal and infant acetaminophen exposure, antioxidant gene polymorphisms, and childhood asthma. J Allergy Clin Immunol [Internet]. 2010 Dec [cited 2017 Aug 26];126(6):1141–8.e7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21051083

50. Perzanowski MS, Miller RL, Tang D, Ali D, Garfinkel RS, Chew GL, et al. Prenatal acetaminophen exposure and risk of wheeze at age 5 years in an urban low-income cohort. Thorax [Internet]. 2010 Feb 1 [cited 2017 Apr 12];65(2):118–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19850963

51. Mazaud-Guittot S, Nicolaz CN, Desdoits-Lethimonier C, Coiffec I, Maamar M Ben, Balaguer P, et al. Paracetamol, Aspirin, and Indomethacin Induce Endocrine Disturbances in the Human Fetal Testis Capable of Interfering With Testicular Descent. J Clin Endocrinol Metab [Internet]. 2013 Nov 1 [cited 2018 Nov 29];98(11):E1757–67. Available from: https://academic.oup.com/jcem/article-lookup/doi/10.1210/jc.2013-2531

52. Avella-Garcia CB, Julvez J, Fortuny J, Rebordosa C, García-Esteban R, Galán IR, et al. Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. Int J Epidemiol [Internet]. 2016 Jun 28 [cited 2017 Apr 12];25(6):dyw115. Available from: https://academic.oup.com/ije/article-lookup/doi/10.1093/ije/dyw115

53. Jedrychowski W, Spengler JD, Maugeri U, Miller RL, Budzyn-Mrozek D, Perzanowski M, et al. Effect of prenatal exposure to fine particulate matter and intake of Paracetamol (Acetaminophen) in pregnancy on eczema occurrence in early childhood. Sci Total Environ [Internet]. 2011 Nov 15 [cited 2017 Apr 12];409(24):5205–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21962593

54. Bauer AZ, Kriebel D. Prenatal and perinatal analgesic exposure and autism: an ecological link. Environ Heal [Internet]. 2013 Dec 9 [cited 2017 Apr 12];12(1):41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23656698

55. Saad A, Hegde S, Kechichian T, Gamble P, Rahman M, Stutz SJ, et al. Is There a Causal Relation between Maternal Acetaminophen Administration and ADHD? Pawluski J, editor. PLoS One [Internet]. 2016 Jun 13 [cited 2017 Dec 6];11(6):e0157380. Available from: http://dx.plos.org/10.1371/journal.pone.0157380

56. Reuter I, Knaup S, Romanos M, Lesch K-P, Drepper C, Lillesaar C. Developmental exposure to acetaminophen does not induce hyperactivity in zebrafish larvae. J Neural Transm [Internet]. 2016 Aug 26 [cited 2018 Sep 28];123(8):841–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27116683

57. Gervin K, Nordeng H, Ystrom E, Reichborn-Kjennerud T, Lyle R. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. Clin Epigenetics [Internet]. 2017 [cited 2017 Sep 15];9:77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28785368

58. Mazaud-Guittot S, Nicolaz CN, Desdoits-Lethimonier C, Coiffec I, Maamar M Ben, Balaguer P, et al. Paracetamol, Aspirin, and Indomethacin Induce Endocrine Disturbances in the Human Fetal Testis Capable of Interfering With Testicular Descent. J Clin Endocrinol Metab [Internet]. 2013 Nov 1 [cited 2017 Aug 1];98(11):E1757–67. Available from: https://academic.oup.com/jcem/article-lookup/doi/10.1210/jc.2013-2531

59. Thiele K, Solano ME, Huber S, Flavell RA, Kessler T, Barikbin R, et al. Prenatal Acetaminophen Affects Maternal Immune and Endocrine Adaptation to Pregnancy, Induces Placental Damage, and Impairs Fetal Development in Mice. Am J Pathol [Internet]. 2015 Oct [cited 2017 Aug 1];185(10):2805–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26254283

CHAPTER 1: ELEVATED METABOLITES OF ACETAMINOPHEN IN CORDBLOOD OF CHILDREN WITH OBESITY

(Formatted for Pediatric Obesity)

Patricia Sorrow[1], Rachel Maguire[2], Susan K. Murphy[3], Scott M. Belcher[1,2] and Cathrine Hoyo[1,2]

[1] Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, U.S.A

[2] Center for Human Health and the Environment, North Carolina State University, Raleigh, NC 27695, U.S.A

[3] Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC, 27708

Senior authors to whom correspondence may be directed.

Cathrine Hoyo, PhD, Email: choyo@ncsu.edu

Scott Belcher, PhD, Email: smbelch2@ncsu.edu

Center for Human Health and the Environment, and Department of Biological Sciences,

Campus Box 7633, North Carolina State University,

Raleigh, NC 27695-7633

ABSTRACT

Background: High-throughput metabolomics has been used cross-sectionally to evaluate differential metabolic profiles associated with individuals who have obesity.

Objectives: This study longitudinally assessed the cord-blood metabolome to explore if metabolic signatures of obesity at age 3-5 are apparent at birth.

Methods: In a nested case control design, metabolomics analysis was performed on umbilical cord blood of 25 children who developed obesity by age 3-5 years, compared to 25 sex-matched non-obese children enrolled as part of an ongoing birth cohort. Logistic regression models were used to identify significant metabolites, adjusting for maternal pre-pregnancy obesity.

Results: Children who had obesity by age 3-5 years had elevated levels of medium and long chain fatty acids including stearate, oleate, and palmitate at birth. Children with obesity were also more likely to have elevated levels of acetaminophen metabolites at birth, specifically: 3-(N-acetyl-L-cystein-S-yl) acetaminophen, 2-hydroxyacetaminophen sulfate, 2-methoxyacetaminophen glucuronide, and p-acetamidophenyl glucuronide.

Conclusion: Although the observed increases in lipids are consistent with previous metabolomic studies of obesity, this study is the first to report associations between acetaminophen metabolites and obesity in children; however, we lack mechanistic insights for this link. Larger human studies with longer follow-up and laboratory-controlled animal experiments are needed to clarify associations.

Keywords: metabolomics, acetaminophen, epidemiology, children, obesity

INTRODUCTION

The prevalence of childhood obesity in the United States has increased from 10% to 17% in the last 30 years (1). Obesity at younger ages tends to persist into adulthood and is often found concurrent with many metabolic diseases (2,3) In adults, obesity is a consistent risk factor for type 2 diabetes, cardiovascular disease and liver disease, it is associated with reduced quality of life and premature mortality (4). Genetic differences only account for about 5% of the variation in obesity risk (5). While the etiology of obesity is complex, a significant component is driven by complex gene-environment interactions in early life which alter metabolic programming (6–8). Biochemical signatures related to in utero exposures may be apparent at birth, and exploration of these signatures may reveal insights into metabolic pathways involved in the etiology of obesity.

Blood chemistry testing has been used for many years as a clinical measure of metabolic health, but high-throughput "metabolomic" profiling techniques that are now available have made possible simultaneous, agnostic identification, and quantification of hundreds of small molecules, metabolites, and protein products. At different ages, many studies have investigated the metabolic profile associated with obesity and its comorbidities. Those studies found significant differences in metabolites including species of acylcarnitines, branched-chain amino acids (BCAAs) and other amino acid species, as well as lipid species like non-esterified fatty acids (NEFAs), mono and poly-unsaturated fatty acids (MUFAs and PUFAs), phospholipids (PL), phosphatidylcholines (PC), as well as short, medium, and long-chain free fatty acids (FFAs) (9–15). The transient nature of many species of metabolites, and cross-sectional or case-control design of many of these studies has made cause-and-effect between altered metabolites and the onset of metabolic disease difficult to infer. Since umbilical cord-blood is collected prior

to clinical disease manifestation, temporal sequence is maintained, and causal inference is enhanced. Currently, only a few umbilical cord blood metabolomics assessments relating to obesity have been published (16,17).

Reported here are umbilical cord blood metabolite profiles associated with childhood obesity at ages 3-5 years. The metabolomics array revealed 46 metabolites at significantly higher levels in the cord-blood of children with obesity including 25 lipid species similar to those identified in individuals affected by metabolic diseases (10–15). Additionally, an unexpected association was found between acetaminophen metabolites at birth and childhood obesity.

MATERIALS AND METHODS

Metabolomics Analysis

Study Participants

Study participant recruitment methods are described in detail elsewhere(18). Expectant mothers who were 18 years or older, spoke English and intended to receive care within the Duke Obstetrics or Durham Regional Hospital were recruited. Of 601 eligible pregnant women were consented and enrolled between April 2005 and June 2009, 590 participants remained at delivery when umbilical cord blood samples were obtained. Children were contacted to collect growth data once every two years. Characteristics of the study population are presented in Table 1. The study protocol was approved by the Duke University Institutional Review Board.

Childhood Obesity

Twenty-five children classified as "obese" (Weight-Height-Zscore ≥ 95%) and 25 classified as "non-obese" (Weight-Height-Zscore < 85%) were selected at age 3-5 years. Weight and height was obtained from a combination of medical records and measurement by staff during study visits, the latter using a Seca® stadiometer and Tanita BWB-800 scale. Children were

chosen from amongst the first 100 participants enrolled into the cohort at the time of analysis. These did not differ from the remaining 540 participants with respect to ethnicity (p= 0.20), gestational weight gain (p= 0.40), and maternal cigarette smoking (p= 0.76). They did however differ with respect to maternal pre-pregnancy obesity (p= 0.02), but this was adjusted for in regression models.

Specimen collection and processing

Within minutes of delivery, umbilical cord blood (UCB) was collected into a K3EDTA tube by puncturing the umbilical vein. The specimens were kept at 4°C for up to 2 hours, before they were centrifuged at 2,000 rpm for 10 minutes in the laboratory to isolate plasma and buffy coat. Aliquots were frozen and stored at -80°C prior to analysis.

Metabolomic Profiling

Two hundred microliter aliquots were shipped to Metabolon (Durham, NC) for metabolite analysis using a combination of LC and GC mass spectrometry platforms. Sample preparation and mass spectrometry procedures have been published elsewhere (19). Metabolites were identified using Metabolon's Precision Metabolomics Biochemical Reference Library TM. Analyses identified 384 metabolites and the data was presented in area counts. Since exposure to acetaminophen was not ubiquitous the raw area counts were dichotomized into "exposed" or "unexposed". Individuals were classified as "exposed" for each metabolite if they had detectable levels of that metabolite. For lipids, raw area counts were re-scaled so that the median equaled 1 and absent values were assigned the minimum value. These data were then log2-transformed to minimize the effects of outliers, and fold difference was generated (2log2 mean cases/ 2log2 mean controls). Metabolite concentrations for children with and without obesity were compared using regression models and metabolites $p \leq 0.05$ were considered significant.

Statistical Analysis

Covariates

To identify potential confounding factors, we compared children with and without obesity with respect to pre-pregnancy maternal BMI, maternal gestational weight gain, maternal cigarette smoking, maternal age, maternal hypertension, maternal diabetes, delivery route, sex, ethnicity, birth weight, gestational age, breastfeeding for three months or more, and postnatal daily caloric intake. Pre-pregnancy obesity was calculated using self-reported height and weight (to nearest kg) at last menstrual period and analyzed as a continuous variable. The use of self-reported pre-pregnancy weight has been previously validated (20) Maternal gestational weight was assessed at each visit and weight gain was calculated as the difference between final recorded weight (up to 7 days before delivery) and self-reported pre-pregnancy weight. Ethnicity and maternal cigarette smoking during pregnancy were collected by self-report. Smoking status was verified by cotinine levels in infant cord blood samples. Maternal age, delivery route, infant sex, and birthweight were collected from medical records as were maternal morbidity data including gestational and type 2 diabetes and hypertension. Gestational age was determined based on the last menstrual period unless a 14+ day difference was detected in developmental stage using ultrasonography. Breast-feeding data (yes/no) were self-reported for every month for the first year of life, and then dichotomized at 3 months. Caloric intake for the child was assessed using two 24-hour dietary recalls during postnatal visits.

