AWARD NUMBER: W81XWH-16-1-0753

TITLE: Prenatal Polyunsaturated Fatty Acid Levels and Risk of Autism Spectrum Disorders

PRINCIPAL INVESTIGATOR: Kristen Lyall

CONTRACTING ORGANIZATION: Drexel University, Philadelphia, PA

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PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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Prenatal Polyunsaturated Fatty Acid Levels and Risk of Autism Spectrum Disorders

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**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

DREXEL UNIVERSITY
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U.S. Army Medical Research and Development Command
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**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

14. ABSTRACT The causes of autism spectrum disorder (ASD) are not well understood, but research suggests that factors influencing early brain development may be involved. Polyunsaturated fatty acids (PUFAs), which include omega 3 fatty acids, are fats obtained from the diet that play key roles in early fetal brain development. It is not known whether levels of these crucial fats during pregnancy influence risk of ASD. This project will examine the relationship between PUFA levels and ASD, addressing the role of environmental risk factors in ASD (a FY15 priority Area of Interest. Specifically, the goal of this project is to determine whether levels of PUFAs measured from maternal blood samples collected during pregnancy, and in a subgroup group, from newborn blood spots, differ between children with ASD and those without ASD. We will also explore whether the relationship between PUFAs and ASD differs in certain subgroups, such as by race/ethnicity, preterm birth, or child gender. Based on the importance of PUFAs in neurodevelopment, we suspect that lower levels of PUFAs may be related to ASD.

In order to address these questions, we will use data from routine screening programs in the state of California. Children with ASD (cases) will be selected from the California Department of Developmental Services (DDS), a statewide program that coordinates services for children with autism and other disabilities. Children without ASD (controls)will be selected from California birth certificates in the same year as children with ASD. PUFAs will be measured in the previously collected blood samples from pregnancy(500 cases and 500 controls), and in newborn blood spots from a subgroup (200 cases and 200 controls) using sensitive, state-of-the-art technology. Statistical analyses will examine differences in levels of maternal and newborn PUFAs between children with and without ASD, adjusting for demographic and other factors that may influence the association. Subgroup analyses will explore potential differences by major categories of race/ethnicity, gender, preterm birth, and others. Because the samples used in this study were collected during the time when PUFAs may have the greatest influence on the developing brain, associations seen here will inform on the role of PUFAs in risk of ASD.

**15. SUBJECT TERMS**

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRDC |
| Unclassified | Unclassified | Unclassified | Unclassified | 47 | 19b. TELEPHONE NUMBER *(include area code)* |

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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## 1. INTRODUCTION:

The purpose of this project is to determine whether prenatal levels of polyunsaturated fatty acids (PUFAs), as classes (omega 3, omega 6, and total PUFA) as well as individual fatty acids, are associated with offspring autism spectrum disorder (ASD). These fats are critical in neurodevelopmental processes with evidence for disruption in ASD, and thus we hypothesize that altered levels of them during critical windows of neurodevelopment may influence risk. To address this hypothesis, we are conducting a population-based case control study, including 500 cases with ASD identified through the California Department of Developmental Services (DDS) and 500 general population controls identified through state birth certificates and matched by birth year (2011-2013), birth month, and sex, after excluding DDS clients. Using banked prenatal serum specimens collected through routine prenatal screening in California, levels of PUFAs are measured using liquid chromatography-mass spectrometry/high resolution mass spectrometry (LC-MS/MHMS). In a subset of participants (n=400), we will also examine measured levels of PUFAs in neonatal blood spots. Results from this work will provide novel information about the relationship between PUFAs and ASD, in the first study with measured levels of PUFAs during pregnancy.

## 2. KEYWORDS:

Autism, etiology, epidemiology, polyunsaturated fatty acids, prenatal risk factors, nutrition

## 3. ACCOMPLISHMENTS:

**What were the major goals of the project?**

Major goals included in the SOW, and information on target and actual dates and percent completion, are listed below:

1. **Procurement of Maternal and Neonatal Stored Blood Samples (relevant to Aims 1, 2, and 3)**

   Target completion: Year 1, quarter 3-4

   Actual completion/% complete: Year 1, quarter 4- all maternal and neonatal samples have been obtained; thus, this task is 100% complete.

   Description: We have obtained all maternal prenatal serum samples (n=1002; an additional 2 were obtained due to unexpected differences in availability of samples and the need to balance case-control birth years) neonatal blood spots (n=400). This major goal included the following sub-tasks: completing and submitting IRB and vital record use applications (completed by the second quarter of our first year, slightly later than expected due to California review board meeting dates); selecting cases and controls from California databases (projected for quarter 1; completed slightly later than anticipated, due to waiting for approvals); obtaining approval

from the Genetic Disease Screening Program (GDSP) for use of samples (completed as projected); requesting and obtaining samples from GDSP and sample shipment to the Snyder laboratory (obtaining of samples completed approximately one quarter later than expected, due to delays in the California Biobank queue process.)
Major milestones sought and achieved: Local and CPHS IRB approvals; HRPO approval; procurement of samples.

2. **Measurement of PUFAs in maternal serum and newborn blood spots (relevant to Aims 1 and 3)**
   Target completion: Year 1, quarter 4- year 2, quarter 2
   Actual completion/% complete: We have completed laboratory analyses of all samples (100% complete).
   Description: Measurement of PUFAs in maternal samples was completed in the Spring of 2018. Measurement of PUFAs in newborn spots was just recently completed. There had been some delay in shipment of newborn blood spots due to California Biobank administrative delays.
   Major milestones sought and achieved: Completion of biosample assays for PUFAs.

3. **Data analysis of PUFAs in association with ASD (relevant to Aims 1, 2, and 3)**
   Target completion: (Year 2, quarters 2-4.)
   Actual completion/% complete: 100% complete; we have completed analyses of maternal PUFA levels and ASD, as well as analyses of newborn PUFAs and ASD.
   Description: Subtasks 1 (analyses of maternal levels) and subtasks 2 (examination of modifers and subgroups) were completed in the previous grant year. Subtask 3 (analyses of newborn levels) was completed in this grant year, with preliminary statistical analyses completed between November 2018 and January 2019, and analyses finalized between February and May 2019.
   Milestone achieved: Completion of statistical analyses.

4. **Presentation of findings (relevant to Aims 1, 2, and 3)**
   Target completion: (Year 3, quarters 1-4)
   Actual completion/% complete: Year 3- NCE.
   Description: In our last annual report we described several presentations for the prior reporting year. During this NCE year, we finalized and published manuscripts for our primary aims. These have been published in the *American Journal of Epidemiology* and *Autism Research*.
   Milestone achieved: Presentation and publication of findings.

**What was accomplished under these goals**

Major activities during this reporting period include publication of our findings in peer-reviewed scientific journals. Specifically, we published our results of maternal serum PUFA levels in association with child ASD in the American Journal of Epidemiology (Lyall et al., 2020) and our results of newborn bloodspot PUFAs and ASD in Autism Research (Bostwick et al, 2020).

In addition to these publications describing our findings from this work, we also accomplished supplemental analyses of maternal cotinine levels in association with ASD. The results of these additional analyses have been prepared as a third manuscript (Berger et al; under review by the California Department of Public Health prior to submission). Furthermore, we have conducted analyses exploring potential interactions between PUFA levels and cotinine levels in our study population.

**What opportunities for training and professional development has the project provided?**

Under funding from this project, the PI attended the 2018 and 2019 meetings of the International Society for Autism Research (INSAR), where she presented findings from this study. The PI also presented findings on newborn blood spots at the 2019 Society for Pediatric and Perinatal Epidemiologic Research (SPER) conference.

In addition, this project provided training for several students, including:

1. Training for a laboratory analyst who gained additional expertise and training in measurement of PUFAs in two biological matrices using mass spectrometry, under the guidance of Co-Investigator Dr. Snyder.
2. Training and mentoring for three Masters-level students who conducted statistical analyses for this project under the direction of the PI. One of these students contributed to the project as a Research Assistant and coauthor on a manuscript; the other conducted analyses for her master's thesis work, while the final student conducted analyses and led our paper describing the neonatal findings. This student has also been leading analyses of cotinine interactions with PUFAs.

**How were the results disseminated to communities of interest?**

Results from maternal and newborn blood spot analyses from this work were presented to professionals in the field at the INSAR and SPER meetings as described above. We have also disseminated findings through publications in peer-reviewed journals; two manuscripts describing findings from this study are now published. PDFs of these manuscripts are included as attachments to this final report.

**What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to report

## 4. IMPACT:

**What was the impact on the development of the principal discipline(s) of the project?**

Our study provides the first results on the association between measured levels of newborn PUFA levels in association with ASD diagnosis, and is one of only 3 studies to have measured maternal PUFA levels in association with ASD-related outcomes. Because there was some suggestion of increased risk for having a child with ASD with comorbid intellectual disability among mothers with low levels of certain PUFAs, our work suggests the need for continued investigation into the potential relationship between PUFAs and phenotypic subgroups within ASD. The majority of pregnant women do not eat recommended levels of fish, which are a key source of these PUFAs. If our findings are further supported in work seeking to replicate these results specific to ASD with comorbid intellectual disability, there is the potential for risk reduction with dietary modifications for certain subgroups. Furthermore, because few studies have measured levels of these fatty acids in newborn blood spot samples, our study has provided novel information on methods to conduct such measurements, which could be applied in other studies. Because newborn bloodspots are more readily accessible than maternal prenatal samples, demonstration of feasibility of measurement in bloodspots stored up to 5 years has applicability and potential impact for other fields and outcomes.

**What was the impact on other disciplines?**

The suggestion of potentially increased risk with certain PUFA levels for ASD with comorbid intellectual disability from our maternal PUFA analyses suggests the need to further examine these associations in study populations with detailed information on broader neurodevelopmental outcomes in order to tease apart specificity of associations and better understand how risk may differ for different neurodevelopmental conditions and for individuals with comorbid conditions. Thus, our findings could have an impact on other fields of specialty focused on other, non-ASD neurodevelopmental disorders. In addition, as stated above, the measurement of PUFAs in newborn blood spots may have an impact on other disciplines seeking to measure these fatty acids in novel and more readily available matrices.

**What was the impact on technology transfer?**

Nothing to Report

**What was the impact on society beyond science and technology?**

Findings here should be replicated and further studied prior to making wide-scale public health recommendations. However, there is evidence that PUFA intake is below recommended levels for pregnant women in the US; thus, if there is further support for increased risk to certain subgroups with levels outside of average ranges, risk reduction strategies based on dietary recommendations could ultimately be created, and this may have an impact on risk of ASD.

## 5. CHANGES/PROBLEMS:

**Changes in approach and reasons for change**

No significant changes in approach have been made during the reporting period.

**Actual or anticipated problems or delays and actions or plans to resolve them**

We did not experience challenges or delays during this reporting period, nor have we experienced any major problems over the course of the grant. As noted in prior reports, within the newborn bloodspot samples, we were able to measure and quantify the majority of PUFAs of interest, but two fatty acids of the same chain length, ALA and GLA, could not be further resolved and were reported together.

**Changes that had a significant impact on expenditures**

None

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

None

**Significant changes in use or care of vertebrate animals.**

Not applicable

**Significant changes in use of biohazards and/or select agents**

Not applicable

## 6. PRODUCTS:

- **Publications, conference papers, and presentations**

**Journal publications.**

**Publications:**
Bostwick A., Snyder N.W., Windham G.C., Whitman C., Pearl M., Robinson L., Newschaffer C.J., Lyall K. Polyunsaturated fatty acids in newborn bloodspots: Associations with autism spectrum disorder and correlation with maternal serum levels. Autism Research. doi: 10.1002/aur.2365

Lyall K., Windham G.C., Snyder N., Kuskvsky R., Peining X., Bostwick A., Robinson L., Newschaffer C.J. Maternal serum polyunsaturated fatty acid levels in association with autism spectrum disorder: Results from a population-based case control study in California. American Journal of Epidemiology. https://doi.org/10.1093/aje/kwaa171

**Books or other non-periodical, one-time publications.**

None

**Other publications, conference papers, and presentations**. /

**Manuscript:**
Berger K, Pearl M, Kharrazi M, Li Y, DeGuzman J, She J, Behniwal P, Lyall K, Windham GC. The association of *in utero* tobacco smoke exposure, quantified by serum cotinine, and Autism Spectrum Disorder in a case-control study in California.