Modeling Approach

Logistic regression models were used to compare metabolite concentrations of children with and without obesity at age 3-5 years controlling for potential confounding variables. Concentrations of acetaminophen metabolites were dichotomized as “exposed” if acetaminophen

was detected or "unexposed" otherwise. Figure 1 shows the mean difference in the level of each of the acetaminophen metabolites for children who had obesity vs those who did not by ages 3-5 years. Data are presented here continuously and non-detectable levels were imputed with ½ the minimum detected value for each metabolite(21). Additionally, the number of children exposed is reflected below the x-axis of each plot. Lipid metabolites were also analyzed continuously using logistic regression models. Of the factors considered for confounding, only maternal pre-pregnancy obesity changed the odds ratios by greater than 10% and was included in models. Additionally, we explored pathway enrichment using IPA (Qiagen Inc.).

Prenatal Assessment of Acetaminophen Exposure

A separate cohort assembled between 2009 and 2011 was used to determine if therapeutic maternal acetaminophen use during pregnancy was associated with childhood obesity at ages 3 and 5 years. Inclusion and exclusion criteria and recruitment methods for the cohort were similar to the first cohort and have been previously described (22). Pregnant women were recruited during their first prenatal visit at one of five prenatal/delivery clinics in Durham, NC. These analyses are limited to 681 of 1700 total participants who remained in the cohort at age 3 or 5 years and responded to questions about intake of pharmaceuticals including acetaminophen during pregnancy. These 681 were similar to those not included with respect to ethnicity (age 3: Black p=0.24, White p=0.21, and Other p=1.00 , and at age 5: Black p=0.07, White p=1.00, and Other p=1.00), gestational weight gain (p= 0.61, p=0.74 at ages 3 and 5 respectively), cigarette smoking (p=0.41 at age 3 and p=0.88 at age 5) and sex distribution (p=0.28 and p=0.69 at ages 3 and 5), but differed with respect to maternal pre-pregnancy obesity (p=0.01 and p=0.001 at ages 3 and 5 respectively). The effects of maternal pre-pregnancy obesity were adjusted for in logistic regression models.

The use of pharmaceuticals during pregnancy was queried for six categories: cough/cold, pain relief, sleep aid, migraine, back pain, and other. Acetaminophen use was assessed as a binary variable where "1" reflected a single reported use, and "0" referred to no usage. Child obesity status was calculated using Weight-Height(z) (WHz) scores at ages 3 and 5 years, and was defined in this cohort in the same manner as previously described. Logistic regression models were used to adjust for pre-pregnancy obesity.

RESULTS

Table 1 shows that study participants were similar with respect to gestational age, birth weight, breastfeeding, caloric intake, and sex. They did differ by ethnicity ($p=0.01$), gestational weight gain ($p=0.02$), and maternal pre-pregnancy obesity ($p=0.002$). Of these factors, only maternal pre-pregnancy obesity was also found to be related to cord-blood metabolite levels.

Of 384 metabolites analyzed, 68 were found statistically different in children who developed obesity by age 3-5 years. (full list in Supplementary Table 1) Children with obesity had significantly elevated lipid species, including linoleate, myristate, oleate, palmitate, stearate, caprate, and species of acylcarnitines ($P<0.05$) compared to non-obese children. These associations were adjusted for maternal pre-pregnancy BMI, but models remained unchanged when maternal cigarette smoking, gestational weight gain, maternal hypertension, maternal diabetes, gestational diabetes, delivery route, ethnicity, sex, birthweight, and offspring caloric intake were included. IPA analysis revealed associations with biosynthesis of glycine, creatine, glutathione and palmitate.

The analysis revealed that of the eight metabolites of acetaminophen measured, four were significantly elevated in children who developed obesity by age 3-5 years. The odds ratio (OR) for 3-(N-acetyl-L-cystein-S-yl) acetaminophen was 10.42 (95% Confidence Interval (CI) 1.67-

65.26; p=0.01). Two to three-fold increases in risk of obesity were also observed in children with higher concentrations of 2-hydroxyacetaminophen sulfate, 2-methoxyacetaminophen glucuronide, and p-acetamidophenyl glucuronide ($p<0.05$). The association of 4-methoxyacetaminophen sulfate and 4-acetamidophenol with obesity were borderline significant (p=0.07 and p=0.09). These relationships remained unchanged after adjusting for pre-pregnancy maternal BMI. (Table 2) Associations in the same direction, albeit non-significant, were found between 4-acetaminophen sulfate (OR=3.20; p=0.14), 3(cysteine-S-yl)acetaminophen (OR=1.20; p=0.80) and obesity.

To identify potential sources of acetaminophen exposure, the association between acetaminophen intake during pregnancy and obesity in children at age 3-5 years was examined. As expected, maternal obesity was strongly associated with child obesity status at both ages 3 and 5 years, but no association was found with self-reported maternal acetaminophen intake during pregnancy. The odds ratios of the WHz for exposed and unexposed individuals was 1.07 (95%CI 0.29 – 4.02) (p=0.91) and 0.86 (95%CI 0.12 – 6.11) (p=0.86) at ages 3 and 5 years, respectively. No associations were found when stratified by sex [Boys age 3: 1.28 (95%CI 0.44-3.73; p=0.77); Boys age 5: 1.70 (95%CI 0.49-5.52; p=0.43; Girls age 3: 1.18 (95%CI 0.39-3.54); p=0.66; Girls age 5: 0.70 (95%CI 0.23-2.13; p=0.53)]. Additionally, we explored the possibility that women with obesity take acetaminophen more often during pregnancy but found no association. The mean difference in BMI in between mothers who reported they did not take acetaminophen during pregnancy vs those who reported they did, was 0.82kg/m2 (95%CI: (-0.17-1.80) p=0.10).

DISCUSSION

In a nested case-control study of 25 children with obesity and 25 children without obesity at age 3-5 years, we undertook a metabolomics analysis to identify differential metabolite profiles in umbilical cord blood at birth. We found elevated concentrations of 3-(N-acetyl-L-cystein-S-yl) acetaminophen, 2-hydroxyacetaminophen sulfate, 2-methoxyacetaminophen glucuronide, p-acetamidophenyl glucuronide, 2-methoxyacetaminophen sulfate, and 4-acetamidophenol, metabolites of the analgesic/antipyretic medication acetaminophen, in children who developed obesity by age 3-5 years. Associations between childhood obesity and acetaminophen metabolites were of a similar magnitude and direction as those found in a similar size study nested within the Project Viva Cohort of Massachusetts, USA. That study investigated the differential metabolic profile associated with rapid post-natal weight gain at age 0-6 months. These associations were comparable even though the average prenatal BMI of mothers whose children developed obesity in Project Viva was much lower (27.9kg/m2) compared to the prenatal BMI (35.5kg/m2) found in this population. Notably, associations were identified in both studies despite very different ethnic distributions, with 50% Caucasian, 31% African American and 15% Hispanic in Project Viva, compared to 68% African American, and 28% Caucasian in the cohort analyzed here (16).

In addition to acetaminophen metabolites, we also found elevated levels of lipid species associated with childhood obesity at ages 3-5 years. Similar lipid species have previously been linked to obesity, diabetes and/or nonalcoholic fatty liver disease (NAFLD) in adults and children (10–12,14,23). For instance, children born to mothers with gestational diabetes have been shown to have elevations in the same free fatty acids found in this study (myristate, palmitate, stearate, oleate, and linoleate). The specificity of these associations to diabetes is

unclear since maternal diabetes was not found to contribute to elevated lipids in our population (23). It is therefore possible that elevations in these metabolites are an outcome of the obesity phenotype which may be apparent at birth. This makes these lipids a potential target for discovering biomarkers of early detection of obesity and intervention for those at greatest risk.

Age at which obesity is assessed may also play a role in discovery of more disease specific biomarkers. Isganaitis et al. who investigated metabolomic signatures associated with elevated postnatal growth trajectories by six months of age, did not find elevated fatty acid signatures, but instead uncovered enrichment in "Tryptophan Metabolism" and "Excitatory neural signaling through 5HTR4/6/7 and serotonin" (16). Cases in the present study were also significantly larger at age one (1.27 WHz Cases, 0.23 WHz controls), but because of sample size restrictions metabolite analysis at age one could not be examined.

In addition to lipids, this analysis also found acetaminophen metabolites more often in the umbilical cord blood of children who developed obesity vs those who did not by ages 3-5 years. Although acetaminophen is regarded as safe and commonly recommended for use as an analgesic during pregnancy, in utero exposure to acetaminophen has been associated with attention deficit hyperactive disorder, asthma, and endocrine disruption (24–28).

To date, no mouse studies have been conducted linking in utero exposure to acetaminophen and obesity but, chronic acetaminophen exposure to mice during pregnancy has been shown to result in decreased fetal liver stem cells (29). This is thought to result in altered immune function/allergic response later in life. Moreover, this work suggests that the fetal liver may be a target of insult from acetaminophen during pregnancy. Furthermore, acetaminophen hepatoxicity has been shown in mice to correlate with serum levels of liver free fatty acids including palmitate, stearate, and oleate—these lipids have been proposed as biomarkers of

subclinical liver damage(30). Together, these metabolic signatures suggest it is plausible that in-utero exposure to acetaminophen could result in decreased liver function at birth, and that dysfunction in the primary organ of lipid metabolism may lead to obesity later in childhood.

Since data regarding the use of pharmaceuticals during pregnancy was not collected, and acetaminophen was not recorded in patient medical records as administered at delivery, questionnaire data of self-reported intake of acetaminophen from a larger cohort of women assembled from the same geographic location was evaluated to clarify these findings. No association between childhood obesity and reported gestational acetaminophen use was found. These data suggest that in utero exposure to acetaminophen is not associated with obesity. These findings may be due to chance alone, inaccurate recall in the use of this common pharmaceutical agent, or failure of the questionnaire to fully capture critical windows of exposure. Elucidating the timing during gestation when acetaminophen is associated with obesity risk and mechanism by which this occurs will require further study.

Nesting this case control study within an ongoing birth cohort adds considerable strength to these findings. This design eliminates the temporal ambiguity that plagues traditional case-control study designs as blood samples in which metabolites were measured were collected many years prior to the clinical manifestation of obesity, enhancing causal inference. In addition, the agnostic metabolomic assay revealed differential concentrations of lipid metabolites at birth similar in species and pathway to those identified by other obesity studies (10–15,23).

These findings should be interpreted in the context of the study limitations. It is possible that other associations in the comprehensive metabolomics array may have been masked because of limited statistical power. Additionally, although we controlled for maternal obesity, residual confounding by pre-pregnancy obesity or its correlate in this study, African American ethnicity,

cannot be excluded as a potential explanation for our findings; however, others have found associations between acetaminophen presence in cord blood and obesity later in life where maternal obesity was less extreme and ethnicity more homogenous (16). Despite limited statistical power in the metabolomics analysis and population heterogeneity, this data adds to the evidence relating in-utero exposure to a very common analgesic, acetaminophen with adverse effects in children.

In summary, we observed associations between childhood obesity at age 3-5 years with detectable concentrations of acetaminophen and elevated lipid metabolites in infant cord blood. Replication of these findings in controlled laboratory experiments and larger epidemiologic studies will increase confidence in the association and facilitate inquiry into these relationships.

ACKNOWLEDGEMENTS

We thank the parents and children of the Newborn Epigenetics Study, research nurse Tammy Bishop, and research assistants Stacy Murray, Carole Grenier, Darby Kroyer, Natasha Duggan, and Suba Narasimhan for tracing enrolled participants; data manager Francine Overcash for statistical assistance, and the obstetrics faculty and staff at Duke University and Durham Regional Hospitals, Durham, ND.

Conceived the question: PS. Designed nested case-control study for metabolomic analysis: CH. Conducted statistical analysi-s: RM. Interpreted the data: PS, CH, SMB. Oversaw specimen handling: SM. Contributed to writing the manuscript: PS, CH, SMB, RM, SM.

REFERENCES

1. Ogden CL, Carroll MD, Lawman HG, Fryar CD, Kruszon-Moran D, Kit BK, et al. Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988-1994 Through 2013-2014. JAMA [Internet]. 2016 Jun 7 [cited 2017 Jul 20];315(21):2292. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2016.6361
2. Singh AS, Mulder C, Twisk JWR, Van Mechelen W, Chinapaw MJM. Tracking of childhood overweight into adulthood: a systematic review of the literature. Obes Rev [Internet]. 2008 Mar 5 [cited 2017 Sep 23];9(5):474–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18331423
3. Schelbert KB. Comorbidities of Obesity. Prim Care Clin Off Pract [Internet]. 2009 Jun [cited 2017 Sep 28];36(2):271–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19501243
4. Katz DA, McHorney CA, Atkinson RL. Impact of obesity on health-related quality of life in patients with chronic illness. J Gen Intern Med [Internet]. 2000 Nov [cited 2017 Sep 28];15(11):789–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11119171
5. Bouchard C. Childhood obesity: are genetic differences involved? Am J Clin Nutr [Internet]. 2009 May [cited 2018 Mar 20];89(5):1494S–1501S. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19261728
6. Xia Q, Grant SFA. The genetics of human obesity. Ann N Y Acad Sci [Internet]. 2013 Apr [cited 2017 Sep 28];1281(1):178–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23360386
7. van Dijk SJ, Tellam RL, Morrison JL, Muhlhausler BS, Molloy PL. Recent developments on the role of epigenetics in obesity and metabolic disease. Clin Epigenetics [Internet].

2015 [cited 2017 Sep 28];7:66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27408648

8. Swanson JM, Entringer S, Buss C, Wadhwa PD. Developmental origins of health and disease: environmental exposures. Semin Reprod Med [Internet]. 2009 Sep [cited 2017 Sep 28];27(5):391–402. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19711249

9. Rauschert S, Uhl O, Koletzko B, Hellmuth C. Metabolomic Biomarkers for Obesity in Humans: A Short Review. Ann Nutr Metab [Internet]. 2014 [cited 2017 Sep 28];64(3–4):314–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25300275

10. Puri P, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min H-K, et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. Hepatology [Internet]. 2009 Dec [cited 2017 Apr 17];50(6):1827–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19937697

11. Fiehn O, Timothy Garvey W, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS One. 2010;

12. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic Signature Shift in Type 2 Diabetes Mellitus Revealed by Mass Spectrometry-based Metabolomics. J Clin Endocrinol Metab [Internet]. 2013 Jun [cited 2017 Apr 17];98(6):E1060–5. Available from: https://academic.oup.com/jcem/article-lookup/doi/10.1210/jc.2012-4132

13. Yi L-Z, He J, Liang Y-Z, Yuan D-L, Chau F-T. Plasma fatty acid metabolic profiling and biomarkers of type 2 diabetes mellitus based on GC/MS and PLS-LDA [Internet]. Vol. 580, FEBS Letters. 2006 [cited 2017 Apr 17]. Available from: http://www.sciencedirect.com/science/article/pii/S001457930601372X

14. Liu L, Wang M, Yang X, Bi M, Na L, Niu Y, et al. Fasting Serum Lipid and Dehydroepiandrosterone Sulfate as Important Metabolites for Detecting Isolated Postchallenge Diabetes: Serum Metabolomics via Ultra-High-Performance LC-MS. Clin Chem [Internet]. 2013 [cited 2017 Apr 17];59(9). Available from: http://clinchem.aaccjnls.org/content/59/9/1338.long

15. Tulipani S, Palau-Rodriguez M, Miñarro Alonso A, Cardona F, Marco-Ramell A, Zonja B, et al. Biomarkers of Morbid Obesity and Prediabetes by Metabolomic Profiling of Human Discordant Phenotypes. Clin Chim Acta [Internet]. 2016 [cited 2017 Apr 13];463:53–61. Available from: http://www.sciencedirect.com/science/article/pii/S0009898116304028

16. Isganaitis E, Rifas-Shiman SL, Oken E, Dreyfuss JM, Gall W, Gillman MW, et al. Associations of cord blood metabolites with early childhood obesity risk. Int J Obes [Internet]. 2015 Jul 25 [cited 2017 May 10];39(7):1041–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25804930

17. Ivorra C, García-Vicent C, Chaves FJ, Monleón D, Morales JM, Lurbe E. Metabolomic profiling in blood from umbilical cords of low birth weight newborns. J Transl Med [Internet]. 2012 Jul 9 [cited 2017 Sep 21];10(1):142. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22776444

18. Hoyo C, Murtha AP, Schildkraut JM, Forman MR, Calingaert B, Demark-Wahnefried W, et al. Folic acid supplementation before and during pregnancy in the Newborn Epigenetics STudy (NEST). BMC Public Health [Internet]. 2011 Dec 21 [cited 2017 Mar 18];11(1):46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21255390

19. Gall WE, Beebe K, Lawton KA, Adam K-P, Mitchell MW, Nakhle PJ, et al. alpha-

hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. PLoS One [Internet]. 2010 May 28 [cited 2017 May 17];5(5):e10883. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20526369

20. Fuemmeler BF, Wang L, Iversen ES, Maguire R, Murphy SK, Hoyo C. Association between Prepregnancy Body Mass Index and Gestational Weight Gain with Size, Tempo, and Velocity of Infant Growth: Analysis of the Newborn Epigenetic Study Cohort. Child Obes [Internet]. 2016 Jun [cited 2017 Aug 7];12(3):210–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27135650

21. US EPA O. Regional Guidance on Handling Chemical Concentration Data Near the Detection Limit in Risk Assessments. [cited 2018 Apr 2]; Available from: https://www.epa.gov/risk/regional-guidance-handling-chemical-concentration-data-near-detection-limit-risk-assessments

22. Liu Y, Murphy SK, Murtha AP, Fuemmeler BF, Schildkraut J, Huang Z, et al. Depression in pregnancy, infant birth weight and DNA methylation of imprint regulatory elements. Epigenetics [Internet]. 2012 Jul [cited 2017 Jul 3];7(7):735–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22677950

23. Yee JK, Mao CS, Ross MG, Lee WNP, Desai M, Toda A, et al. High oleic/stearic fatty-acid desaturation index in cord plasma from infants of mothers with gestational diabetes. J Perinatol [Internet]. 2014 May [cited 2018 Mar 8];34(5):357–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24577432

24. Avella-Garcia CB, Julvez J, Fortuny J, Rebordosa C, García-Esteban R, Galán IR, et al. Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. Int J Epidemiol [Internet]. 2016 Jun 28 [cited 2017 Apr

12];25(6):dyw115. Available from: https://academic.oup.com/ije/article-lookup/doi/10.1093/ije/dyw115

25. Sordillo JE, Scirica C V, Rifas-Shiman SL, Gillman MW, Bunyavanich S, Camargo CA, et al. Prenatal and infant exposure to acetaminophen and ibuprofen and the risk for wheeze and asthma in children. J Allergy Clin Immunol [Internet]. 2015 Feb [cited 2017 Apr 12];135(2):441–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25441647

26. de Fays L, Van Malderen K, De Smet K, Sawchik J, Verlinden V, Hamdani J, et al. Use of paracetamol during pregnancy and child neurological development. Dev Med Child Neurol [Internet]. 2015 Aug [cited 2017 Aug 1];57(8):718–24. Available from: http://doi.wiley.com/10.1111/dmcn.12745

27. Mazaud-Guittot S, Nicolaz CN, Desdoits-Lethimonier C, Coiffec I, Maamar M Ben, Balaguer P, et al. Paracetamol, Aspirin, and Indomethacin Induce Endocrine Disturbances in the Human Fetal Testis Capable of Interfering With Testicular Descent. J Clin Endocrinol Metab [Internet]. 2013 Nov 1 [cited 2017 Aug 1];98(11):E1757–67. Available from: https://academic.oup.com/jcem/article-lookup/doi/10.1210/jc.2013-2531

28. Andersen ABT, Farkas DK, Mehnert F, Ehrenstein V, Erichsen R. Use of prescription paracetamol during pregnancy and risk of asthma in children: a population-based Danish cohort study. Clin Epidemiol [Internet]. 2012 Feb [cited 2017 Aug 1];4:33–40. Available from: http://www.dovepress.com/use-of-prescription-paracetamol-during-pregnancy-and-risk-of-asthma-in-peer-reviewed-article-CLEP

29. Karimi K, Keßler T, Thiele K, Ramisch K, Erhardt A, Huebener P, et al. Prenatal acetaminophen induces liver toxicity in dams, reduces fetal liver stem cells, and increases airway inflammation in adult offspring. J Hepatol [Internet]. 2015 May [cited 2017 Mar

29];62(5):1085–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25529619

30. Suciu M, Gruia AT, Nica D V., Azghadi SMR, Mic AA, Mic FA. Acetaminophen-induced liver injury: Implications for temporal homeostasis of lipid metabolism and eicosanoid signaling pathway. Chem Biol Interact [Internet]. 2015 Dec [cited 2017 Mar 18];242:335–44. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0009279715300971

Table 1: Characteristics of Study Participants
Maternal and early childhood characteristics for 50 children recruited via NExT cohort. This analysis includes 25 children who became obese by age 3-5 years and 25 who were not classified as obese (non-obese) by age 3-5 years.

	Obese (n=25)	Non-Obese (n=25)	P-value
Maternal Characteristics	Mean (SD) or N (%)		
Pre-pregnancy BMI, kg/m²	35.57 (7.82)	26.27 (8.12)	0.002
Maternal Smoking, N (%)			0.07
Yes	12 (50)	6 (25)	
No	12 (50)	18 (75)	
Gestational Weight Gain, kg	12.23 (6.86)	16.94 (6.69)	0.02
Maternal Age, years	30.2 (8.19)	30.3 (7.03)	0.97
Chronic Hypertension, N (%)			0.64
Yes	3 (12)	2 (8)	
No	22 (88)	23 (92)	
Pregnancy Hypertension, N (%)			1.00
Yes	1 (4)	1 (4)	
No	24 (96)	24 (96)	
Diabetes, N (%)			1.00
Yes	8 (32)	8 (32)	
No	17(68)	17(68)	
Gestational Diabetes, N (%)			0.68
Yes	3 (12)	4(16)	
No	22 (88)	21(84)	
Preeclampsia, N (%)			0.29
Yes	3 (12)	1 (4)	
No	22 (88)	24 (96)	
Delivery Route, N (%)			0.16
Vaginal	11 (41)	16 (61)	
Caesarian	14 (59)	9 (39)	
Ethnicity, N (%)			
African American	17 (68)	8 (32)	0.01
Caucasian	7 (28)	17 (68)	
Child Characteristics			
Gestational Age, weeks	38.3 (1.66)	38.3 (2.25)	0.96
Birth Weight, grams	3256 (665)	3250 (563.7)	0.97
Breastfeeding, N (%)			0.16
Yes	11 (44)	12 (48)	
No	10 (40)	4 (16)	
Missing	4 (16)	9 (36)	
Caloric Intake, kCal	1630 (464)	1642 (414)	0.94
WHZ-Age 1	1.27 (1.3)	0.23 (1.1)	0.01
Sex, N (%)			1.00
Male	14 (56)	14 (56)	
Female	11 (44)	11 (44)	
Age at Assessment, N (%)			0.0003
3 years	7 (14)	1 (2)	
4 years	10 (20)	2 (4)	
5 years	8 (16)	22 (44)	

Table 2: Odds Radios and 95% Confidence Intervals for the Association between Acetaminophen Metabolites and Childhood Obesity
Adjusted for maternal pre-pregnancy BMI.
Maternal Smoking, Breastfeeding 3+ months, Caloric Intake, Gestational Age, Birthweight, Sex, Race, Maternal Hypertension, Maternal Diabetes, Delivery Route, and Child Age at Obesity Assessment were not significant confounders.