**Conference poster and abstract presentations (from prior reporting years):**
Lyall K, Windham GC, Whitman C, Snyder N, Newschaffer C. (2019). Neonatal levels of polyunsaturated fatty acids in association with autism spectrum disorder. Society for Pediatric and Perinatal Epidemiologic Research (SPER), Minneapolis, MN. June 2019.

Lyall K, Windham GC, Snyder N, Whitman C, Newschaffer C. (2019). Neonatal levels of polyunsaturated fatty acids in association with autism spectrum disorder. INSAR, Montreal, Canada, May 2019.

Lyall K, Windham GC, Snyder N, Newschaffer C. (2018). Prenatal levels of polyunsaturated fatty acids in association with autism spectrum disorder. Society for Epidemiologic Research (SER), Baltimore, MD. June 2018.

Acknowledgement of federal support was given in all presentations/publications.

**Website(s) or other Internet site(s)**

Nothing to report

**Technologies or techniques**

Nothing to report

**Inventions, patent applications, and/or licenses**

**Other Products**

Nothing to report

Nothing to report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Name: Dr. Kristen Lyall
Project Role: Principle Investigator
Researcher Identifier
Nearest person month worked: 2
Contribution to Project: Oversaw all project activities, conducted and/or supervised data analyses. Led maternal serum manuscript and edited neonatal manuscript; contributed to cotinine manuscript.

Name: Dr. Nathaniel Snyder
Project Role: Co-Investigator
Researcher Identifier
Nearest person month worked: 1
Contribution to Project: Performed laboratory analysis of PUFAs in maternal serum samples and newborn blood spots. Reviewed and contributed to manuscripts.

Name: Dr. Gayle Windham
Project Role: Co-Investigator
Researcher Identifier
Nearest person month worked:1
Contribution to Project: Oversaw data linkage and aided in preparation of data files and original coordination of case control selection process. Reviewed and contributed to manuscripts.

Name: Casey Whitman
Project Role: Data Analyst
Researcher Identifier
Nearest person month worked: 1
Contribution to Project: Under supervision of PI, Kristen Lyall, performed data analyses. Contributed to neonatal manuscript.

Name: Jasmine Carver
Project Role: Data Analyst
Researcher Identifier
Nearest person month worked: 1
Contribution to Project: Under supervision of Co-I, Gayle Windham, performed data management, and data linkage.

Name: Anna Bostwick
Project Role: Data Analyst
Researcher Identifier
Nearest person month worked: 1
Contribution to Project: Under supervision of PI, Kristen Lyall, performed data analyses. Prepared neonatal manuscript.

Please see attached appendix. Active support has changed for some investigators, but there is no overlap and support changes have not substantially impacted effort on the current project.

**What other organizations were involved as partners?**

(NO CHANGE)
Organization name: California Department of Public Health (CDPH)
Location: Richmond, CA
Contribution: Collaboration- Co-Investigator Dr. Gayle Windham and her study staff at CDPH collaborated with the study PI to ensure data linkage and study sample selection necessary for this project. As noted in the previous annual report for this project, Dr. Windham and her team have extensive experience with California birth certificate and DDS data, and conducting data linkages for similar projects. Dr. Windham and her staff have maintained close communication with the PI of this project, Dr. Lyall, including through attending project meetings via conference calls. Facilities have not been exchanged.

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:**

**QUAD CHARTS:**

N/A

## 9. APPENDICES:

Please see attached copies of published manuscripts from this study. (Lyall et al., 2020 is provided as an online advance copy).

**Kristen Lyall**

## Other Support

3/1/2020-12/31/2024
NIH R01ES029511 (subcontract)
Metals dysregulation, brain development, and autism disorder (PI: Volk)
Role: Co-Investigator/site PI

3/1/2020-2/28/2021
Epidemiology Dept Pilot Funding
Development and validation of scores indexing pesticide residues in pregnant women (PI: Lyall)
Role: PI

1/24/2020-10/31/2024
NIH R24ES030893 (subcontract)
Expanding the value of the EARLI study: Small cohort with big data (PI:Fallin)
Role: Co-Investigator/site PI

7/1/2019-6/30/2023
NIH R01NS107607-01A1
Maternal epilepsy, anti-epileptic drug use during pregnancy, and risk of autism (PI: Lee)
Role: Co-Investigator

4/1/2019-9/30/2021
Eagles Foundation
Maternal dietary patterns during pregnancy in association with autism and autism spectrum disorder traits
Role: PI

8/15/2018-8/15/2021 (NCE)
NIH R21 HD096356-01
Oxidative stress pathways and placental pathology in association with autism spectrum disorder and neurodevelopment (PI: Lyall)
Role: PI

4/1/2018-2/29/21 (NCE)
NIH/ECHO OIF 1U2COD023375-02
Optimizing social communication measurement with the Social Responsiveness Scale (PI: Lyall)
Role: PI

9/15/2017-8/31/2021
NIH R01ES026903
Prenatal exposure to endocrine disrupting chemical mixtures and ASD risk (PI: Newschaffer)
Role: Co-Investigator

9/30/2016-8/31/2021
NIH R01ES025531 (subcontract)
Prenatal exposure to metals and risk for Autism Spectrum Disorder in MARBLES and EARLI (PI: Fallin)
Role: Co-Investigator

9/30/2016-9/30/2020 (NCE)
DoD/CDMRP/USAMRAA AR150143
Prenatal polyunsaturated fatty acid levels and risk of autism spectrum disorder (PI: Lyall)
Role: PI

09/21/2016 – 08/31/2023
NIH UH3OD023342
An ASD-Enriched Risk (ASD-ER) ECHO Cohort (PI: Newschaffer)
Role: Co-Investigator

6/01/2019-5/31/2021
NJ Governor's Council (subcontract)
Building a streamlined birth cohort to study autism risk factors and biomarkers (PIs: Librizzi, Newschaffer)
Role: Co-Investigator

7/1/2015-6/29/2021
NIH R01ES025574 (subcontract)
Folic acid prevention pathways for ASD in high-risk families (PI: Schmidt)
Role: Co-Investigator/site PI

Pending support:

7/1/2021-6/30/2026
NIH PA-18-401
A multi-cohort prospective investigation of prenatal pesticide mixtures and diet in association with autism-related outcomes.
Role: PI

7/1/2021-6/30/2026
NIH PA-18-401
Examining dietary modifiers of associations between air pollution and autism-related outcomes in two cohorts.
Role: Co-PI

## Other Support

**SNYDER, NATHANIEL**

ACTIVE

R01 GM132261 (Snyder) 07/01/2019-06/30/2024 1.8 calendar months (15%)
NIH (Yr 1 Direct Costs)
Quantification of compartmentalized metabolism in eukaryotic systems
First, we will quantify the kinetics and fate of major carbon substrates for compartmentalized acyl-CoAs in cells and heart tissue. Second, we will quantify the effect of perturbing compartment specific processes with histone deacetylase inhibitors, electron transport chain inhibitors, and genetic models on acyl-CoAs, downstream metabolites, and compartment specific PTMs.

R01 DK116005 (Wellen) 12/06/2018-11/30/2022 0.3 calendar months (2.5% FTE)
NIH (Subcontract Direct Costs)
Acetyl-CoA metabolism and nutrient sensing in adipocytes
We propose to test the hypothesis that glucose-dependent acetyl-CoA production by ACLY enables nutrient-dependent gene regulation in adipocytes, serving as a key control mechanism for carbohydrate handling and insulin response, as well as for thermogenesis. Dr. Snyder will be responsible for acyl-CoA profiling related to the aims of Dr. Wellen's submission.

R01 CA228339 (Wellen) 07/01/2018-06/30/2023 0.6 calendar months (5% FTE) NIH (Subcontract)
Defining an acetyl-CoA-sensing mechanism as a form of intra-organelle communication in cancer
The goals of this proposal are to quantify the dynamics of mitochondrial, cytoplasmic and nuclear acetyl-CoA in cancer. Dr. Snyder will be responsible for acyl-CoA profiling related to the aims of Dr. Wellen's submission.

R01 ES029336 (Adibi) 03/01/2018-02/28/2023 0.3 calendar months (2.5% FTE)
NIH (Subcontract)
The role of the placenta in mediating the exposure and the hormonal effects of phthalates on fetal development
In completing the aims of the project, we will generate novel urinary biomarkers that can be applied prospectively and retrospectively to birth cohort studies to increase precision in estimating associations of prenatal exposures and postnatal outcomes, adjusting for the role of the placenta. We will develop a human-specific in vitro model to test biologic placental mediation of fetal endocrine disrupting effects, and use statistical mediation analysis to evaluate these relationships in pregnancy. With this, we will develop the potential to identify high-risk pregnancies earlier and within a timeframe to reduce long-term risks to the reproductive health of the child. Dr. Snyder will be responsible for phthalate and steroid profiling related to the aims of Dr. Adibi's submission.

R01 DK094004 (Guertin) 04/01/2019-03/31/2024 0.3 calendar months (2.5%)
NIH (Subcontract)
Mechanistic Target of Rapamycin Pathways in Metabolism and Energy Expenditure
Aim 1, investigate the mechanistic similarities and potential connection between mTORC2 loss and high fat diet on adipocyte metabolism in Aim 2, and investigate a novel transcriptional circuit under direct mTORC2 control that regulates adipocyte lipid metabolism in Aim 3. Elucidating how nutrient-sensing signaling pathways like the mTOR pathway link nutritional signals to metabolic regulation in adipocytes has important implications for advancing therapies to treat obesity, type 2 diabetes, and related metabolic diseases. Dr. Snyder will be responsible for acyl-CoA profiling related to the aims of Dr. Guertin's submission.

OVERLAP

There is NO OVERLAP on existing active projects. If any future proposed projects are funded containing overlap, Dr. Snyder will work directly with program officers to reduce his effort as needed.

TOTAL COSTS (Anticipated Direct + Anticipated Indirect for Yr1)

PENDING

R37 (Aird) TBD
Investigating p16 Loss in Pro-tumorigenic Metabolism
This study will investigate the loss of the p16 tumor suppressor on cancer metabolism.

COMPLETED

NARSAD (Snyder) 01/15/2018-01/14/2020 0.6 calendar (5% FTE) Brain & Behavior Research Foundation (Y1 total cost)
Steroid metabolism in a high-risk autism spectrum disorder prospective pregnancy cohort
This study will quantify and characterize the molecular lipid content of meconium from a high-risk autism spectrum disorder (ASD) pregnancy cohort to identify biomarkers of risk for ASD.

K22ES026235 (Snyder) 02/01/2016-01/31/2019 8.5 calendar (71% FTE) NIH
Prenatal biomarkers of exposure and individual susceptibility to endocrine disrupting compounds
The goal of this proposal is to support a new investigator in overcoming major challenges in quantification of exposure and metabolism in the prenatal environment.

W81XWH-16-1-0753 (Lyall) 07/01/16-06/30/19 1.2 calendar (10% FTE) DOD
Prenatal polyunsaturated fatty acid levels and risk of autism spectrum disorder
The goal of this project is to determine whether levels of PUFAs measured from maternal blood samples collected during pregnancy, and in a subgroup group, from newborn blood spots, differ between children with ASD and those without ASD.

R03HD092630 (Snyder) 09/01/2017-08/31/2019 0.3 calendar (2.5% FTE) NIH

Metabolism of propionic acid
This research will elucidate and quantify this new branch of metabolism in the following three aims; Specific Aim 1. Characterize and quantify metabolites of propionate through 2M2PE. Specific Aim 2. Identify cellular localization and enzymology of metabolism through 2M2PE. Specific Aim 3. Establish and validate a quantitative method for novel propionic acid metabolites in urine, saliva, and serum. These aims are limited in scope and focused on early-stage research meant to address gaps in knowledge necessary before studies in animal model systems and human subjects.