	Odds Ratio	95% Confidence Interval	P-Value
3-(N-acetyl-L-cystein-S-yl) acetaminophen	10.42	(1.67 – 65.26)	0.01
2-methoxyacetaminophen glucuronide	9.06	(1.19 – 68.97)	0.03
2-hydroxyacetaminophen sulfate	5.94	(1.07 – 33.10)	0.04
p-acetamidophenyl glucuronide	6.09	(1.05 – 35.33)	0.04
2-methoxyacetaminophen sulfate	4.54	(0.89 – 23.33)	0.07
4-acetamidophenol	3.65	(0.82 – 16.33)	0.09
4-acetaminophen sulfate	3.20	(0.67 – 15.27)	0.14
3-(cystein-S-yl) acetaminophen	1.20	(0.23 – 4.93)	0.80

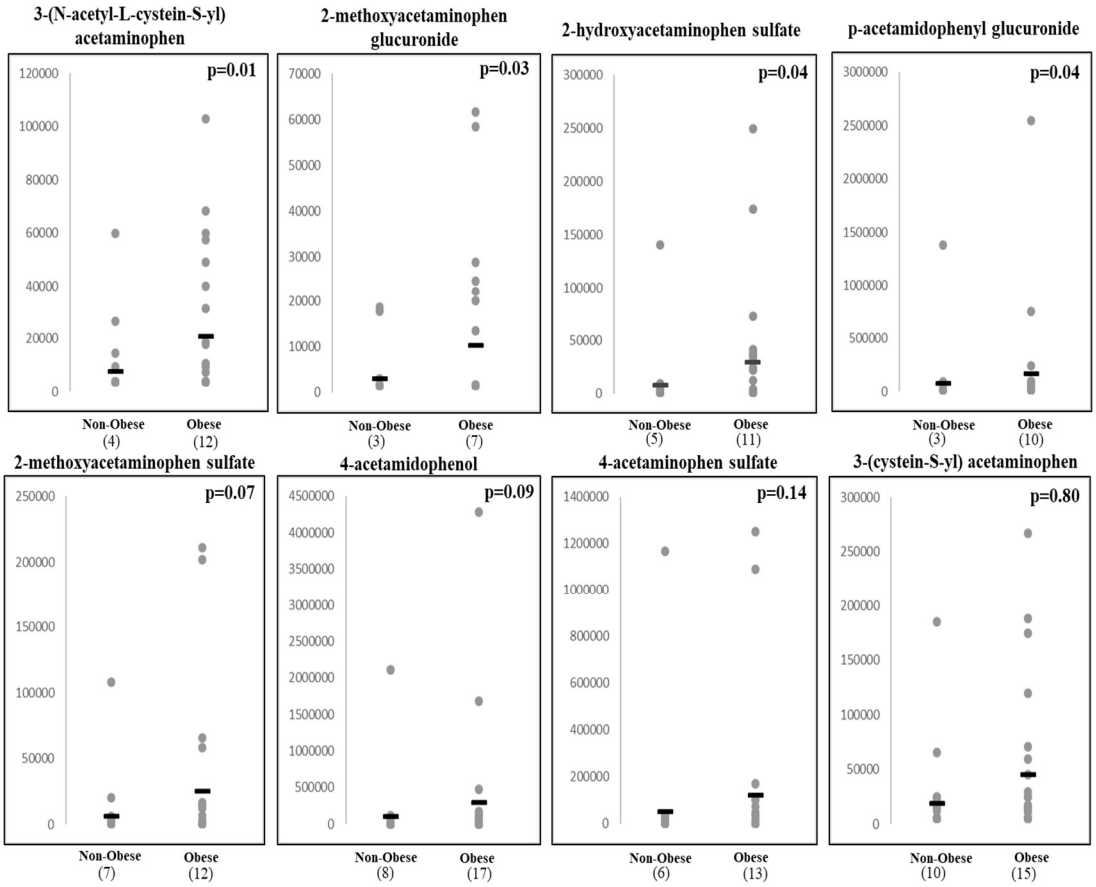

Figure 1: Mean Difference between Children with and without Obesity.
Shown are 8 metabolites of acetaminophen measured through agnostic metabolomics array (Metabolon Inc.) P-values calculated using logistic regression corrected for maternal pre-pregnancy BMI.
Y-axis = arbitrary units derived from Area Under the Curve (AUC) values. Non-detect values were imputed as ½ the lowest detectable level for each metabolite and number of children with detectable levels is reflected below each plot.

Supplementary Table 1: Metabolites found at significantly different levels in children who became obese by age 3-5
These values are not adjusted for confounding.

Major Metabolic Pathway	Metabolite	Odds Ratio	p-value
Amino acid	glycine	1.56	0.0668
	serine	1.42	0.0884
	beta-alanine	1.42	0.0739
	glutamate	1.82	0.0487
	N-acetyltyrosine	0.74	0.0735
	kynurenate	0.73	0.0292
	tryptophan	0.92	0.0584
	indolepropionate	0.5	0.0169
	cysteine	1.24	0.0605
	S-methylcysteine	1.26	0.0787
	cystine	1.49	0.0112
	N-acetylmethionine	0.87	0.074
	2-hydroxybutyrate (AHB)	0.76	0.0518
	ornithine	1.71	0.0573
	2-aminobutyrate	0.83	0.0576
Peptide	pro-hydroxy-pro	0.89	0.0596
	gamma-glutamylvaline	0.88	0.0514
Carbohydrate	mannitol	0.4	0.0617
	3-phosphoglycerate	1.43	0.041
	arabinose	1.12	0.0869
Energy	citrate	1.15	0.0893
Lipid	linoleate (18:2n6)	1.39	0.0491
	linolenate [alpha or gamma; (18:3n3 or 6)]	1.47	0.0602
	pelargonate (9:0)	0.83	0.0474
	caprate (10:0)	1.33	0.0205
	laurate (12:0)	1.64	0.0064
	myristate (14:0)	1.32	0.0508
	palmitate (16:0)	1.22	0.0315

Supplementary Table 1: Metabolites found at significantly different levels in children who became obese by age 3-5 Continued
These values are not adjusted for confounding.

Major Metabolic Pathway	Metabolite	Odds Ratio	p-value
Lipid	10-heptadecenoate (17:1n7)	1.56	0.0634
	stearate (18:0)	1.16	0.0454
	oleate (18:1n9)	1.53	0.0428
	10-nonadecenoate (19:1n9)	1.57	0.0686
	eicosenoate (20:1n9 or 11)	1.51	0.0682
	3-hydroxydecanoate	1.31	0.0677
	2-hydroxypalmitate	1.14	0.0228
	2-aminooctanoate	0.83	0.0905
	Carnitine	1.11	0.087
	Acetylcarnitine	1.2	0.0737
	hexanoylcarnitine	1.24	0.0559
	octanoylcarnitine	1.35	0.0568
	decanoylcarnitine	1.31	0.0295
	Laurylcarnitine	1.45	0.0454
	Oleoylcarnitine	1.4	0.0149
	glycochenodeoxycholate	1.49	0.0217
	Ethanolamine	2.1	0.0216
	phosphoethanolamine	1.47	0.0448
	2-oleoylglycerophosphoethanolamine*	1.36	0.0463
	5alpha-pregnan-3alpha,20beta-diol disulfate 1*	1.43	0.0624
	pregnenolone sulfate	0.83	0.0877
Cofactors and vitamins	Threonate	0.88	0.0434
	Pantothenate	0.58	0.0534
	pyridoxate	0.59	0.0064
	gamma-tocopherol	1.47	0.0354
Xenobiotics	catechol sulfate	0.56	0.0248
	benzoate	0.85	0.024
	glycolate (hydroxyacetate)	0.88	0.0927
	glycerol 2-phosphate	1.63	0.087
	2-piperidinone	1.38	0.0553
	4-acetaminophen sulfate	2.26	0.0441
	4-acetamidophenol	2.95	0.02
	2-hydroxyacetaminophen sulfate*	3.47	0.0152

Supplementary Table 1: Metabolites found at significantly different levels in children who became obese by age 3-5 Continued
These values are not adjusted for confounding.

Major Metabolic Pathway	Metabolite	Odds Ratio	p-value
Xenobiotics	2-methoxyacetaminophen sulfate*	3.49	0.0357
	3-(cystein-S-yl)acetaminophen*	2.11	0.0721
	2-methoxyacetaminophen glucuronide*	2.7	0.0472
	3-(N-acetyl-L-cystein-S-yl) acetaminophen*	2.15	0.0243
	cinnamoylglycine	0.56	0.0012
	3,7-dimethylurate	0.31	0.0247
	3-methylxanthine	0.42	0.0489

CHAPTER 2: ASSOCIATION BETWEEN DETECTABLE LEVELS OF ACETAMINOPHEN METABOLITES IN CORD BLOOD AND OBESITY AND BLOOD PRESSURE IN MALES AND FEMALES AGED 3-5 YEARS.

(Formatted for The International Journal of Obesity)

Patricia Sorrow[1], Jeffery Enders[2], Rachel Maguire[3], Cynthia Moylan[4], Michael Cowley[1,2], Susan K. Murphy[4], Scott M. Belcher[1,2] and Cathrine Hoyo[1,2]

[1] Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, U.S.A

[2] Molecular Education, Technology, and Research Innovation Center (METRIC), North Carolina State University, Raleigh, NC 27695, U.S.A

[3] Center for Human Health and the Environment, North Carolina State University, Raleigh, NC 27695, U.S.A

[4] Department of Medicine Division of Gastroenterology, Duke University, Durham NC 27710

[5] Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC, 27708

Senior author to whom correspondence may be directed.

Cathrine Hoyo, PhD, Email: choyo@ncsu.edu Phone: 919-515-0540

Center for Human Health and the Environment, and Department of Biological Sciences,

Campus Box 7633, North Carolina State University,

Raleigh, NC 27695-7633

ABSTRACT

Background: Thus far, two reports from untargeted metabolomics have described prospective associations between acetaminophen and its metabolites in umbilical cord blood (UCB) and obesity in young children. In mice, gestational exposure to APAP is associated with male-specific metabolic and endocrine dysfunction. With ~65% of women estimated to take acetaminophen (APAP) during pregnancy, even modest associations have the potential to sizably increase obesity risk. However, beyond untargeted approaches, these findings remain unverified.

Methods: Among 120 children enrolled as part of an ongoing birth cohort, 56% of them obese, LC-MS was used to quantify concentrations of APAP, and metabolites 4-Acetaminophen Sulfate Potassium Salt (APAP-S), 4-Acetamidophenyl β-D-Glucuronide Sodium Salt (APAP-G), and Acetaminophen Glutathione Disodium Salt (APAP-GSH) in umbilical cord blood (UCB). Regression models were used to evaluate the relationships between the presence of APAP, APAP-S, APAP-G, and APAP-GSH with two indicators of metabolic dysfunction - weight for height z-score (WHz), and systolic and diastolic blood pressure at age 3 years.

Results: Twenty three percent of newborn UCB specimens had detectable levels (<5ng/mL) of APAP, APAP-S, or APAP-G metabolites and one individual had detectable levels of APAP-GSH. After adjusting for pre-pregnancy obesity, APAP, APAP-S and APAP-G concentrations at birth were associated with higher systolic and diastolic blood pressure in males at age 3 years (APAP β= 4.32, se=1.70, p=0.01; APAP-S β= 4.51, se=1.72, p=0.01 and APAP-G β= 4.32, se=1.70, p=0.002). APAP metabolites were also associated with higher WHz-score at 3 years of age (APAP β= 1.00, se=0.43, p=0.02; APAP-S β= 0.95, se=0.44, p=0.03 and APAP-G β= 0.99, se=0.46, p=0.04). Dehydroepiandrosterone, the metabolic precursor of testosterone and estrogen, was also lower in males exposed to APAP (p=0.04).

Conclusions: Our data suggest that APAP exposure is associated with higher weight for height z-score and blood pressure at 3 years of age, and associations may be stronger in males. These data are consistent with the hypothesis that APAP may elicit endocrine disruption after prenatal exposure.

INTRODUCTION

Approximately 17% of children aged two to five years are classified as obese, and this estimate is even higher in western societies [1]. Although energy imbalance where energy input exceeds output contributes substantially, several lines of evidence now suggest these factors alone are unlikely to explain the increase in the obesity prevalence in children. Moreover, genetic predisposition alone is estimated to explain only 5% of the variation in obesity. It is imperative that research efforts focus on identifying additional risk factors in order to prevent the long term health consequences associated with this metabolic disease [2].