R21HD087866 (Snyder) 04/01/2016-03/31/2018 1.3 calendar NIH
Lipidomics of meconium in neurodevelopment
This research will use cutting edge analytical technology to quantify the lipid composition of meconium in relation to development of autism spectrum disorder. Since meconium begins accumulation around the 12th week of gestation and is passed as the first bowel movements of a newborn, meconium will provide a window into the early origins of autism. This will facilitate understanding of the causes of autism and other developmental disorders of prenatal origin.

R03CA211820 (Snyder) 09/15/2016-08/31/2017 0.45 calendar NIH
The influence of prenatal maternal exposures on fetal sterol metabolics
This proposal will quantify differences in sterol metabolites from meconium in children from a prospective enriched risk cohort of early events in autism spectrum disorder etiology by levels of maternal exposure to 1) PBDEs, 2) major phenols, 3) PBDE/phenol mixtures.

## OTHER SUPPORT

**ROBINSON, LUCY F.**

**ACTIVE:**

1R01DK124388-01 (Harhay) 08/01/2020-05/31/2025 0.6 CM
NIH-NIDDKD
Identifying Healthy and High-Risk Weight Loss Phenotypes to Optimize Obesity Management in End Stage Kidney Disease
The main goal of this project is to define healthy and high-risk weight loss among people with obesity on dialysis.

1R01ES030717-01 (Clougherty and Sheffield) 08/1/2019-04/30/2024 0.6 CM
NIH
Pediatric Health and Extreme Weather - Health Effects of Ambient Temperature (PHEW - HEAT)
The main goal of this project is to study the effect of high temperature on children's health.

1R01DC017181-01A1 (Vivanti) 04/15/2019 – 03/31/2022 1.2 CM
NIH
Prevalence and profile of treatment non-responders in Autism Early Intervention
Estimate prevalence of children responding sub-optimally to ASD early intervention and create a detailed profile of non-responders.

R01 (Newschaffer) 09/15/2017 – 08/31/2021 0.9 CM
NIH
Prenatal Exposure to Endocrine Disrupting Chemical Mixtures and ASD Risk
The major goal of this project is to study the association between prenatal maternal serum levels of endocrine disrupting chemicals and ASD.

W81XWH-16-1-0753 (Lyall) 09/15/2017 – 08/31/2021 0.6 CM Department of Defense
Prenatal polyunsaturated fatty acid levels and risk of autism spectrum disorder
The major goal of this project is to study the association between prenatal maternal serum levels of polyunsaturated fatty acids and ASD.

R21OH011740-01 (Huynh) 07/01/2020 – 06/30/2022 1.2 CM
NIOSH
A feasibility study to develop a multilevel occupational health intervention program for nail salon employees and owners
The major goal of this project is to develop a multilevel occupational health intervention program for nail salon employees and owners.

**INACTIVE:**

K23DK105207 (PI: Harhay MN) 01/01/2020-04/30/2020 0.8 CM
NIH/NIDDK
Patterns and Implications of Functional Decline Among Kidney Transplant Candidates
The objectives of this study are to determine how metrics of physical and cognitive function explain variation in access to the kidney transplant waiting list and other adverse outcomes among transplant candidates with kidney disease.

PA CURE FY18 (Harhay) 06/1/2018-01/31/2020 0.6 CM
Pennsylvania Department of Health
Implications of Unstable Cerebral Oxygenation on the Safety and Tolerability of Hemodialysis
The major goal of this project is to evaluate a novel neuroimaging technique (fNIRS) to study cerebral deoxygenation during dialysis and its association with recovery time and performance on cognitive tests.

15-2 (Clougherty) 06/30/2018-09/30/2019 0.6 CM Health
Effects Institute (HEI)
Susceptibility to Multiple Air Pollutants in Cardiovascular Disease
The major goal of this project is to study the socio economic and psychosocial factors which influence susceptibility to negative health effects of air pollutants.

R41CA221595-DRXL (Shih) 06/1/2018 -09/30/2019 .36 CM
NIH
Molecular, Cancer-Specific, Intraoperative Imaging of Breast Surgical Margin,
The major goal of this project is to evaluate the sensitivity and specificity of a new intraoperative imaging technique for testing the margins of a surgical breast lumpectomy.

**OVERLAP**:
None

American Journal of Epidemiology

Vol. 00, No. 00
DOI: 10.1093/aje/kwaa171
Advance Access publication:

## Original Contribution

# Association Between Midpregnancy Polyunsaturated Fatty Acid Levels and Offspring Autism Spectrum Disorder in a California Population-Based Case-Control Study

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Polyunsaturated fatty acids (PUFAs) are critical for brain development and have been linked with neurodevelopmental outcomes. We conducted a population-based case-control study in California to examine the association between PUFAs measured in midpregnancy serum samples and autism spectrum disorder (ASD) in offspring. ASD cases ($n = 499$) were identified through the California Department of Developmental Services and matched to live-birth population controls ($n = 502$) on birth month, year (2010 or 2011), and sex. Logistic regression models were used to examine crude and adjusted associations. In secondary analyses, we examined ASD with and without co-occurring intellectual disability (ID; $n = 67$ and $n = 432$, respectively) and effect modification by sex and ethnicity. No clear patterns emerged, though there was a modest inverse association with the top quartile of linoleic acid level (highest quartile vs. lowest: adjusted odds ratio = 0.74, 95% confidence interval: 0.49, 1.11; *P* for trend = 0.10). Lower levels of total and ω-3 PUFAs were associated with ASD with ID (lowest decile of total PUFAs vs. deciles 4–7: adjusted odds ratio = 2.78, 95% confidence interval: 1.13, 6.82) but not ASD without ID. We did not observe evidence of effect modification by the factors examined. These findings do not suggest a strong association between midpregnancy PUFA levels and ASD. In further work, researchers should consider associations with ASD with ID and in other time windows.

autism; autism spectrum disorder; intellectual disability; maternal diet; polyunsaturated fatty acids

Abbreviations: AOR, adjusted odds ratio; ASD, autism spectrum disorder; CI, confidence interval; DDS, Department of Developmental Services; DHA, docosahexaenoic acid; ID, intellectual disability; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition defined by deficits in social communication and the presence of restricted, repetitive behaviors (1). Evidence supports the hypothesis of prenatal origins for ASD (2) and the possibility that both environmental and genetic factors play a role in its etiology (3, 4). Emerging research suggests that certain maternal dietary factors may be inversely associated with ASD. Most of this work has focused on the role of prenatal vitamin supplements, folic acid, and vitamin D. Yet, polyunsaturated fatty acids (PUFAs) also play a critical role in fetal brain development (5). PUFAs are required for neurodevelopmental processes beginning early in gestation, including neurogenesis, differentiation, connectivity, and synaptogenesis (6). There is evidence of disruption of these processes in ASD (7–9). PUFAs also play roles in signal transduction, gene expression and methylation, and placental function and as components of cell membranes (10–13), and they have effects on inflammatory markers and immune responses (14, 15), suggesting a number of potential pathways relevant to ASD (16–21).

PUFAs are fatty acids with multiple double bonds in the hydrocarbon chain; depending on the positioning of the double bond from the methyl end of the molecule, they are classified as either ω-3 or ω-6 fatty acids. Key dietary sources of PUFAs are fish (for ω-3 PUFAs) and nuts, seeds,

and oils (particularly for ω-6 PUFAs). Both linoleic acid (LA; an ω-6 PUFA) and α-linolenic acid (an ω-3 PUFA) are known as essential fatty acids because they cannot be synthesized in the body and must be obtained from the diet. These fatty acids are metabolized into long-chain PUFAs, which can then be further metabolized to both pro- and antiinflammatory molecules or incorporated into membrane lipids (22). Docosahexaenoic acid (DHA) is an ω-3 PUFA of particular interest given its high content in the human brain and evidence for its role in neuronal growth and differentiation processes (6). Given the rapid growth of the brain during gestation, the prenatal period represents a key window for a potential impact of PUFAs on child health outcomes. Furthermore, because the supply of PUFAs to the developing fetus is dependent on maternal diet (23–25), there is the potential for modification if associations are observed.

Although there have been studies reporting differences in circulating PUFA levels in children already diagnosed with ASD (26–29), as well as suggested improvements in certain symptoms in children with ASD following ω-3 PUFA supplementation, these findings do not necessarily address the role of PUFAs in ASD etiology. Existing research considering PUFA levels, or intake, during critical prenatal windows of neurodevelopment in association with ASD has yielded conflicting findings. In the first published study, Lyall et al. (30) found a general pattern of decreased risk of ASD among children of mothers with higher total PUFA and total ω-6 PUFA levels according to reported diet during or surrounding pregnancy, and an increased risk of ASD among those with the very lowest total ω-3 PUFA levels. Two studies suggested protective associations between maternal fish intake and autism or autism symptom scores (31, 32), though in another, Suren et al. (33) reported no association with fish oil supplementation. In one of the only studies with PUFA levels measured during gestation, Steenweg-de Graaff et al. (34) reported an association between higher prenatal plasma ω-6 PUFA levels and more autism-related traits in the child. In the other study, which included only 57 cases from a cohort with high familial risk of ASD, Cohen et al. (35) did not report an association with third-trimester levels and ASD diagnosis, but they did find an inverse association with ω-3 PUFAs according to reported diet. These limited and conflicting findings stand in contrast to a wider body of literature supporting a positive association between PUFAs and broader neurodevelopmental outcomes (36, 37), suggesting the need to clarify associations with ASD specifically.

Our goal in this study was to determine the relationship between levels of PUFAs, measured in prospectively collected midpregnancy samples, and offspring ASD in a large case-control study with participants drawn from the general population. We also sought to further prior work by examining whether results differed by ASD with and without comorbid intellectual disability (ID), by child sex, and by ethnicity.

## METHODS

### Study population

Study subjects were drawn from women who delivered a live infant in the state of California in 2010 or 2011, participated in routine prenatal screening, and had banked serum samples available and whose infants were not known to have died in the first year of life. Approximately 70% of women in California participate in prenatal screening, with samples from various diverse counties throughout the state being stored as part of the California Biobank Program. (For this study, mothers were residents of Fresno, Madero, Kings, Tulare, Kern, Orange, and San Diego counties at the time of their children's births.) Birth years included here were chosen to allow sufficient time for identification of an ASD diagnosis while optimizing measurement of PUFAs in stored biospecimens. Information on covariates was obtained from California vital statistics data. All study procedures were approved by the institutional review board of Drexel University (Philadelphia, Pennsylvania), as well as the Committee for the Protection of Human Subjects of California.

### Determination of case/control status

Information from the California Department of Developmental Services (DDS), which provides services to individuals with autism, ID, and other developmental disabilities, was used to identify children with ASD and potential general population controls. Cases were defined according to the presence of ASD in DDS records at the time of data linkage (mean child age at linkage = 5.24 years; range, 4.1–6.2 years). Following exclusion of DDS clients for any diagnosis, controls were randomly sampled from prenatal screening-program-linked birth and infant death certificates and frequency-matched to ASD cases on sex, birth month, and birth year to create eligible pools to select for biospecimen retrieval. We aimed to include 500 cases and 500 controls; however, because of issues regarding biospecimen retrievability, 499 cases and 502 controls were included in the final analyses (with 1 control not being matched on sex). DDS case status has been utilized in multiple other epidemiologic investigations of ASD (38–41), with previous work supporting a high validity of ASD diagnosis in DDS data. DDS records have also been shown to capture the majority of cases who remain in the state (an estimated 84%, with milder cases being more likely to be missed (38, 41)). In addition to ASD diagnoses, we obtained information on co-occurring ID, defined according to composite scores less than 70 on standardized cognitive and functional tests in DDS records.

### Specimens and laboratory analysis of PUFAs

Maternal second-trimester serum specimens were retrieved from the California Department of Public Health's California Biobank Program. The archive includes maternal serum collected for routine prenatal screening at 15–19 weeks' gestation. Maternal specimens were collected in serum separator tubes by obstetrical care service providers; leftover specimens following screening were stored at −20°C. Consent forms for the screening program were distributed at the time of the blood collection, which stipulated that specimens and results from prenatal testing could be used for legitimate research purposes given appropriate institutional

**Table 1.** Levels and Detection Rates of Polyunsaturated Fatty Acids Measured in Maternal Midpregnancy Serum Samples in a Population-Based Case-Control Study, by Case/Control Status, California, 2010–2011

| Class and PUFA | % Below LOQ[a] | Cases ($n$ = 499) | | Controls ($n$ = 502) | |
|---|---|---|---|---|---|
| | | Geometric Mean (SD) | IQR | Geometric Mean (SD) | IQR |
| ω-3 PUFAs | | | | | |
| α-Linolenic acid | 12 | 0.99 (1.30) | 0.59–1.97 | 0.95 (1.29) | 0.53–1.94 |
| Stearidonic acid | 0 | 0.16 (0.06) | 0.13–0.20 | 0.16 (0.06) | 0.13–0.19 |
| Eicosapentaenoic acid[b] | 0 | 0.30 (0.52) | 0.18–0.49 | 0.27 (0.36) | 0.16–0.45 |
| Docosapentaenoic acid | 0 | 15.60 (7.34) | 11.70–20.30 | 15.50 (7.97) | 11.20–20.20 |
| Docosahexaenoic acid | 0 | 1.49 (0.56) | 1.20–1.79 | 1.48 (0.61) | 1.20–1.76 |
| Total ω-3 PUFAs[c] | | 19.10 (8.59) | 14.57–24.84 | 18.80 (9.00) | 14.60–24.00 |
| ω-6 PUFAs | | | | | |
| Linoleic acid | 0 | 35.90 (18.50) | 26.80–48.10 | 36.90 (20.70) | 27.00–49.40 |
| γ-Linolenic acid | 0 | 2.24 (1.66) | 1.50–3.56 | 2.15 (1.77) | 1.50–3.33 |
| Eicosadienoic acid[d] | 79 | | | | |
| Dihomo-γ-linolenic acid | 0 | 1.63 (0.88) | 1.20–2.24 | 1.63 (0.94) | 1.19–2.31 |
| Arachidonic acid | 0 | 6.50 (1.61) | 2.26–5.00 | 6.43 (1.57) | 2.15–4.74 |
| Total ω-6 PUFAs | | 47.10 (21.00) | 36.76–60.80 | 48.00 (23.30) | 36.20–62.40 |
| Total PUFAs[c,e] | | 66.7 (27.80) | 52.0–84.30 | 67.40 (30.70) | 51.80–85.30 |

Abbreviations: IQR, interquartile range; LOQ, limit of quantification; PUFA, polyunsaturated fatty acid; SD, standard deviation.
[a] The LOQ is not applicable for totals; numbers shown are the percentage of the study population below the LOQ for that fatty acid.
[b] $P < 0.05$ for comparison between case and control levels, according to Student's $t$ test.
[c] Eicosapentaenoic acid was not included because of the low detection rate.
[d] Not included in further analyses because of the low detection rate.
[e] Sum of all individual PUFAs shown.

review board approval unless participants formally opted out.

Nonesterified PUFAs in maternal serum were measured by analysts who were blinded to sample identity using isotope dilution liquid chromatography–high-resolution mass spectrometry following protein precipitation in a randomized order (extraction, analysis, and quality control data are provided in the Web Appendix and Web Table 1, available at https://academic.oup.com/aje). For values below the lower limit of quantification, that value was reported if possible, to provide a value based on a detected peak (42). Where no laboratory-derived value could be provided, values were imputed using multiple-imputation models including all available covariates, assuming sufficient detection of the PUFA in the study population (>60%, so as to not base analyses on primarily imputed levels). Persons with samples that failed quality control for a given PUFA were excluded from analyses. Information on the PUFAs measured here and their detection rates is shown in Table 1.

### Statistical methods

PUFA levels and covariates were examined in univariate analyses. Total PUFA levels were calculated by summing levels across all individual measured PUFAs (Table 1); likewise, totals for ω-3 and ω-6 PUFAs were created by summing levels of individual PUFAs measured within these classes. We compared basic demographic and covariate information by case/control status and according to quartiles of total PUFA levels (highest and lowest) in bivariate analyses.

Conditional logistic regression models were used to examine crude and adjusted associations between prenatal PUFA levels and ASD. Unconditional logistic regression, adjusting for matching factors, was used in analyses of effect modifiers (described below) in order to maintain sample size. Covariates examined in adjusted models were selected on the basis of a priori knowledge of associations with maternal diet and ASD status. These included: maternal age (years; continuous), maternal race/ethnicity and educational level (both in categories, as shown in Table 2), an indicator for short interpregnancy interval (defined as having a birth within 2 years prior to the current child under study, relative to all others), and prepregnancy body mass index (calculated as weight (kg)/height $(m)^2$; continuous). Additional variables examined but not retained in the final models because they did not substantially (e.g., <10%) change the estimates included maternal smoking during pregnancy (yes/no), metabolic conditions (gestational diabetes, hypertension, or preeclampsia), parity, maternal birthplace outside the United States, and paternal age.

In order to allow for comparison with our earlier work based on dietary intake (30), our primary analysis examined PUFAs in quartiles, using the lowest quartile (quartile 1) as

**Table 2.** Selected Characteristics of Participants in a Study of Midpregnancy Serum Polyunsaturated Fatty Acid Levels and Offspring Autism Spectrum Disorder, by Case/Control Status, California, 2010–2011[a]

| Characteristic | ASD Cases (*n* = 499) | | | Controls (*n* = 502) | | |
|---|---|---|---|---|---|---|
| | Mean (SD) | No. | % | Mean (SD) | No. | % |
| Maternal age, years[b] | 29.4 (5.8) | | | 28.3 (5.8) | | |
| Paternal age, years[b] | 32.6 (6.9) | | | 31.1 (6.7) | | |
| Gestational age, days | 274.5 (13.0) | | | 273.8 (13.0) | | |
| Birth weight, g | 3,382.1 (496.0) | | | 3,381.8 (538.0) | | |
| Prepregnancy body mass index[b,c] | 27.2 (6.9) | | | 25.8 (5.9) | | |
| Parity | 1.9 (1.2) | | | 2.0 (1.1) | | |
| Child's sex | | | | | | |
| Male | | 412 | 83 | | 416 | 83 |
| Female | | 87 | 17 | | 86 | 17 |
| Maternal education | | | | | | |
| Less than high school (no diploma)[b] | | 86 | 17 | | 115 | 23 |
| High school diploma | | 112 | 22 | | 109 | 22 |
| Some college or 2-year degree | | 161 | 32 | | 115 | 23 |
| College degree | | 79 | 16 | | 93 | 19 |
| Graduate degree | | 44 | 9 | | 46 | 9 |
| Missing data | | 17 | 3 | | 24 | 5 |
| Maternal race/ethnicity | | | | | | |
| Non-Hispanic White | | 126 | 25 | | 128 | 26 |
| Asian | | 88 | 18 | | 67 | 13 |
| Black | | 17 | 3 | | 20 | 4 |
| Hispanic | | 251 | 50 | | 265 | 53 |
| Other or missing data[d] | | 17 | 3 | | 22 | 4 |
| Maternal birthplace outside United States | | 228 | 46 | | 223 | 44 |
| Heath insurance status at delivery | | | | | | |
| Private | | 216 | 43 | | 221 | 44 |
| Government program | | 274 | 55 | | 270 | 54 |
| Other | | 9 | 2 | | 11 | 2 |
| Smoking during pregnancy[e] | | 12 | 2 | | 10 | 2 |
| Short interpregnancy interval (<2 years) | | 103 | 21 | | 86 | 17 |
| Any pregnancy complication | | 117 | 23 | | 103 | 21 |
| Metabolic pregnancy complication[b,f] | | 48 | 10 | | 30 | 6 |
| Preterm birth[g] | | 33 | 7 | | 45 | 9 |
| Low birth weight[h] | | 21 | 4 | | 26 | 5 |

Abbreviations: ASD, autism spectrum disorder; SD, standard deviation.

[a] Mean values and SDs are shown for continuous variables, numbers and percentages for categorical variables.

[b] Statistically significant *P* value ($P < 0.05$) from Student's *t* test (continuous variables) or a $\chi^2$ test (categorical variables). For parental age and body mass index, $P = 0.001$; for maternal education, $P = 0.01$; for pregnancy complications, $P = 0.03$.

[c] Weight (kg)/height $(m)^2$.

[d] Fewer than 2% of participants were missing information on race/ethnicity.

[e] Defined as any smoking from 3 months prior to conception through pregnancy.

[f] Defined as gestational diabetes, hypertension, or preeclampsia.

[g] Defined as birth at less than 37 weeks' gestation according to California vital statistics data.

[h] Defined as birth weight less than 2,500 g according to California vital statistics data.

the referent. We also examined distributional extremes of levels according to deciles (relative to middle deciles 4–7) and the highest and lowest fifth percentiles of the distribution (relative to the interquartile range). The distribution of values in controls was used to determine category cutpoints. We also considered potential nonlinear associations with continuous PUFA levels, using cubic spline analyses (43, 44) adjusting for covariates.

In order to explore whether PUFAs may be related to phenotypic subgroups within ASD, we conducted secondary analyses of the associations between PUFAs and ASD with and without comorbid ID as recorded in DDS records ($n = 67$ with ID and $n = 432$ without ID). We also examined potential modification by offspring sex, given the skewed sex ratio in ASD and reports of sex-specific findings for certain ASD risk factors (45–47), and by maternal ethnicity (Hispanic vs. non-Hispanic), given potential differences in dietary patterns. Interaction terms (with continuous total PUFAs as well as ω-3 and ω-6 PUFA levels) and stratified models were used to assess differences according to these factors.

## RESULTS

With the exception of eicosadienoic acid, all PUFAs were sufficiently detected in the maternal serum samples (Table 1). When PUFA levels were compared by case status, geometric mean levels for all PUFAs, except eicosapentaenoic acid, did not differ. Basic characteristics of the study population are shown in Table 2. Case mothers (and fathers) were slightly older than control mothers and had a higher prepregnancy body mass index. Our study population had a high proportion (∼50%) of Hispanic participants, which did not vary by case status. As would be expected given the sex ratio in ASD and our study's matching, approximately 80% of the children were male. Associations between PUFA levels and demographic covariates differed for total PUFAs and total ω-3 PUFAs; higher levels of ω-3 PUFAs, but not total PUFAs, were more common among women with higher education, while women in the highest quartile of total PUFAs, but not total ω-3 PUFAs, were more likely to be Hispanic (Web Table 2).

In multivariate-adjusted models, overall, we did not find evidence for associations between PUFA levels in quartiles and ASD (Table 3). However, the adjusted odds ratio estimate for the top quartile of LA was below the null value (adjusted odds ratio (AOR) = 0.74, 95% confidence interval (CI): 0.49, 1.11), and a nonsignificant trend ($P$ for trend = 0.10) of decreasing odds of ASD was observed across quartiles. Total PUFA, total ω-6, DHA, and dihomo-γ-linolenic acid levels also demonstrated similar point estimates for quartile 3 or 4 (with confidence intervals overlapping the null) but showed no evidence of a monotonic trend. Evidence of stronger associations for these or other fatty acids was generally not found when examining further extremes of the distribution. While persons with linoleic acid levels in the eighth decile had reduced odds of ASD relative to those with levels in the middle of the distribution (deciles 4–7) (AOR = 0.44, 95% CI: 0.26, 0.75), corresponding decreases were not seen for the highest 2 deciles (Web Table 3). Similar results were observed for total PUFAs in decile 8 (AOR = 0.60, 95% CI: 0.37, 0.98), though the adjusted odds ratio for decile 9 was also below the null. In addition, there was no evidence of potentially stronger associations for persons with the very highest and lowest levels (fifth percentiles) relative to the interquartile range (Web Table 4). When examining associations using cubic splines, we did not find evidence for nonlinearity; the general pattern observed for total PUFA levels was a nonsignificant linear decrease in odds of ASD with increasing total PUFA level (Web Figure 1), with similar findings for LA and total ω-3 PUFAs, and flatter curves with wide confidence intervals for most others.