Metabolic profiling, utilizing discovery style metabolomics --a high-throughput method enabling a non-targeted profiling of small molecules in biological matrices, has been used to identify novel patterns that discriminate obese and non-obese children [3]. Similar to what has been shown in cross-sectional studies among adults, long and medium chain fatty acids have been longitudinally linked to childhood obesity [4,5]. In addition, recent untargeted metabolomics data also identified associations between detectable levels of multiple acetaminophen metabolites in umbilical cord blood (UCB) at birth and obesity at ages 6 months and 3-5 years [4,6]. These associations were independent of persistent risk factors including maternal pre-pregnancy obesity; however, the statistical power was limited as each study was limited to 50 participants.

Acetaminophen (APAP) is an antipyretic/analgesic widely recommended as safe for use during pregnancy. Mothers were first encouraged to use APAP as a safer alternative to the nonsteroidal anti-inflammatory drugs, aspirin and ibuprophen, which were found to be causally linked with placental hemorrhaging and less seriously with moderate gastrointestinal distress [7]. Today, reports cite APAP's "long record of safety" noting no evidence of miscarriage, pre-term birth, low birth weight, or gross fetal malformations associations in pregnant women hospitalized

for APAP overdose [8,9]. Now it is estimated that more than 50% of pregnant women report taking APAP, at least once, during pregnancy [10].

In recent years, the use of APAP during pregnancy has been correlated with elevated risk for adverse child health outcomes including asthma, attention deficit hyperactive disorder, and endocrine disruption; yet long-term safety data are limited. Although mechanisms by which APAP increases the risk of obesity in humans remains unclear, Karimi et al. found that prenatal exposure to APAP in mice resulted in loss of fetal liver stem cells thereby altering immune function/allergic response later in life [11]. Others have linked gestational exposure to APAP to lower circulating hormones, impaired reproductive development and maternal immune response to pregnancy, and altered placental morphology [12,13].

To clarify the association between prenatal APAP exposure and obesity identified via untargeted metabolomics approaches, we employed a targeted high-performance liquid chromatography- mass spectrometry (LC-MS) assay to measure APAP and its metabolites 4-acetaminophen sulfate potassium salt (APAP-S), 4-acetamidophenyl β-d-glucuronide sodium salt (APAP-G), and acetaminophen glutathione disodium salt (APAP-GSH). We measured these metabolites in the UCB samples of 114 obese and non-obese children aged 3, 4 and 5 years. These specific metabolites are representative of APAP metabolism pathways: sulfation, glucuronidation, and monooxygenation followed by glutathione conjugation. The data presented herein support that APAP exposure peripartum may increase the risk of childhood obesity – an association that may be more pronounced in males.

SUBJECTS AND METHODS

STUDY PARTICIPANTS

Recruitment methods for NEST participants have been described in detail elsewhere [14,15]. Briefly, more than 2,000 participants were recruited in one of six prenatal/delivery clinics in Durham, North Carolina between 2005 and 2011. English and Spanish speaking, expectant mothers who were 18 years or older were recruited. Median gestational age at enrollment was 12-13 weeks. Children were contacted for weight and height data at age 3, 4 and 5 years. The study protocol was approved by the Duke University and North Carolina State University Institutional Review Boards.

Individuals for LC-MS analysis (n=114 were randomly selected from NEST participants and were independent of the n=50 used analyzed using high-throughput metabolomics (3). The 114 individuals used in the LC-MS determination of APAP, APAP-S, APAP-G, and APAP-GSH were representative of the remainder of the cohort with respect to gestational weight gain (p= 0.89) but differed with respect to sex (p=0.0002) and maternal pre-pregnancy obesity (p=0.002), which were accounted for in regression models. Additionally, 20 individuals were chosen from among those (n= 50) formerly analyzed by Metabolon Inc. to confirm the accuracy of the non-targeted approach that was previously reported [4].

CHILDHOOD OBESITY AND BLOOD PRESSURE

Weight and height data were collected from medical records and measurement by staff during follow-up visits with a Seca® stadiometer (Hanover, MD) and Tanita BWB-800 scale (Arlington Heights, IL). Additionally, blood pressure data were measured at every visit averaged, and then blood pressure (BP) percentiles were calculated using the norms recommended by the

National Education Program Working Group on High Blood Pressure in Children and Adolescents [16].

SPECIMEN COLLECTION AND PROCESSING

Immediately following delivery, UCB was collected into a K_3EDTA tube by puncturing the umbilical vein. Specimens were kept at 4°C for up to 2 hours, before being centrifuged in a Beckman Coulter X22R refrigerated centrifuge using a SX4250 rotor at 2,000 rpm (544 x g) for 10 minutes to separate plasma and buffy coat. Aliquots were frozen and stored at -80°C prior to analysis.

ACETAMINOPHEN DETERMINATION VIA LC-MS

Sample Preparation

Plasma samples were prepared for LC-MS analysis using a cold methanol protein precipitation [17]. UCB plasma was thoroughly mixed with cold methanol and centrifuged at 10,000 x G for 15 minutes and the supernatant was kept for analysis. Samples were evaporated using a Savant SpeedVac™ (Hyannis, MA) at 45°C and 1atm for 45 minutes then reconstituted in LC grade water (Fisher, W51). Deuterated internal standard solutions containing 250ng/mL of 4-Acetaminophen Sulfate D3 (A161227) and Acetaminophen D4 (A161222) (Toronto Research Chemical, Ontario, CA) were added to each sample.

LC-MS Method

All mass spectrometry measurements were made in the Molecular Education, Technology, and Research Innovation Center (METRIC) at NC State University using a Thermo Scientific TSQ Altis Triple Quadrupole Mass Spectrometer (Waltham, MA) complexed with a Thermo Scientific Vanquish Horizon UHPLC system. The gradient consisted of the following steps (time/%B): 0 min/5%, 0.25 min/5%, 3.5 min/15%, 3.75 min/95%, 4.5 min/95%, 4.51

min/5%, 5 min/5%; where mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in methanol. The flow rate was 0.4 mL/min throughout.

Analyte concentrations were calculated via external calibration using labeled and unlabeled standards. Briefly, calibration curves are prepared from purchased unlabeled standards. To every calibration curve point, a known amount of internal standards (i.e., APAP-D4 and APAP-Sulfate-D3) are added. The calibration curve is then reported as an area ratio (analyte chromatographic peak area/internal standard chromatographic peak area). All unknown samples are doped with the same identity and amount of internal standards and this ratio is used to calculate the amount of unlabeled analyte in the unknown samples. These practices help control for instrument and injection variability across the sample batch.

Calibration curves were run before and after the batch and quality controls (containing all compounds of interest spiked it at 250 ng/mL) were run every 11^{th} injection to insure instrument performance. Additionally, two transitions are monitored for each analyte and a consistent ratio between the chromatographic peak areas of these transitions is required for an acceptable result. This helps control for potential unknown interferences that may be present in the samples.

STATISTICAL ANALYSIS

Regression models were used to determine if trends existed between child WHz scores, systolic and diastolic blood pressure and at birth detectable levels of APAP and its metabolites. Each APAP metabolite concentrations measured was dichotomized as detected or not-detected at the LC-MS low detection limit (5ng/mL). Linear regression models were fit determine associations between each measured APAP metabolite and WHz scores and blood pressure at age 3 years only, as available data at ages 4 and 5 years showed departure from normality.

We compared potential confounders by child outcomes of WHz, systolic, and diastolic blood pressure. Variables considered were pre-pregnancy maternal BMI, maternal gestational weight gain, maternal age, maternal hypertension, maternal diabetes, pre-eclampsia status, delivery route, sex, ethnicity, birth weight, gestational age, breastfeeding, and postnatal daily caloric intake. Pre-pregnancy obesity was determined by self-reported height and weight (to nearest kg) at last menstrual period. This approach to maternal pre-pregnancy BMI estimation has been previously validated [18]. Additionally, maternal weight was assessed at each visit and gestational weight gain was determined as the difference between the self-reported pre-pregnancy weight and the final recorded weight (up to 7 days before delivery). Ethnicity data were also collected by self-report. Maternal age, delivery route, infant sex, birthweight, gestational and type 2 diabetes, chronic and pregnancy hypertension, and pre-eclampsia status were obtained via medical records and verified by self-report. Unless a 14+ day difference was detected in developmental stage using ultrasonography, gestational age was determined based on the last menstrual period. Self-reported breast-feeding data were collected for every month from the first year of life and were then dichotomized into breastfeeding for at least 3 months (yes/no). Additionally, caloric intake for the child was assessed during follow-up visits using two 24-hour dietary recalls. Of these factors, only maternal pre-pregnancy obesity altered regression estimate by more than 10% and so was adjusted for in statistical models.

RESULTS

Linear regression models were used to compare metabolite concentrations of APAP and cardiometabolic child outcomes, controlling for potential confounding variables. Study participants were similar with respect to maternal gestational weight gain, maternal age, maternal hypertension during pregnancy, maternal diabetes, pre-eclampsia status, delivery route, sex,

ethnicity, gestational age, breastfeeding, and postnatal daily caloric intake. Obese children were significantly more likely to have a mother with pre-pregnancy obesity (mean BMI 33kg/m2 vs 28.5kg/m2; p=0.002), have a higher birth weight (3640g vs 3261g; p=0.001), and be of male sex (47% vs 24%; p=0.01) as shown in Table 1.

Comparison of Non-Targeted and Targeted APAP Determination

The accuracy of the non-targeted metabolomics results was confirmed by repeating the analysis of samples previously analyzed by Metabolon (n=20). Interrater reliability (Cohen's Kappa statistic) was calculated for the similarity between the metabolites identified by Metabolon Inc. and our targeted LC method. Substantial agreement (0.6+) was found for the metabolites.

Association of Detectible APAP Metabolites with Obesity and Cardiometabolic Phenotypes in Children

Twenty-seven children had detectable metabolites; and concentrations ranged from 5.07ng/mL to 4350 ng/ml for APAP, 5.32ng/mL to 8550 ng/ml for APAP-G and 5.76ng/mL to 4463ng/mL for APAP-S. The mean and interquartile range for APAP, APAP-G, and APAP-S are 772ng/mL; 9.06ng/mL – 621ng/mL, 2120ng/mL; 73.8ng/mL – 3190ng/mL, and 1220ng/mL; 84.8ng/mL – 2190ng/mL respectively. At age 3 years, the average WHz score was 1.73 with a standard deviation of 0.93. Figure 1 shows the mean difference in the WHz at age 3 years and in systolic and diastolic blood pressure for children categorized by metabolite detection in males and females.

Among all children, the presence of APAP metabolites in UCB was not associated with elevated WHz score. However, among males, we found associations between APAP, APAP-S, and APAP-G and childhood obesity (WHz) at age 3 years (β= 0.77, 95% CI = 1.1-5.0, p= 0.04),

APAP- G (β= 0.76, 95%CI 1.1-5.2, p= 0.05) APAP-S (β= 0.76, 95%CI = 1.1-5.17, p=0.05). These associations were not apparent in females for APAP (β = -0.05, 95%CI = 0.6-1.5, p= 0.82), APAP- G (β= -0.23, 95%CI = 0.5-1.3, p= 0.35) APAP-S (β= -0.17, 95%CI = 0.5-1.5, p= 0.52) (Figure 2). Results of regression analyses are presented in Table 2.

As a measure of cardiometabolic disease, we also assessed blood pressure and its association with APAP metabolite levels. We found that at age 3 years, systolic and diastolic blood pressure were significantly higher in children with detectable levels of APAP metabolites (Table 2). In children with detectable levels of APAP (systolic blood pressure: (β= 09, 95%CI = 1.02-1.017, p = 0.07) and diastolic blood pressure (β= 0.1, 95%CI =1.02 – 1.2, p = 0.02)). In children with detectable levels of APAP-S (systolic blood pressure: (β= 0.10, 95%CI = 1.03 – 1.2, p= 0.007) and diastolic blood pressure (β= 0.14, 95%CI = 1.05 – 1.28, p= 0.004)) and in children with detectable levels of APAP-G (systolic blood pressure: (β= 0.10, 95%CI = 1.03 – 1.18 , p= 0.009) and diastolic blood pressure: (β= 0.26, 95%CI = 1.03 – 1.08, p= 0.009) (Figure 2).