Examining associations between maternal PUFA levels and ASD with and without comorbid ID, we observed mostly null associations with quartiles of PUFA levels (Table 4). There was some suggestion of differences in ω-3 and ω-6 PUFA associations between these groups, with the highest quartiles of the ω-3 PUFAs DHA and eicosapentaenoic acid being below the null for ASD with ID (but not ASD without ID) and the highest quartiles of total PUFAs and total ω-6 PUFAs being below the null for ASD without ID (but not ASD with ID). However, confidence intervals were wide. When examining deciles, the lowest deciles of docosapentaenoic acid, arachidonic acid, total PUFAs, and total ω-3 PUFAs were all associated with increased odds of ASD with ID (for the lowest decile of total PUFAs vs. deciles 4–7, AOR = 2.78 (95% CI: 1.13, 6.82); similar associations were observed for the other PUFAs), though reductions in odds were generally not observed with higher deciles (Web Table 3). In contrast, a higher decile (decile 8) of total PUFAs and total ω-6 PUFAs was associated with reduced odds of ASD without ID (total PUFAs: AOR = 0.55 (95% CI: 0.33, 0.93); total ω-6 PUFAs: AOR = 0.48 (95% CI: 0.28, 0.83)), though deciles 9 and 10 for these groups were closer to the null (Web Table 3, Web Figure 2). Small numbers in individual categories for the ASD-with-ID group precluded us from examining further distributional extremes.

We did not find evidence for statistically significant interactions of total PUFAs with sex and ethnicity ($P = 0.98$ and $P = 0.78$, respectively; similar results were seen for interactions with ω-3 or ω-6 PUFAs). While a few statistically significant estimates were observed in analyses of PUFA quartiles stratified by these factors, including reduced odds of ASD in Hispanics with higher total ω-6 PUFA levels (quartile 3 vs. quartile 1: AOR = 0.54, 95% CI: 0.30, 0.98) (Web Tables 5 and 6), no clear trends across PUFAs or patterns of association with ethnicity or sex emerged.

## DISCUSSION

In this population-based case-control study drawn from pregnant women in California, overall, we did not find strong evidence for an association between PUFA levels measured in midpregnancy and offspring ASD. We examined PUFAs according to several parameterizations and also explored potential effect modification by sex and ethnicity. Secondary analyses did suggest potentially stronger associations between LA and ASD without ID, as well as between some ω-3 PUFAs and ASD with ID, but these findings were

**Table 3.** Associations Between Maternal Midpregnancy Serum Polyunsaturated Fatty Acid Levels and Offspring Autism Spectrum Disorder in a Population-Based Case-Control Study, California, 2010–2011

| PUFA | Median Value, ng/mL | No. of Cases (*n* = 499) | No. of Controls (*n* = 502) | OR[a] | 95% CI | AOR[b] | 95% CI | *P* for Trend[c] |
|---|---|---|---|---|---|---|---|---|
| ALA (18:3)[d] | | | | | | | | 0.74 |
| Q1 (lowest) | 0.30 | 109 | 128 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 0.79 | 132 | 119 | 1.29 | 0.91, 1.84 | 1.26 | 0.87, 1.82 | |
| Q3 | 1.48 | 125 | 125 | 1.18 | 0.82, 1.69 | 1.10 | 0.76, 1.60 | |
| Q4 (highest) | 2.73 | 124 | 122 | 1.21 | 0.83, 1.75 | 1.15 | 0.78, 1.70 | |
| SA (18:4) | | | | | | | | 0.96 |
| Q1 | 0.11 | 90 | 101 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 0.14 | 138 | 135 | 1.16 | 0.79, 1.69 | 1.08 | 0.73, 1.60 | |
| Q3 | 0.17 | 122 | 115 | 1.21 | 0.81, 1.83 | 1.13 | 0.74, 1.73 | |
| Q4 | 0.22 | 149 | 151 | 1.13 | 0.76, 1.69 | 1.03 | 0.70, 1.56 | |
| EPA (20:5) | | | | | | | | 0.86 |
| Q1 | 0.12 | 107 | 133 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 0.22 | 136 | 117 | 1.45 | 1.02, 2.08 | 1.28 | 0.88, 1.87 | |
| Q3 | 0.34 | 116 | 130 | 1.12 | 0.78, 1.60 | 0.88 | 0.58, 1.31 | |
| Q4 | 0.71 | 140 | 122 | 1.45 | 1.00, 2.09 | 1.16 | 0.76, 1.77 | |
| DPA (22:5) | | | | | | | | 0.73 |
| Q1 | 9.64 | 125 | 125 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 13.50 | 120 | 131 | 0.91 | 0.64, 1.30 | 0.81 | 0.56, 1.17 | |
| Q3 | 17.70 | 127 | 123 | 1.03 | 0.72, 1.48 | 0.88 | 0.60, 1.28 | |
| Q4 | 24.50 | 127 | 123 | 1.04 | 0.72, 1.50 | 0.88 | 0.60, 1.30 | |
| DHA (22:6) | | | | | | | | |
| Q1 | 1.05 | 118 | 120 | 1.00 | Referent | 1.00 | Referent | 0.76 |
| Q2 | 1.31 | 132 | 126 | 1.06 | 0.75, 1.51 | 0.96 | 0.67, 1.38 | |
| Q3 | 1.58 | 118 | 136 | 0.88 | 0.61, 1.26 | 0.79 | 0.54, 1.15 | |
| Q4 | 2.11 | 131 | 120 | 1.10 | 0.77, 1.59 | 0.95 | 0.63, 1.43 | |
| LA (18:2) | | | | | | | | 0.10 |
| Q1 | 21.90 | 125 | 124 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 31.70 | 132 | 120 | 1.08 | 0.76, 1.55 | 0.95 | 0.65, 1.38 | |
| Q3 | 42.00 | 123 | 126 | 0.96 | 0.67, 1.38 | 0.83 | 0.56, 1.22 | |
| Q4 | 58.20 | 119 | 132 | 0.89 | 0.61, 1.28 | 0.74 | 0.49, 1.11 | |
| GLA (18:3)[d] | | | | | | | | 0.75 |
| Q1 | 1.10 | 121 | 122 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 1.91 | 118 | 137 | 0.87 | 0.61, 1.24 | 0.82 | 0.57, 1.19 | |
| Q3 | 2.80 | 126 | 123 | 1.02 | 0.71, 1.46 | 0.98 | 0.67, 1.43 | |
| Q4 | 4.45 | 134 | 117 | 1.14 | 0.80, 1.64 | 0.98 | 0.67, 1.44 | |
| DGLA (20:3) | | | | | | | | 0.16 |
| Q1 | 0.93 | 121 | 125 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 1.42 | 131 | 123 | 1.09 | 0.77, 1.55 | 1.01 | 0.70, 1.45 | |
| Q3 | 1.92 | 126 | 123 | 1.05 | 0.73, 1.51 | 0.88 | 0.60, 1.29 | |
| Q4 | 2.76 | 121 | 131 | 0.94 | 0.65, 1.36 | 0.77 | 0.52, 1.16 | |

**Table continues**

**Table 3.** Continued

| PUFA | Median Value, ng/mL | No. of Cases (*n* = 499) | No. of Controls (*n* = 502) | OR[a] | 95% CI | AOR[b] | 95% CI | *P* for Trend[c] |
|---|---|---|---|---|---|---|---|---|
| AA (20:4) | | | | | | | | 0.90 |
| Q1 | 4.94 | 118 | 131 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 5.92 | 123 | 127 | 1.09 | 0.76, 1.56 | 0.96 | 0.66, 1.40 | |
| Q3 | 6.94 | 127 | 123 | 1.17 | 0.81, 1.71 | 0.97 | 0.65, 1.44 | |
| Q4 | 8.44 | 131 | 121 | 1.24 | 0.85, 1.80 | 1.02 | 0.68, 1.51 | |
| Total PUFAs[e] | | | | | | | | 0.16 |
| Q1 | 44.10 | 123 | 125 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 60.00 | 140 | 126 | 1.12 | 0.79, 1.60 | 1.07 | 0.74, 1.55 | |
| Q3 | 75.40 | 116 | 125 | 0.94 | 0.65, 1.36 | 0.77 | 0.52, 1.15 | |
| Q4 | 101.00 | 120 | 126 | 0.96 | 0.66, 1.40 | 0.81 | 0.54, 1.21 | |
| Total ω-3 PUFAs[f] | | | | | | | | 0.87 |
| Q1 | 12.30 | 124 | 135 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 16.70 | 131 | 124 | 0.85 | 0.59, 1.23 | 0.76 | 0.52, 1.11 | |
| Q3 | 21.20 | 119 | 122 | 0.98 | 0.68, 1.41 | 0.83 | 0.57, 1.21 | |
| Q4 | 29.00 | 125 | 121 | 1.06 | 0.73, 1.53 | 0.90 | 0.61, 1.33 | |
| Total ω-6 PUFAs[g] | | | | | | | | 0.18 |
| Q1 | 30.30 | 124 | 127 | 1.00 | Referent | 1.00 | Referent | |
| Q2 | 42.20 | 142 | 125 | 1.25 | 0.88, 1.79 | 1.11 | 0.77, 1.62 | |
| Q3 | 55.00 | 118 | 125 | 0.91 | 0.63, 1.32 | 0.78 | 0.52, 1.17 | |
| Q4 | 73.50 | 115 | 125 | 0.97 | 0.67, 1.40 | 0.80 | 0.53, 1.20 | |

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; AOR, adjusted odds ratio; CI, confidence interval; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; OR, odds ratio; PUFA, polyunsaturated fatty acid; Q, quartile; SA, stearidonic acid.

[a] Crude OR from a conditional logistic regression model accounting for study matching factors only (sex, birth month, and year of birth).

[b] Adjusted OR from a conditional logistic regression model including maternal education, maternal race/ethnicity, prepregnancy body mass index, maternal age, health insurance status at delivery, and an indicator for short interpregnancy interval.

[c] *P* value from a Wald test for trend across the ordinal score variable for the PUFA, defined using the median value within each quartile.

[d] Numbers in ALA and GLA quartiles do not sum to the total sample sizes because of the exclusion of 17 samples that failed quality control for ALA and 3 that failed for GLA.

[e] Sum of all individual PUFAs shown.

[f] Sum of ALA, SA, EPA, DPA, and DHA.

[g] Sum of LA, GLA, DGLA, and AA.

based on very small numbers in the ASD-with-ID group. Taken together, and considering multiple comparisons, the results of this study do not offer much evidence implicating these PUFAs in ASD etiology, though directions for future research are noted below.

A primary goal of this work was to determine whether associations we previously observed (30) on the basis of reported maternal diet in Nurses' Health Study II could be replicated when using measured PUFA levels from midpregnancy. Despite a larger number of cases in our present analysis, we did not replicate the previous statistically significant findings (30) suggesting inverse associations between the highest quartiles of total PUFA level and LA and ASD overall. Potential reasons for differences could be measurement error in estimated PUFA levels according to reported diet or differences in the time periods covered (with the earlier work spanning a broader period overlapping with pregnancy and analyses here timed more specifically to the second trimester). However, it is of some note that the point estimates for the top quartile of LA were quite similar to those of the previous work based on reported diet (30). Taken together, these studies might suggest a potential benefit of higher levels of prenatal LA in child ASD risk. A potential beneficial role of LA in reducing risk of ASD is biologically plausible, perhaps through an immune-mediated pathway; studies in humans have consistently demonstrated reductions in cholesterol levels and in the risk of cardiovascular disease with higher levels of LA and total ω-6 PUFAs (48). However, these findings conflict with those from a Dutch study that found increases in ASD-related traits in association with higher midpregnancy plasma total ω-6 PUFA and LA levels (34) and with those from a high-familial-risk cohort, the Markers of Autism Risk in Babies (MARBLES) cohort (35). In the latter study, Cohen et al.

**Table 4.** Associations Between Maternal Midpregnancy Serum Polyunsaturated Fatty Acid Levels (Quartiles) and Offspring Autism Spectrum Disorder With and Without Co-Occurring Intellectual Disability in a Population-Based Case-Control Study, California, 2010–2011[a]

| PUFA | ASD With ID (*n* = 67) | | | | ASD Without ID (*n* = 432) | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of Cases | AOR[b] | 95% CI | *P* for Trend | No. of Cases | AOR[b] | 95% CI | *P* for Trend |
| ALA (18:3)[c] | | | | 0.76 | | | | 0.71 |
| Q1 (lowest) | 13 | 1.00 | Referent | | 96 | 1.00 | Referent | |
| Q2 | 20 | 1.43 | 0.65, 3.16 | | 112 | 1.21 | 0.83, 1.77 | |
| Q3 | 18 | 1.33 | 0.68, 3.01 | | 107 | 1.06 | 0.72, 1.56 | |
| Q4 (highest) | 14 | 1.02 | 0.42, 2.48 | | 110 | 1.15 | 0.77, 1.71 | |
| SA (18:4) | | | | 0.56 | | | | 0.93 |
| Q1 | 16 | 1.00 | Referent | | 74 | 1.00 | Referent | |
| Q2 | 13 | 0.64 | 0.28, 1.49 | | 125 | 1.20 | 0.80, 1.80 | |
| Q3 | 22 | 1.04 | 0.46, 2.40 | | 100 | 1.14 | 0.73, 1.78 | |
| Q4 | 16 | 0.69 | 0.29, 1.66 | | 133 | 1.10 | 0.71, 1.70 | |
| EPA (20:5) | | | | 0.52 | | | | 0.39 |
| Q1 | 17 | 1.00 | Referent | | 90 | 1.00 | | |
| Q2 | 17 | 1.04 | 0.57, 2.26 | | 119 | 1.34 | 0.91, 1.99 | |
| Q3 | 18 | 0.85 | 0.36, 1.70 | | 98 | 0.92 | 0.60, 1.40 | |
| Q4 | 15 | 0.78 | 0.32, 1.91 | | 125 | 1.30 | 0.83, 2.03 | |
| DPA (22:5) | | | | 0.24 | | | | 0.56 |
| Q1 | 18 | 1.00 | Referent | | 107 | 1.00 | Referent | |
| Q2 | 15 | 0.83 | 0.37, 1.88 | | 105 | 0.80 | 0.54, 1.17 | |
| Q3 | 12 | 0.67 | 0.28, 1.62 | | 115 | 0.92 | 0.62, 1.36 | |
| Q4 | 22 | 1.48 | 0.67, 3.24 | | 105 | 0.83 | 0.56, 1.25 | |
| DHA (22:6) | | | | 0.54 | | | | 0.96 |
| Q1 | 16 | 1.00 | Referent | | 102 | 1.00 | Referent | |
| Q2 | 21 | 1.18 | 0.54, 2.57 | | 111 | 0.93 | 0.63, 1.37 | |
| Q3 | 14 | 0.62 | 0.26, 1.45 | | 104 | 0.82 | 0.55, 1.22 | |
| Q4 | 16 | 0.88 | 0.37, 2.10 | | 115 | 1.00 | 0.65, 1.54 | |
| LA (18:2) | | | | 0.94 | | | | 0.08 |
| Q1 | 17 | 1.00 | Referent | | 108 | 1.00 | Referent | |
| Q2 | 12 | 0.67 | 0.28, 1.62 | | 120 | 1.00 | 0.68, 1.48 | |
| Q3 | 22 | 1.04 | 0.47, 2.30 | | 101 | 0.78 | 0.52, 1.17 | |
| Q4 | 16 | 0.83 | 0.34, 2.02 | | 103 | 0.74 | 0.48, 1.12 | |
| GLA (18:3) | | | | 0.76 | | | | 0.79 |
| Q1 | 19 | 1.00 | Referent | | 102 | 1.00 | Referent | |
| Q2 | 12 | 0.55 | 0.24, 1.27 | | 106 | 0.90 | 0.61, 1.32 | |
| Q3 | 19 | 1.13 | 0.52, 2.45 | | 107 | 0.98 | 0.77, 1.46 | |
| Q4 | 17 | 0.95 | 0.43, 2.08 | | 117 | 1.01 | 0.68, 1.50 | |
| DGLA (20:3) | | | | 0.82 | | | | 0.13 |
| Q1 | 15 | 1.00 | Referent | | 106 | 1.00 | Referent | |
| Q2 | 19 | 1.34 | 0.62, 2.90 | | 112 | 0.97 | 0.66, 1.42 | |
| Q3 | 17 | 1.01 | 0.44, 2.34 | | 109 | 0.86 | 0.58, 1.29 | |
| Q4 | 16 | 1.01 | 0.42, 2.40 | | 105 | 0.74 | 0.49, 1.13 | |

**Table continues**

**Table 4.** Continued

| PUFA | ASD With ID (*n* = 67) | | | | ASD Without ID (*n* = 432) | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of Cases | AOR[b] | 95% CI | *P* for Trend | No. of Cases | AOR[b] | 95% CI | *P* for Trend |
| AA (20:4) | | | | 0.79 | | | | 0.89 |
| Q1 | 18 | 1.00 | Referent | | 100 | 1.00 | Referent | |
| Q2 | 18 | 0.91 | 0.42, 1.97 | | 105 | 0.94 | 0.63, 1.38 | |
| Q3 | 13 | 0.74 | 0.31, 1.77 | | 114 | 1.01 | 0.67, 1.52 | |
| Q4 | 18 | 1.12 | 0.48, 2.64 | | 113 | 1.00 | 0.66, 1.52 | |
| Total PUFAs[d] | | | | 0.92 | | | | 0.12 |
| Q1 | 17 | 1.00 | Referent | | 106 | 1.00 | Referent | |
| Q2 | 15 | 0.87 | 0.38, 2.01 | | 125 | 1.12 | 0.76, 1.65 | |
| Q3 | 17 | 0.85 | 0.37, 1.96 | | 99 | 0.76 | 0.50, 1.16 | |
| Q4 | 18 | 1.01 | 0.43, 2.38 | | 102 | 0.79 | 0.52, 1.21 | |
| Total ω-3 PUFAs[e] | | | | 0.35 | | | | 0.74 |
| Q1 | 21 | 1.00 | Referent | | 103 | 1.00 | Referent | |
| Q2 | 18 | 0.75 | 0.32, 1.76 | | 113 | 0.76 | 0.51, 1.13 | |
| Q3 | 11 | 0.71 | 0.30, 1.71 | | 108 | 0.85 | 0.57, 1.26 | |
| Q4 | 17 | 1.28 | 0.58, 2.80 | | 108 | 0.87 | 0.58, 1.30 | |
| Total ω-6 PUFAs[f] | | | | 0.90 | | | | 0.07 |
| Q1 | 17 | 1.00 | Referent | | 107 | 1.00 | Referent | |
| Q2 | 15 | 1.05 | 0.45, 2.41 | | 127 | 1.14 | 0.77, 1.67 | |
| Q3 | 17 | 0.89 | 0.38, 2.07 | | 101 | 0.76 | 0.50, 1.16 | |
| Q4 | 18 | 1.10 | 0.45, 2.69 | | 97 | 0.77 | 0.51, 1.18 | |

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; AOR, adjusted odds ratio; ASD, autism spectrum disorder; CI, confidence interval; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; ID, intellectual disability; LA, linoleic acid; PUFA, polyunsaturated fatty acid; Q, quartile; SA, stearidonic acid.

[a] Cases were identified in California Department of Developmental Services records.

[b] AOR from a conditional logistic regression model (as in Table 3) accounting for study matching factors (sex, birth month, and year of birth), maternal education, maternal race/ethnicity, prepregnancy body mass index, maternal age, health insurance status at delivery, and an indicator for short interpregnancy interval.

[c] Numbers for ALA do not sum to the total sample sizes because some samples failed quality control.

[d] Sum of all individual PUFAs shown.

[e] Sum of ALA, SA, EPA, DPA, and DHA.

[f] Sum of LA, GLA, DGLA, and AA.

found no association with ω-6 PUFA and LA levels but suggestive findings for ω-3 PUFAs according to measured levels of eicosapentaenoic acid and total ω-3 PUFAs based on reported diet (35). However, the study's small sample size limited statistical power, and it is not known whether or how associations may differ in a high-familial-risk setting. The Dutch investigation (34) considered only a subset of items from a common ASD trait parent-report measure, the Social Responsiveness Scale, and it may be that associations differ by phenotypic aspects of ASD (as potentially suggested by our results for ASD with and without ID). Differences in fish intake, overall diet, and levels of other environmental exposures potentially interacting with PUFAs across the different study populations may also account for discrepant findings.

A wealth of literature suggests neurocognitive benefits of ω-3 PUFAs; contrary to our hypotheses, our findings were generally not supportive of strong associations with DHA or other ω-3 PUFAs. We did observe somewhat stronger findings for an association with the lowest deciles of total PUFAs and ω-3 PUFAs in association with ASD with ID; however, we did not observe corresponding associations with DHA specifically, nor were patterns clear across all deciles or PUFAs. In contrast, there were several ω-6 PUFAs for which reductions in odds of ASD were suggested with higher (but not the highest) deciles. These results should be interpreted with caution given the small number of persons with ASD with ID in our study population and the potential underrepresentation in the DDS records of ID in the presence of ASD. These findings should be examined in other, larger study populations with more complete information on ID status. Because of the roles of ω-3 PUFAs in neurodevelopmental processes like synaptogenesis, neurogenesis, and differentiation, future mechanistic work might also consider

whether ω-3 mechanisms act more directly on fetal brain development and whether ω-6 mechanisms might be mediated through immune system pathways.

Lack of strong associations with maternal levels here does not preclude a potential role of PUFAs in ASD etiology. Abnormalities in placental structure and function (for which there is some emerging evidence in ASD (49, 50)) may impact placental fatty acid transfer to the developing fetus (51), and we were not able to measure fetal levels. In addition, PUFAs may serve as modifiers of other ASD risk factors; for example, recent work suggested that PUFAs may mitigate the adverse associations of the pesticide dichlorodiphenyltrichloroethane (DDT) with child neurodevelopment as measured by the Bayley Scales (52). It may also be that other time windows of rapid brain growth and PUFA uptake (including the late third trimester or infancy) represent critical windows for a relationship between PUFAs and ASD. If diet changed over the course of this time, such relationships might be missed. Future work with repeated measures should consider the role of PUFAs in these additional time periods, as neither diet nor development is static. Finally, it is possible that PUFAs relate to specific phenotypic aspects of ASD, given associations with other developmental outcomes (8, 22, 53) (and suggestive findings for differences by co-occurring ID here). Each of these areas represents potential directions for future research.