Dehydroepiandrosterone (DHEA), the metabolic precursor of testosterone and estrogen, was also lower in males exposed to APAP (p=0.04) (n=5) (Figure 3). Selection criteria, sample processing, and data collection for this cohort has previously been reported 4.

DISCUSSION

The prevalence of acetaminophen use during pregnancy has been estimated to be greater than 50% of all pregnancies in the United States [10]. At questionnaire completion at gestational age 20 weeks, 47% of mothers in this cohort reported to have taken acetaminophen at least once during pregnancy. Since the proportion of the population that is exposed is sizable, the potential for widespread impacts on child development is high even with a modest effect size. In mice,

gestational exposure to APAP is associated with a loss of fetal liver stem cells, altered immune function, lower circulating estrogen and testosterone, impaired reproductive development and altered placental morphology [11].

Herein, we report persistent associations between elevated WHz in males at age 3 years and levels of acetaminophen metabolites. WHz data suggest a sex specific mechanism in which males with detectable levels of acetaminophen UCB metabolites are more likely to become obese by 3 years of age. These sex specific trends could be explained by an endocrine disruption mechanisms including feminized genitalia and decreased levels of male hormones previously proposed by others [12,19–21]. Interestingly, APAP metabolite detection in UCB was also associated with elevated systolic and diastolic blood pressure in both males and females at age 3 years.

Although the mechanisms by which vascular and metabolic dysfunction effects linked to APAP remain unclear, prenatal exposure to acetaminophen has been associated with other adverse child health outcomes including asthma, attention deficit hyperactive disorder and endocrine disruption[19–24]. Animal studies investigating these relationships have found evidence of decreased number of fetal liver stem cells suggesting that the fetal liver may suffer insult because of prenatal exposure to acetaminophen. It is plausible then that liver dysfunction is apparent at birth, and that this dysfunction may lead to inefficient lipid metabolism, contributing to obesity via increased deposition of intramuscular and visceral fat, fatty liver and fatty pancreas, all of which are commonly found in individuals with obesity [25].

In Europe, epidemiological evidence is mounting in support of APAP eliciting endocrine disruption properties [12,19–21]. Intrauterine exposure to acetaminophen has been shown to result in reduced anogenital distance in male mice as well as decreased testosterone production – specifically a decrease in the principal adrenal hormone DHEA [12,26]. Anogenital distance is a

commonly used diagnostic measure of intrauterine endocrine disruption –more specifically a measure of prenatal androgen deficit resulting in feminization of the genitalia [19]. A similar relationship exists within our previously published metabolomics assay **(Figure 3)**[4]. Males with detectable levels of acetaminophen metabolites had a mean area under the curve of 1,947,744 units while the mean area under the curve for males without detectable levels of acetaminophen metabolites was 3,104,731units (p=0.04).

We also found sex specific associations between APAP metabolites and indicators of vascular and metabolic dysfunction. This may be because DHEA or dehydroepiandrosterone, primarily produced by the adrenal cortex, is a metabolic precursor of testosterone and estrogen [27]. The abundance of this hormone varies over time with high levels occurring during fetal development followed by a sharp decline in production during the first month of life until prepuberty (age 6-11 years) when levels begin to increase gradually until peaking in the mid-twenties: a time, which is known as adrenarche. During fetal development the placenta may play a major role in processing DHEA into estrone and oestradiol 17B, maintaining a positive feedback loop for the continuous production of DHEA by the fetus [26]. DHEA conversion generates the majority of human sex hormones, thus DHEA abundance is a reasonable proxy for testosterone levels in males [28]. Low levels of DHEA in adults is with diabetes. Supplementation has been shown to decrease the rate of gluconeogenesis in the liver and reduce fat mass in elderly men [27]. Hypogonadism and low testosterone in ageing men is often accompanied by obesity [29]. Data relating low DHEA at birth with obesity or other symptoms of low testosterone later in life could clarify our findings.

A major strength of these analyses is the prospective design with a sample size large enough to evaluate sex-specific effects. Our data suggesting possible sex-specific endocrine

disruption are supported by finding decreased DHEA in males with detectable levels of APAP at birth. While associations were not found in females, it is unknown whether similar associations exist in the female population but are masked by residual confounding by maternal pre-pregnancy obesity and related yet unmeasured factors.

In summary, our targeted analysis of three APAP metabolites at birth, measured using LC mass spectrometry in relation to vascular and/or metabolic dysfunction at age 3 years found novel associations between the presence of metabolites that include APAP parent, APAP-G, AAP-S and early dysfunction. Although larger sample sizes may help clarify these associations, causal inference will be enhanced by using a murine model with gestational exposure to acetaminophen.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

We thank the parents and children of the Newborn Epigenetics Study, research nurse Tammy Bishop, and research assistants Stacy Murray, Carole Grenier, Darby Kroyer, Natasha Duggan, and Suba Narasimhan for tracing enrolled participants; data manager Francine Overcash for statistical assistance, and the obstetrics faculty and staff at Duke University and Durham Regional Hospitals, Durham, ND. P30ES025128, R01ES016772, R21ES014947

REFERENCES

1 Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of Childhood and Adult Obesity in the United States, 2011-2012. *JAMA* 2014; **311**: 806.

2 Bouchard C. Childhood obesity: are genetic differences involved? *Am J Clin Nutr* 2009; **89**: 1494S–1501S.

3 Fuhrer T, Zamboni N. High-throughput discovery metabolomics. *Curr Opin Biotechnol* 2015; **31**: 73–78.

4 Sorrow P, Maguire R, Murphy SK, Belcher SM, Hoyo C. Elevated metabolites of acetaminophen in cord blood of children with obesity. *Pediatr Obes* 2018. doi:10.1111/ijpo.12465.

5 Yee JK, Mao CS, Ross MG, Lee WNP, Desai M, Toda A *et al.* High oleic/stearic fatty-acid desaturation index in cord plasma from infants of mothers with gestational diabetes. *J Perinatol* 2014; **34**: 357–63.

6 Isganaitis E, Rifas-Shiman SL, Oken E, Dreyfuss JM, Gall W, Gillman MW *et al.* Associations of cord blood metabolites with early childhood obesity risk. *Int J Obes* 2015; **39**: 1041–1048.

7 Mazaleuskaya LL, Sangkuhl K, Thorn CF, FitzGerald GA, Altman RB, Klein TE. PharmGKB summary: pathways of acetaminophen metabolism at the therapeutic versus toxic doses. *Pharmacogenet Genomics* 2015; **25**: 416–26.

8 McElhatton PR, Sullivan FM, Volans GN. Paracetamol overdose in pregnancy analysis of the outcomes of 300 cases referred to the Teratology Information Service. *Reprod Toxicol*; **11**: 85–94.

9 Rebordosa C, Kogevinas M, Bech BH, Sorensen HT, Olsen J. Use of acetaminophen

during pregnancy and risk of adverse pregnancy outcomes. *Int J Epidemiol* 2009; **38**: 706–714.

10 Liew Z, Ritz B, Rebordosa C, Lee P-C, Olsen J. Acetaminophen Use During Pregnancy, Behavioral Problems, and Hyperkinetic Disorders. *JAMA Pediatr* 2014; **168**: 313.

11 Karimi K, Keßler T, Thiele K, Ramisch K, Erhardt A, Huebener P *et al.* Prenatal acetaminophen induces liver toxicity in dams, reduces fetal liver stem cells, and increases airway inflammation in adult offspring. *J Hepatol* 2015; **62**: 1085–1091.

12 Holm JB, Chalmey C, Modick H, Jensen LS, Dierkes G, Weiss T *et al.* Aniline Is Rapidly Converted Into Paracetamol Impairing Male Reproductive Development. *Toxicol Sci* 2015; **148**: 288–298.

13 Thiele K, Solano ME, Huber S, Flavell RA, Kessler T, Barikbin R *et al.* Prenatal Acetaminophen Affects Maternal Immune and Endocrine Adaptation to Pregnancy, Induces Placental Damage, and Impairs Fetal Development in Mice. *Am J Pathol* 2015; **185**: 2805–2818.

14 Hoyo C, Murtha AP, Schildkraut JM, Forman MR, Calingaert B, Demark-Wahnefried W *et al.* Folic acid supplementation before and during pregnancy in the Newborn Epigenetics STudy (NEST). *BMC Public Health* 2011; **11**: 46.

15 Liu Y, Murphy SK, Murtha AP, Fuemmeler BF, Schildkraut J, Huang Z *et al.* Depression in pregnancy, infant birth weight and DNA methylation of imprint regulatory elements. *Epigenetics* 2012; **7**: 735–46.

16 NIH. The Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents. https://www.nhlbi.nih.gov/files/docs/resources/heart/hbp_ped.pdf (accessed 29 Nov2018).

17 Cook SF, King AD, van den Anker JN, Wilkins DG. Simultaneous quantification of acetaminophen and five acetaminophen metabolites in human plasma and urine by high-performance liquid chromatography?electrospray ionization?tandem mass spectrometry: Method validation and application to a neonatal pharmacokinetic study. *J Chromatogr B* 2015; **1007**: 30–42.

18 Fuemmeler BF, Wang L, Iversen ES, Maguire R, Murphy SK, Hoyo C. Association between Prepregnancy Body Mass Index and Gestational Weight Gain with Size, Tempo, and Velocity of Infant Growth: Analysis of the Newborn Epigenetic Study Cohort. *Child Obes* 2016; **12**: 210–218.

19 Fisher BG, Thankamony A, Hughes IA, Ong KK, Dunger DB, Acerini CL. Prenatal paracetamol exposure is associated with shorter anogenital distance in male infants. *Hum Reprod* 2016; **31**: 2642–2650.

20 Mazaud-Guittot S, Nicolaz CN, Desdoits-Lethimonier C, Coiffec I, Maamar M Ben, Balaguer P *et al.* Paracetamol, Aspirin, and Indomethacin Induce Endocrine Disturbances in the Human Fetal Testis Capable of Interfering With Testicular Descent. *J Clin Endocrinol Metab* 2013; **98**: E1757–E1767.

21 Kristensen DM, Hass U, Lesné L, Lottrup G, Jacobsen PR, Desdoits-Lethimonier C *et al.* Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. *Hum Reprod* 2011; **26**: 235–244.

22 Avella-Garcia CB, Julvez J, Fortuny J, Rebordosa C, García-Esteban R, Galán IR *et al.* Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. *Int J Epidemiol* 2016; **25**: dyw115.

23 Sordillo JE, Scirica C V, Rifas-Shiman SL, Gillman MW, Bunyavanich S, Camargo CA *et*

al. Prenatal and infant exposure to acetaminophen and ibuprofen and the risk for wheeze and asthma in children. *J Allergy Clin Immunol* 2015; **135**: 441–8.

24 de Fays L, Van Malderen K, De Smet K, Sawchik J, Verlinden V, Hamdani J *et al.* Use of paracetamol during pregnancy and child neurological development. *Dev Med Child Neurol* 2015; **57**: 718–24.

25 Raz I, Eldor R, Cernea S, Shafrir E. Diabetes: insulin resistance and derangements in lipid metabolism. Cure through intervention in fat transport and storage. *Diabetes Metab Res Rev* 2005; **21**: 3–14.

26 Quinn T, Greaves R, Badoer E, Walker D. DHEA in Prenatal and Postnatal Life: Implications for Brain and Behavior. *Vitam Horm* 2018; **108**: 145–174.

27 Aoki K, Terauchi Y. Effect of Dehydroepiandrosterone (DHEA) on Diabetes Mellitus and Obesity. *Vitam Horm* 2018; **108**: 355–365.

28 Miller WL. Disorders of Androgen Synthesis-from Cholesterol to Dehydroepiandrosterone Steroid Hormones. *Princ Pr* 2005; **14**: 58–68.

29 Mammi C, Calanchini M, Antelmi A, Cinti F, Rosano GMC, Lenzi A *et al.* Androgens and adipose tissue in males: a complex and reciprocal interplay. *Int J Endocrinol* 2012; **2012**: 789653.