This study had a number of strengths representing advances over prior work, most notably the use of measured PUFA levels in samples collected during a time frame suspected to be biologically relevant. Additional strengths include examination of associations in approximately 1,000 mother-child pairs drawn from the general population with representation of ethnic diversity, use of a reliable source to obtain child diagnostic information, and examination of not just classes of PUFAs or total PUFAs but also individual fatty acids. However, a number of limitations should be noted. As stated, our ASD-with-ID case group was smaller than would be expected given comorbidity estimates (54); milder ID in particular is often not recognized until offspring reach an age older than that of the children included here (55) and therefore may not have been accounted for in DDS records at the time of data linkage. We did not have information on levels of mercury, which co-occurs with PUFAs in some fish and may have opposing effects on neurodevelopment (56). In future work, researchers should consider combined impacts of nutrient and environmental toxicant levels on ASD outcomes. We also did not have the ability to examine dietary sources of PUFAs or adjust for other dietary factors. However, adjustment for short interpregnancy interval (which has been associated with ASD in prior work (57–61)) may serve as a proxy for nutrient depletion. Although prior work supports the stability of PUFAs stored under our conditions (62, 63), we cannot rule out potential degradation of PUFAs, though this would not have been differential by case status because of the matching on time of birth. Low fish intake in the United States may have affected our ability to examine potential benefits with high levels of ω-3 PUFAs like DHA. Finally, we cannot rule out potential chance findings observed in secondary analyses.

Given the known importance of PUFAs in neurodevelopment and their involvement in multiple pathways relevant to ASD (6, 13, 64), this class of nutrients should be further considered for associations with ASD-related outcomes and phenotypes. Even in the face of no or limited direct associations with ASD, future work should also consider the potential modifying effects these fatty acids may exert on the impact of other ASD risk factors that may act through the same pathways. Because ASD is currently estimated to occur in 1 out of every 58 children, identifying modifiable factors that have the potential to ameliorate risks and associated disabilities is critical.

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# Polyunsaturated Fatty Acids in Newborn Bloodspots: Associations With Autism Spectrum Disorder and Correlation With Maternal Serum Levels

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We conducted a population-based case–control study to examine newborn polyunsaturated fatty acid (PUFA) levels in association with autism spectrum disorder (ASD) and assess PUFA correlation across two time points. ASD cases ($n$ = 200) were identified through the Department of Developmental Services and matched to live-birth population controls ($n$ = 200) on birth month, year (2010–2011), and sex. Nonesterified PUFAs were measured by isotope dilution liquid chromatography-high resolution mass spectrometry from archived newborn dried blood spots and maternal mid-pregnancy serum samples. Crude and adjusted conditional logistic regression models were used to examine the association between neonatal PUFA levels, categorized in quartiles and according to distributional extremes, and ASD. Cubic splines were utilized to examine nonlinear relationships between continuous neonatal PUFAs and ASD. The correlation between neonatal and maternal levels was examined using Pearson correlation coefficients. In adjusted analyses of neonatal PUFA levels, no clear trends emerged, though there was an elevated odds ratio of ASD for the third quartile of linoleic acid, relative to the first (adjusted odds ratio = 2.49, 95% confidence interval: 1.31, 4.70). Cubic spline analysis suggested a nonlinear association between linoleic acid and ASD, though this was not robust to sensitivity analyses. While individual PUFAs were significantly correlated with one another within a given time point, aside from docohexaseanoic acid, PUFAs were not correlated across maternal and neonatal samples. Overall, our findings do not support an association between neonatal PUFA levels and ASD. Future work should confirm and expand these findings by examining associations with phenotypic subgroups and considering PUFAs in other time points. ***Autism Res*** *2020, 13: 1601–1613.* © 2020 International Society for Autism Research and Wiley Periodicals LLC

**Lay Summary:** In this study, we examined whether levels of fats known as polyunsaturated fatty acids, measured in newborns, were related to later child diagnosis of autism spectrum disorder (ASD). Overall, we did not find strong evidence for hypothesized reduction in risk of ASD based on newborn levels of these fats. Future studies in larger samples and considering other time points may be useful to explain whether these fats are important in brain development related to ASD.

**Keywords:** autism; linoleic acid; newborn bloodspots; polyunsaturated fatty acids

## Background

Etiologic factors underlying the complex neurodevelopmental condition autism spectrum disorder (ASD) are poorly understood. With an estimated prevalence of 1 in 59 children in the United States [Baio et al., 2018], ASD has a growing public health importance. Evidence supports the prenatal origins of ASD [Courchesne et al., 2019], with both genetic and environmental factors known to play a role [Saunders, Woodland, & Gander, 2019]. Maternal diet is known to impact fetal development; established examples include the associations between folic acid and neural tube defects and nutrient deprivation and schizophrenia [McGrath, Brown, & St Clair, 2010; McNulty et al., 2019]. Emerging evidence has linked certain maternal dietary factors with ASD as well, including reports of protective associations between prenatal vitamin supplements/folic acid, and vitamin D and ASD [B. K. Lee et al., 2019; McNulty et al., 2019; Saunders et al., 2019]. However, other critical nutrients may also influence risk of ASD. Polyunsaturated fatty acids (PUFAs) in particular have evidence for associations with neurodevelopmental outcomes related to ASD (with both suggested improvements in cognitive outcomes with higher PUFA levels and increases in adverse outcomes with lower levels reported) [Agostoni

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et al., 2017; Cohen, Bellinger, Connor, & Shaywitz, 2005; de Jong et al., 2015; J. C. J. Steenweg-de Graaff et al., 2015]. PUFAs are also needed in neurodevelopmental processes, such as neurogenesis and neural migration, which may be impaired in ASD [Julvez et al., 2016; Wegiel et al., 2010], suggesting plausibility for a contribution to ASD etiology.

PUFAs include both omega 3 (n-3) and omega 6 (n-6) fatty acids. These fats serve as components of neuronal membranes and as factors required for synapse formation, neural migration, neurotransmission, and connectivity [Liu, Green, John Mann, Rapoport, & Sublette, 2015; Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010]. PUFAs also play a role in neuronal and immune function, gene expression and methylation, placental function, and as components of cell membranes [Deckelbaum, Worgall, & Seo, 2006; Gottrand, 2008; H. S. Lee et al., 2014; Megan, Peter, & Brendan, 2014]. The fetus obtains PUFAs by placental transport from the maternal supply, which comes from the diet [Hornstra, 2000]. Linoleic acid (LA) and alpha-linolenic (ALA) acid are known as essential fatty acids [Coletta, Bell, & Roman, 2010], as they cannot be synthesized by the body, so amounts of each of these are entirely dependent on maternal diet [Hornstra, 2000]. Longer chain PUFAs can be synthesized in the body, as well as derived from the diet [Coletta et al., 2010].

To date, only a handful of studies have considered potential associations between measured levels of PUFAs during fetal development and ASD. One study found higher mid-pregnancy maternal serum docosahexaenoic acid (DHA) was associated with fewer childhood emotional and behavioral problems [J. C. J. Steenweg-de Graaff et al., 2015]. In a cohort study in Spain, protective associations were found between fish intake during pregnancy, which contain n-3 and n-6 PUFAs, and a reduction in the number of traits on the Childhood Asperger Syndrome Test [Julvez et al., 2016]. Another study conducted in a large U.S. cohort, found reduction in risk of child ASD with higher reported maternal LA intake during and surrounding pregnancy, as well as higher risk of offspring ASD for mothers with the lowest 5% of total n-3 PUFAs [Lyall, Munger, O'Reilly, Santangelo, & Ascherio, 2013]. However, other studies of prenatal PUFAs have reported no overall associations [Huang, Iosif, Hansen, & Schmidt, 2020; Lyall et al., 2020], or effects in the opposite direction [J. Steenweg-de Graaff et al., 2016]. PUFAs are known to preferentially transfer to the fetus [Jørgensen, Nielsen, Michaelsen, Lund, & Lauritzen, 2006], particularly in the third trimester of pregnancy during more rapid fetal brain development and uptake of PUFAs [Dyall, 2015; Haggarty, 2004]. Thus, work focused on PUFAs earlier in pregnancy may have missed effects if diet changed over pregnancy.

One prior study examined third trimester PUFA levels in a high familial risk cohort [Huang et al., 2020], though only 57 ASD cases were included, few fatty acids were examined, and it is not known how results may differ in families drawn from the general population. In addition to biosamples from late pregnancy, neonatal samples are also expected to represent PUFAs during late pregnancy since PUFAs measured in blood represent intake from the past several weeks to month [Rapoport, Chang, & Spector, 2001]. Given near-universal collection of newborn blood spots, examining PUFAs in newborn blood spot samples presents the opportunity for potential future screening capabilities should strong associations be identified.

The goal of this study was therefore to examine the association between ASD and PUFAs measured in newborns, using dried blood spot (DBS) samples. To our knowledge, this is the first study to examine neonatal PUFA levels in association with ASD, and one of only a few that has measured PUFAs in newborn bloodspots [Bell et al., 2011; Hewawasam, Liu, Jeffery, Muhlhausler, & Gibson, 2017; Metherel et al., 2019]. We also sought to examine the correlation between PUFA levels in maternal serum samples collected during mid-pregnancy and those in newborn bloodspots, to address potential variability in timing of measurement. (In separate work, we examine associations with maternal PUFA levels independently).

## Methods

### *Study Population*

Study subjects were mother–child pairs, with children drawn from births in California between the years of 2010–2011. Mothers were selected from those participating in routine prenatal screening, with banked maternal serum samples available, whose infant was not known to have died in the first year of life; for analyses here, we also required availability of the child's neonatal dried bloodspots (DBS). Approximately 70% of women in California participate in prenatal screening, with samples stored as part of the California Biobank Program (CBP) from 7–10 diverse counties throughout the State [Currier et al., 2012]. (For 2010–2011 birth years, mothers were residents of Fresno, Madero, Kings, Tulare, Kern, Orange and San Diego Counties at the time of their children's births). Information on covariates was obtained from Vital Statistics data.

### *Determination of Case/Control Status*

Information from the California Department of Developmental Services (DDS), which provides services to individuals with autism, intellectual disability, and other developmental disabilities, was used to define children with ASD and general population (GP) controls. Cases

were defined according to presence of ASD in DDS records at time of data linkage (mean child age at linkage = 5.24 years). Following exclusion of DDS clients, controls were randomly sampled from prenatal screening program-linked birth certificates, and frequency matched to ASD cases on sex, birth month, and birth year to create eligible pools to select for biospecimen retrieval. DDS case status has been utilized in multiple other epidemiologic investigations of ASD, with previous work supporting high validity of ASD diagnosis in DDS [Hertz-Picciotto et al., 2006; Lord et al., 2000; Lord, Rutter, & Le Couteur, 1994; Windham et al., 2011]. DDS records have also been shown to capture the majority of cases in the state (an estimated 84%, with milder cases being more likely to be missed) [Windham et al., 2011]. Two hundred cases–control pairs were randomly sampled from the larger study including 1002 participants (in which associations with maternal PUFA levels and child ASD were examined [Lyall et al., 2020]).

*Dried Blood Spot Collection*

DBS were retrieved from California's Newborn Screening Program. As part of routine newborn screening, five 14-mm diameter blood spots were collected from nearly all live newborns in California on filter paper (Schleicher & Schuell BioScience, GmbH, Keene, NH) by heel stick after 12 h and usually no later than 6 days of age (median age at collection in our study = 25 h after birth). Blood spots were dried at room temperature and stored at ambient conditions for approximately 1–3 days. Remaining specimens following routine screening were packed and stored at −20°C. Prior to testing, parents were provided with a privacy notification that described the possible research use of these specimens with the option to request that specimens not be used for such purposes [Kharrazi et al., 2012].

*Specimens and Laboratory Analysis of PUFAs in DBS*

Nonesterified PUFAs from DBS were measured by isotope dilution liquid chromatography-high resolution mass spectrometry (LC-HRMS) following extraction from the DBS punch. The 6 mm punches were taken using an Analytical Sales and Services, Inc. Dried Blood Spot Pneumatic Card Punch. Whatman 903 Protein Saver US cards were used for extraction testing, 4/5 sets of the quality controls, and standard curves. Punches were deposited directly into 1.5 ml plastic eppendorf tubes. For both samples and standards, 10 µl of internal standard (ISTD) mix (containing 0.1% (w/v) beta-hydroxytoluene, D4-Alpha-linolenic (ALA), D5-Eicosapentaenoic (EPA), D5-Docosahexaenoic (DHA), D4-Linoleic (LA), D6-Dihomo-gamma linolenic (DGLA), D8-Arachidonic (AA), and D5-Docosapentaenoic (DPA)) was pipetted directly onto the punch and allowed to air-dry for 10 min. Two hundred microliters of water was added and the vials were vortexed for 15 min and then centrifuged at 16000×*g* for 5 min.