Table 1 Characteristics of Study Participants

Maternal and early childhood characteristics for children recruited via NEsT cohort. This analysis includes children who became obese by age 3-5 years and who were not obese (non-obese) by age 3-5 years. Obese = (Weight for Height Z-Score ≥85%). Non-Obese = (Weight for Height Z-Score < 85%)..

p-value refers to the relationship between obese and non-obese children.

	Obese (n=47)			Non-Obese (n=67)			
	All	Male	Female	All	Male	Female	
Maternal Characteristics	Mean (SD) or N (%)						
Pre-pregnancy BMI, kg/m²	33.6(8.8)	33.5 (8.9)	33.7 (8.9)	28.5 (7.6)	32.2 (7.7)	27.4 (7.2)	0.002
Gestational Weight Gain, kg	14.8 (7.4)	13.8 (7.2)	15.75 (7.5)	13.0 (8.7)	15.0 (10.5)	12.4 (8.1)	0.25
Maternal Age, years	29 (6)	29.3 (5.5)	29.2 (6.7)	28 (6)	29.9 (7.2)	27.3 (6.0)	0.26
Chronic Hypertension, N (%)							0.21
Yes	7 (17.5)	3 (13.6)	3 (12)	4 (5.6)	0 (0)	4 (8)	
No	33 (82.5)	19 (86.4)	22 (88)	68 (94.4)	16 (100)	47 (92)	
Pregnancy Hypertension, N (%)							0.33
Yes	2(5)	1 (4.5)	1 (4)	6 (8.3)	2 (12.5)	4 (8)	
No	38 (95)	21 (95.5)	24 (96)	66 (91.7)	14 (87.5)	47 (92)	
Diabetes, N (%)							0.47
Yes	7 (17.9)	2 (9.1)	5 (20)	3 (4.3)	2 (12.5)	1 (2)	
No	32 (82.1)	20 (90.9)	20 (80)	67 (95.7)	14 (87.5)	50 (98)	
Preeclampsia, N (%)							0.93
Yes	5 (12.5)	2 (9.1)	2 (8)	6 (8.3)	1 (6.25)	5 (10)	
No	35 (87.5)	20 (90.9)	23 (96)	66 (91.7)	15 (93.75)	46 (90)	
Delivery Route, N (%)							0.31
Vaginal	20 (50)	13 (59)	16 (64)	48 (67)	7 (31.8)	33 (64)	
Caesarian	20 (50)	9 (41)	9 (36)	24(33)	9 (56.2)	18 (36)	
Ethnicity, N (%)							0.31
African American	26 (55)	11 (50)	15 (60)	31 (46)	10 (62.5)	21 (41)	
Caucasian	11 (23)	6 (27.2)	5 (20)	12 (18)	5 (31.25)	7 (14)	
Other	10 (21)	5 (22.7)	5 (20)	24 (36)	1 (6.25)	23 (45)	
Child Characteristics							
Gestational Age, weeks	39 (1.4)	39.0 (1.7)	38.9 (1.2)	39 (1.3)	38.8 (1.0)	39.2 (1.4)	0.59
Birth Weight, grams	3640 (557)	3532 (510)	3583 (529)	3261 (446)	3461 (395)	3199 (446)	0.001
Breastfeeding, N (%)							0.59
Yes	18 (38)	10 (45.5)	9 (36)	22 (33)	6 (37.5)	17 (33)	
No	16 (34)	7 (31.8)	10 (40)	16 (24)	5 (31.25)	11 (22)	
Missing	13 (35)	5 (22.7)	6 (24)	29 (43)	5 (31.25)	23 (45)	
Caloric Intake, kCal	1322 (395)	142 (352)	1225 (418)	1229 (597)	1396 (437)	1176 (635)	0.63
Sex, N (%)							0.01
Male	22 (47)			16 (24)			
Female	25 (53)			51 (76)			
Weight for Height Z-Score							
Age 3	1.71 (0.93)	1.64 (1.16)	1.77 (0.67)	-0.12 (0.94)	0.01 (0.5)	-0.16 (1.05)	0.0001
Age 4	1.78 (0.65)	1.81 (0.66)	1.75 (0.65)	-0.26 (1.1)	-0.16 (0.5)	-0.3 (1.25)	0.0001
Age 5	1.95 (0.63)	1.96 (0.59)	1.94 (0.67)	0.04 (0.84)	0.25 (0.49)	-0.02 (0.92)	0.0001
Systolic BP Percentile	70.9 (14.4)	69.3 (16.3)	72.1 (12.8)	63.4 (16.1)	59.4 (17)	64.9 (15.7)	0.01
Systolic Blood Pressure	99.8 (6.0)	98.3 (6.4)	101.0 (5.4)	96.5 (6)	95.2 (5.0)	97.0 (6.3)	0.004
Diastolic BP Percentile	78.1 (10.9)	80.9 (10)	75.8 (11.3)	74.5 (14.2)	73.7 (11.4)	74.7 (15.2)	0.01
Diastolic Blood Pressure	62.3 (4.9)	61.7 (5.6)	62.8 (4.3)	59.8 (4.5)	58.9 (3.0)	60.2 (4.9)	0.01

Table 2: Association between Acetaminophen Metabolites and Cardiometabolic Phenotypes in Children Age 3 Years.
Adjusted for Maternal pre-pregnancy BMI.
Breastfeeding 3+ months, Caloric Intake, Gestational Age, Birthweight, Sex, Race, Maternal Hypertension, Maternal Diabetes, and Delivery Route were note significant confounders.
Number of individuals with detectable concentrations of Acetaminophen Glutathione were note sufficient for statistical analysis.

	All			Males			Females		
	Estimate	**95% CI**	**P-Value**	**Estimate**	**95% CI**	**P-Value**	**Estimate**	**95% CI**	**P-Value**
Childhood Obesity									
APAP	0.18	(0.83 - 1.78)	0.34	0.77	(0.1 - 1.61)	0.069	-0.05	(-0.53 - 0.44)	0.82
APAP_G	0.05	(0.72 - 1.58)	0.79	0.77	(0.05 - 1.64)	0.051	-0.27	(-0.73 - 0.47)	0.65
APAP_S	0.10	(0.74 - 1.67)	0.64	0.77	(0.05 - 1.64)	0.51	-0.17	(-0.69 - 0.37)	0.52
Systolic Blood Pressure									
APAP	0.09	(1.02 - 1.17)	0.01	0.07	(-0.04 - 0.23)	0.26	0.10	(-0.02 - 0.18)	0.06
APAP_G	0.10	(1.03 - 1.18)	0.009	0.09	(-0.03 - 0.23)	0.19	0.09	(-0.01 - 0.18)	0.04
APAP_S	0.10	(1.03 - 1.2)	0.007	0.09	(-0.03 - 0.24)	0.19	0.11	(0.02 - 0.2)	0.06
Diastolic Blood Pressure									
APAP	0.10	(1.02 - 1.2)	0.018	0.01	(-0.12 - 0.15)	0.84	0.14	(0.04 - 0.47)	0.01
APAP_G	0.26	(1.03 - 1.08)	0.009	0.05	(-0.09 - 0.22)	0.52	0.09	(0.05 - 0.47)	0.01
APAP_S	0.14	(1.05 - 1.28)	0.004	0.05	(-0.09 - 0.22)	0.52	0.18	(0.07 - 0.34)	0.006

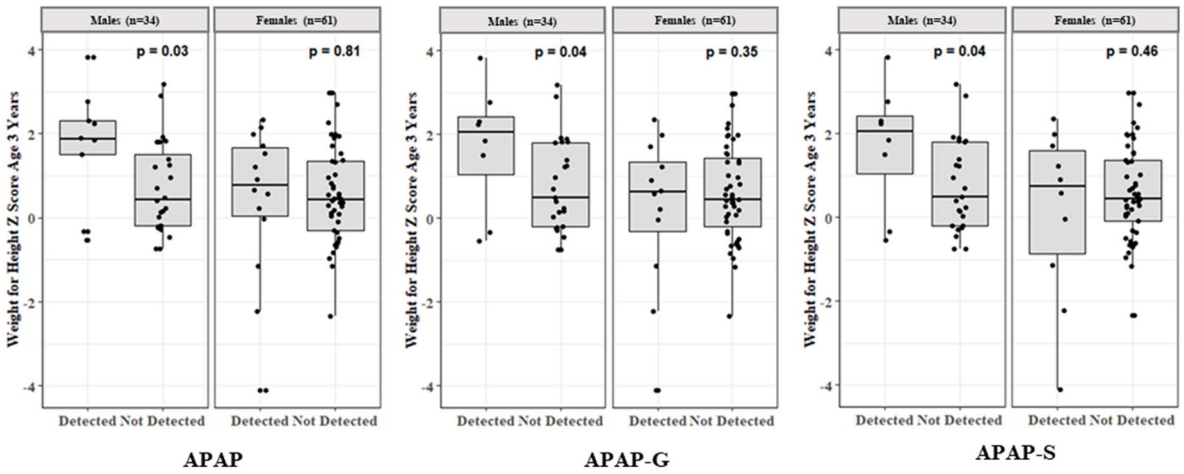

Figure 1: Mean Difference in WHz Score at 3 years of age Separated by Sex
Average weight for height z scores (WHz scores) of those with and without detectible levels of APAP metabolites. Presented separately for males and females at age 3 years.

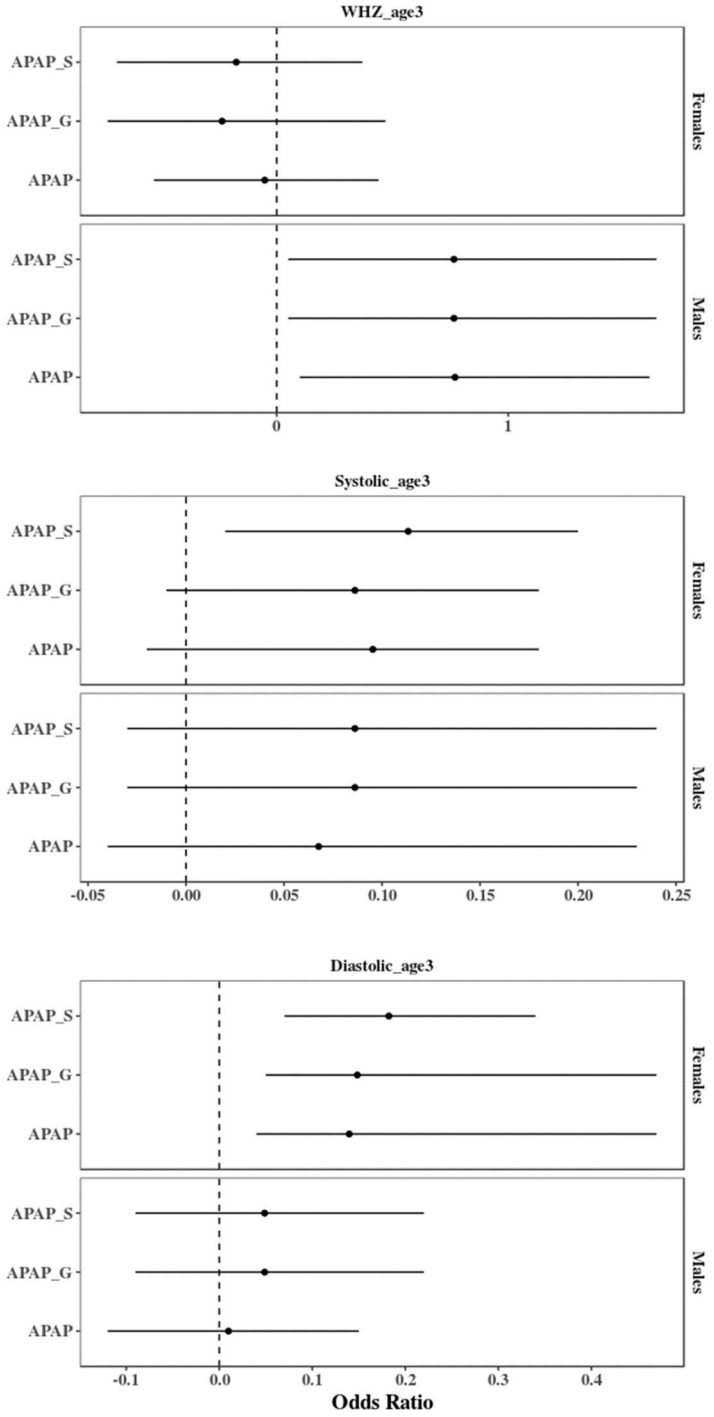

Figure 2: Relationship between Presence of APAP and Metabolites in Cord Blood and Cardiometabolic Phenotypes in Children at age 3 Years.
Shown are regression coefficients and 95% confidence intervals for the relationship between detection of APAP, APAP-S, and APAP-G in umbilical cord blood and offspring WHZ, systolic and diastolic blood pressure at age 3 years.

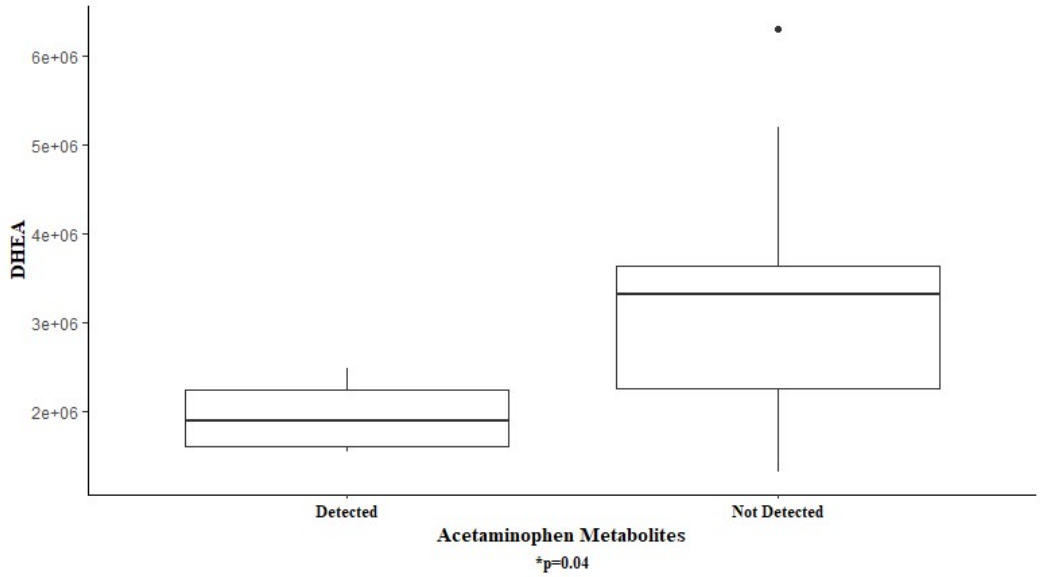

Figure 3: Decrease in DHEA in Cord Blood of Males with Detectable Levels of Acetaminophen Metabolites at Age 3 Years.
Shown are relative levels of DHEA in males with detectable and non-detectable levels of Acetaminophen Metabolites. DHEA determined by discovery metabolomics (Metabolon Inc.) and reported in AUC values.

CONCLUSION

In the future, the prevalence of childhood obesity, including that not attributable to energy imbalance and for which lifestyle interventions fail, is expected to continue to rise in the United States (1,2). This necessitates the development of methods for early detection of children at greatest risk so that preventative strategies can be promptly implemented before the disease is wholly manifest. Non-targeted metabolomics is a promising technology for novel biomarker discovery because blood chemistry profiling of children with and without obesity in a longitudinal design can unravel mechanisms of insult specific to the disease pathology. Presented in chapter one, an unexpected association was found between childhood obesity at ages 3-5 years and the presence of four acetaminophen metabolites (3-(N-acetyl-L-cystein-S-yl) acetaminophen, 2-hydroxyacetaminophen sulfate, 2-methoxyacetaminophen glucuronide, and p-acetamidophenyl glucuronide) in umbilical cord blood as determined by untargeted metabolomics (Metabolon Inc.). These associations were adjusted for the role of maternal obesity, a known driver of childhood adverse metabolic conditions, as well as African American ethnicity which was oversampled in this cohort. Similar associations have previously been noted with elevated post-natal growth trajectory in Project Viva Cohort of Boston Massachusetts, a birth cohort study where maternal obesity was less extreme, and ethnicity was not overrepresented (3).

In chapter two, HPLC-MS assays were developed to further investigate this relationship in a targeted fashion. We first were able to confirm that the exposure, as reported by metabolon, was reproducible by determining the concentration of Acetaminophen, and its metabolites 4-Acetaminophen Sulfate Potassium Salt, 4-Acetamidophenyl β-D-Glucuronide Sodium Salt, and Acetaminophen Glutathione Disodium Salt in 9 individuals previously determined to be exposed

and 11 individuals previously determined to be unexposed. Using a larger population (n=20), we confirmed the association between cord blood concentrations of acetaminophen and its metabolites, and the development of childhood obesity in males by 3 years of age among 111 children. Additionally, individuals with detectable levels of acetaminophen and its metabolites were also more likely to have elevated blood pressure. These relationships were adjusted for the role of maternal pre-pregnancy BMI. These data suggest the potential for an endocrine disruption activity for acetaminophen, but similar associations in females could also be strongly masked by maternal pre-pregnancy obesity which is a major confounder for childhood obesity in this cohort.

Acetaminophen is commonly recommended for use during pregnancy, but recently evidence drawn from epidemiology studies correlates prenatal exposure to acetaminophen with postnatal adverse health outcomes in children including asthma, attention deficit hyperactive disorder, and endocrine disruption (4–12). To these, the data presented in this thesis suggests that in addition to these outcomes, prenatal acetaminophen exposure may also increase the risk of obesity and elevated blood pressure in children (13).

Acetaminophen is commonly recommended for use during pregnancy, but recently evidence drawn from epidemiology studies correlates prenatal exposure to acetaminophen with adverse health outcomes in children including asthma, attention deficit hyperactive disorder, and endocrine disruption (4–11). To these, the data presented above contribute obesity and elevated blood pressure in children (12).

Animal studies investigating mechanistic causality between prenatal exposure to acetaminophen and adverse child outcomes of asthma have shown increased sensitivity to hepatoxicity at therapeutic doses in mothers as well as evidence of fetal liver damage and alterations in placental morphology(5,7). These data suggest that the fetal liver may be a target

for hepatoxic insult leading to alterations in lipid metabolism that develops into obesity (elevated WHz, systolic and diastolic blood pressure) later in life. Alternatively, the sex specific nature of the association between detectable acetaminophen metabolites and elevated WHz suggests the possibility of an endocrine disruption mechanism. Animal studies focused on the endocrine disruption potential of prenatal exposure to acetaminophen have shown evidence of decreased anogenital distance and similar changes in the abundance of circulating hormones (6,9–11). None of the previously mentioned studies reported changes in fat mass, body weight, organ function, or gene expression related to childhood obesity, so further exploration of this relationship would require an animal study using an appropriate model organism.

These data and other reports associating developmental acetaminophen exposure with other adverse child outcomes should be taken within the context of their respective study designs and limitations. Together though, these data support the need for research investigating the possibility of developmental insult conferred by chronic low dose drug administration of over-the-counter pharmaceuticals such as acetaminophen, in pregnant women. Furthermore, considering their pharmacological properties, obstetricians should urge greater caution when making pharmaceutical recommendations to their patients.

Epidemiology is an excellent hypothesis generation tool for toxicology. In the human-centric discipline of medicine, epidemiology has the advantage of directly measuring disease prevalence in the human population. The results from these types of studies can be interpreted directly since they utilize the collection of human tissue and fluid samples that are then used to compare and contrast individuals with and without disease using a variety of molecular biology techniques. However, as the human population is not homogenous, nor exposure controlled, it is not possible to define all unknown confounders or combinations of confounders that may

contribute to risk of obesity. For this reason, it is necessary to test epidemiologically derived hypotheses in a primary reductionist environment using model organisms.

REFERENCES

1. Ogden CL, Carroll MD, Lawman HG, Fryar CD, Kruszon-Moran D, Kit BK, et al. Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988-1994 Through 2013-2014. JAMA [Internet]. 2016 Jun 7 [cited 2017 Jul 20];315(21):2292. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2016.6361
2. Burguera B, Jesús Tur J, Escudero AJ, Alos M, Pagán A, Cortés B, et al. An Intensive Lifestyle Intervention Is an Effective Treatment of Morbid Obesity: The TRAMOMTANA Study-A Two-Year Randomized Controlled Clinical Trial. Int J Endocrinol [Internet]. 2015 [cited 2018 Sep 28];2015:194696. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26257780
3. Isganaitis E, Rifas-Shiman SL, Oken E, Dreyfuss JM, Gall W, Gillman MW, et al. Associations of cord blood metabolites with early childhood obesity risk. Int J Obes [Internet]. 2015 Jul 25 [cited 2017 May 10];39(7):1041–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25804930
4. Mazaleuskaya LL, Sangkuhl K, Thorn CF, FitzGerald GA, Altman RB, Klein TE. PharmGKB summary: pathways of acetaminophen metabolism at the therapeutic versus toxic doses. Pharmacogenet Genomics [Internet]. 2015 Aug [cited 2017 Mar 28];25(8):416–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26049587
5. Karimi K, Keßler T, Thiele K, Ramisch K, Erhardt A, Huebener P, et al. Prenatal acetaminophen induces liver toxicity in dams, reduces fetal liver stem cells, and increases airway inflammation in adult offspring. J Hepatol [Internet]. 2015 May [cited 2017 Mar 29];62(5):1085–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25529619

6. Holm JB, Chalmey C, Modick H, Jensen LS, Dierkes G, Weiss T, et al. Aniline Is Rapidly Converted Into Paracetamol Impairing Male Reproductive Development. Toxicol Sci [Internet]. 2015 Nov 1 [cited 2018 Nov 29];148(1):288–98. Available from: https://academic.oup.com/toxsci/article-lookup/doi/10.1093/toxsci/kfv179
7. Thiele K, Solano ME, Huber S, Flavell RA, Kessler T, Barikbin R, et al. Prenatal Acetaminophen Affects Maternal Immune and Endocrine Adaptation to Pregnancy, Induces Placental Damage, and Impairs Fetal Development in Mice. Am J Pathol [Internet]. 2015 Oct [cited 2017 Aug 1];185(10):2805–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26254283
8. Reuter I, Knaup S, Romanos M, Lesch K-P, Drepper C, Lillesaar C. Developmental exposure to acetaminophen does not induce hyperactivity in zebrafish larvae. J Neural Transm [Internet]. 2016 Aug 26 [cited 2018 Sep 28];123(8):841–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27116683
9. Fisher BG, Thankamony A, Hughes IA, Ong KK, Dunger DB, Acerini CL. Prenatal paracetamol exposure is associated with shorter anogenital distance in male infants. Hum Reprod [Internet]. 2016 Nov [cited 2018 Dec 13];31(11):2642–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27609981
10. Mazaud-Guittot S, Nicolaz CN, Desdoits-Lethimonier C, Coiffec I, Maamar M Ben, Balaguer P, et al. Paracetamol, Aspirin, and Indomethacin Induce Endocrine Disturbances in the Human Fetal Testis Capable of Interfering With Testicular Descent. J Clin Endocrinol Metab [Internet]. 2013 Nov 1 [cited 2018 Nov 29];98(11):E1757–67. Available from: https://academic.oup.com/jcem/article-lookup/doi/10.1210/jc.2013-2531

11. Kristensen DM, Hass U, Lesné L, Lottrup G, Jacobsen PR, Desdoits-Lethimonier C, et al. Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. Hum Reprod [Internet]. 2011 Jan 1 [cited 2018 Nov 29];26(1):235–44. Available from: https://academic.oup.com/humrep/article-lookup/doi/10.1093/humrep/deq323
12. Sorrow P, Maguire R, Murphy SK, Belcher SM, Hoyo C. Elevated metabolites of acetaminophen in cord blood of children with obesity. Pediatr Obes [Internet]. 2018 Sep 25 [cited 2018 Sep 28]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/30253079