Potassium ($K^+$) concentration was then determined as a surrogate for hematocrit as supported in prior work using a modified procedure from Petrick et al. [2017] and Capiau, Stove, Lambert, and Stove [2013]. Potassium ion selective microelectrode (MI-442) and reference electrode (MI-401) were purchased from Microelectrodes, Inc. and paired with an Accumet AB150 meter (Fisher Scientific). The calibration curve consisted of 0.01 N, 0.008 N, 0.006 N, 0.004 N, 0.002 N, and 0.001 N KCl with 0.1 N NaCl as a potentially interfering species. After $K^+$ measurement, 800 µl of MeOH was added to each vial followed by 15 min of vortexing and incubation at −20°C overnight. Following incubation, samples were centrifuged at 17000×*g* for 15 min and the supernatant was removed into a 96-deepwell plate. The supernatant was dried under N2 gas flow for at least 6 h and resuspended in 5% ACN in water. The plate was vortexed for 15 min and 40 µl was transferred from each well to a new 96-well plate for analysis.

Samples were block randomized within each 96-well plate for injection onto an ultimate 3000 UHPLC coupled to a Q Exactive Plus high resolution mass spectrometer (Thermo Scientific, San Jose, CA) with separation on a XBridge C18 2.1x150mm, 3.5 µm pore size column (Waters, Milford, MA) using a reversed phase gradient of water containing 0.2 mM ammonium fluoride to methanol. LC–MS grade Optima solvents were obtained from Fisher and analytical and internal standards from Cayman Chemical. Quantification was performed by interpolation from linear 12-point standard curve extracted from blank DBS cards in the same manner as the samples. Absolute levels were interpolated in Xcalibur software from standard curves constructed of the ratio of the area under the curve for the signal of the analyte/internal standard from the integration of the full scan peak (within a 5 ppm window) with qualifying ions of the major fragments in MS/HRMS mode. For analytes with no available stable isotope labeled analog, the internal standard with the most similar retention time was used. Five sets of quality controls (QCs) were prepared independently across the expected range of PUFA levels and coefficient of variation for all QC levels was below 20% with all but two below 10%. The fifth set of QC samples were blank punches taken from the area adjacent to spotted blood (blank QC punches not containing dried blood) from five randomly selected cards. All such blanks were below 10-times the lowest samples. This was done to account for lack of commercial traceability for all components of original card material.

Limits of quantification were set at the lowest standard curve point for all analytes as the noise level in the standard curve point containing only stable isotope labeled

internal standard was at least 10-times less than that lowest point. Values corresponding to below the lower limit of quantification (LOQ) with a laboratory-provided value based on a detected peak were reported [Succop, Clark, Chen, & Galke, 2004]. Where no laboratory-derived value could be provided, values were imputed using multiple imputation (which included all available data). This imputation was performed only if the majority of the study population (e.g., >60%) had measurements above the limit of quantification (LOQ), in order to ensure analyses were not based primarily on imputed data. As 47.8% of individuals had values falling below the LOQ for eicosadienoic acid, this fatty acid was excluded from further analysis. Twenty-two percent of the study population had LA levels below the LOQ. Levels were above the LOQ in all individuals for all other PUFAs (Table S1). PUFAs were expressed in concentration values (ng); percent totals were not calculated for primary analysis due to high background levels on the DBS filter paper impairing accurate quantification of other major pools of esterified fatty acids (as has been reported elsewhere [Gunash, Henao, & Stark, 2017]).

Maternal PUFAs from mid-pregnancy serum samples were also measured using LC-HRMS following protein precipitation. Further details on these laboratory analyses are provided elsewhere [Lyall et al., 2020].

### *Statistical Methods*

***Neonatal PUFAs.*** We examined PUFA levels and covariates in univariate analyses. Levels were normalized by 1-K+, in order to account for hematocrit as noted above. Total PUFA levels were calculated by summing across all individual measured PUFAs; likewise, variables for total n-3 and total n-6 were created by summing individual PUFAs measured within these classes. ALA, an n-3 PUFA, and GLA, an n-6 PUFA, could not be analytically separated; combined ALA/GLA levels were examined and included in both the n-3 and n-6 totals. Secondary analyses examined these class totals without ALA/GLA. Geometric mean levels and distributions of PUFAs (measured in ng in the DBS) were compared between cases and controls.

Crude and adjusted conditional logistic regression models were used to examine the association between neonatal PUFA levels and ASD. Matching factors (child sex, child month and year of birth) were accounted for in strata of the conditional models. Covariates examined in adjusted models were selected on the basis of a priori knowledge of associations with maternal diet and ASD status. These included: maternal age (continuous in years), maternal race/ethnicity, maternal education level, an indicator for short interpregnancy interval (defined as having a birth within 2 years of the current child under study), and prepregnancy body mass index (BMI; continuous). Additional variables examined, but not retained in final models as they did not substantially change estimates (e.g., <10%), included: age at newborn bloodspot collection, maternal smoking during pregnancy (defined as yes/no), pregnancy complications including gestational diabetes, gestational hypertension, or preeclampsia, parity, maternal birth place outside the United States, and paternal age.

We categorized PUFAs in quartiles, using the lowest quartile (Q1) as the referent, in order to compare to prior work [Lyall et al., 2013]. Tests of trend across quartiles were conducted with the ordinal score test, using the median value in each quartile. In secondary analyses, in order to explore whether associations may differ for those with the highest and lowest levels, and again to enable comparisons to our prior work suggesting associations with these parameterizations [Lyall et al., 2013], we assessed associations with distributional extremes of levels according to the highest and lowest deciles (relative to middle deciles 3–8) and the highest and lowest 5th percentile of the distribution (relative to the interquartile range, IQR). For all analyses categorizing PUFA levels, the distribution in controls was used to determine category cut points. We also considered potential nonlinear associations with continuous PUFA levels, using cubic spline analyses [Durrleman & Simon, 1989] adjusted for covariates. Spline models used the default of three knot points. Tests of nonlinearity used the likelihood ratio test, comparing the model with only the linear term to the model with the linear term and the cubic spline terms. In sensitivity analyses, we examined the impact of excluding individuals with imputed PUFA values (i.e., the 22% with LA levels <LOQ).

***Maternal–neonatal PUFAs.*** Pearson correlation coefficients were calculated among individual neonatal PUFAs, and across maternal (in ng/ml from serum) and neonatal (in ng from DBS) samples for each PUFA. In order to assess potential associations with cumulative PUFA levels across pregnancy, we compared individuals with PUFA levels in the lowest quartile for both maternal and neonatal time points, and those in the highest quartile for both, relative to all others, using conditional logistic regression adjusted as outlined above.

## Results

Basic demographic information of the study population can be found in Table 1. Eighty-two percent of the case and control children were male, as expected given the study's matching on sex and the ~4:1 sex ratio in ASD [Baio et al., 2018]. Maternal and paternal age were slightly higher in cases than in controls, and case mothers also had a higher prepregnancy BMI.

**Table 1. Basic Characteristics of the Study Population by ASD Case Status**

| | ASD cases ($n$ = 200) | Controls ($n$ = 200) | $p$-Value |
|---|---|---|---|
| *Continuous variables (mean, SD)* | | | |
| Maternal age (years) | 29.6 (6.0) | 27.92 (5.7) | 0.005 |
| Paternal age (years) | 33.2 (7.3) | 30.7 (6.5) | 0.001 |
| Gestational age (days) | 274.0 (13.9) | 273.8 (14.1) | 0.90 |
| Birth weight (g) | 3393.3 (487.7) | 3370.1 (492.9) | 0.64 |
| Prepregnancy BMI ($kg/m^2$) | 27.3 (6.8) | 25.5 (5.7) | 0.006 |
| Age at NBS collection (h) | 30.5 (18.9) | 28.5 (13.4) | 0.26 |
| *Categorical variables (n, %)* | | | |
| Offspring sex | | | |
| Male | 164 (82%) | 164 (82%) | |
| Female | 36 (18%) | 36 (18%) | |
| Maternal education | | | |
| <High school (no diploma) | 36 (18%) | 50 (25%) | 0.08 |
| High school diploma | 37 (19%) | 37 (19%) | |
| Some college or 2 year degree | 68 (34%) | 43 (22%) | |
| College degree | 35 (18%) | 40 (20%) | |
| Graduate degree | 19 (10%) | 20 (10%) | |
| Missing | 5 (3%) | 10 (5%) | |
| Maternal race/ethnicity | | | |
| Non-Hispanic White | 46 (23%) | 46 (23%) | 0.89 |
| Asian | 35 (18%) | 29 (15%) | |
| Black | 9 (5%) | 7 (4%) | |
| Hispanic | 102 (51%) | 110 (55%) | |
| Other | 8 (4%) | 8 (4%) | |
| Maternal birthplace outside the United States | 86 (43%) | 93 (47%) | 0.48 |
| Insurance status at delivery | | | |
| Private | 86 (43%) | 89 (45%) | 0.59 |
| Government program | 111 (56%) | 110 (55%) | |
| Other | 3 (2%) | 1 (1%) | |
| Smoking[a] | 5 (3%) | 2 (1%) | 0.25 |
| Short interpregnancy interval[b] | 37 (19%) | 29 (15%) | 0.28 |
| Preterm birth | 16 (8%) | 17 (9%) | 0.86 |
| Low birth weight | 10 (5%) | 7 (4%) | 0.46 |

ASD: autism spectrum disorder; BMI: body mass index; NBS: newborn bloodspot.
[a]Defined as any smoking 3 months prior to conception through pregnancy.
[b]Defined as prior pregnancy within 2 years.

Approximately 50% of our sample had maternal Hispanic ethnicity, and this did not differ between cases and controls. These factors generally did not significantly differ according to high and low quartiles of total PUFA levels, though there was a greater proportion of Hispanic mothers in the lowest quartile of total neonatal PUFAs and of mothers with short IPI in the highest quartile (Table S2).

PUFAs were consistently detected in the neonatal blood spots, with the exception of eicosadienoic acid as noted above. Geometric mean levels of PUFAs did not differ between cases and controls (Table 2), though the geometric mean level of LA was somewhat lower in cases ($p$ = 0.08).

In adjusted analyses, no clear patterns with quartiles of PUFA levels and ASD emerged (Table 3). Those with LA levels in the third quartile had an elevated odds of ASD relative to those with levels in the lowest/first quartile (adjusted odds ratio [AOR] = 2.49, 95% confidence interval [CI] 1.31, 4.70), but there was no evidence of a trend across quartiles ($p$ for trend = 0.82). All other estimates had CIs including the null and no evidence of a trend across quartiles. Associations with class totals (n-3 and n-6 PUFA) did not differ when excluding the combined ALA/GLA levels from the totals (Table S3).

In secondary analyses examining associations with further extremes of the distribution, relative to mid-range values (-Tables S4 and S5), we observed some statistically significant associations, but, as in primary analyses, no clear patterns emerged. In analyses of deciles (Table S3), aside from total n-6 and total PUFAs, similar estimates were observed for highest and lowest values. Exploring further extremes in levels (Table S5), we found significant reductions in odds of ASD for those with the top 5th percentile of total neonatal PUFA levels (AOR = 0.24, 95% CI 0.06, 0.96), as well as class totals of n-6 PUFAs (AOR = 0.24, 95% CI 0.06, 0.93), and n-3 PUFAs (AOR = 0.17, 95% CI 0.03, 0.82), though these estimates were based on only a few individuals.

**Table 2. Distribution of Polyunsaturated Fatty Acids Measured in Newborn Blood Spots in ASD Cases and Controls**

| | | Geometric mean | | Range | | *p*-Value[a] |
|---|---|---|---|---|---|---|
| PUFA | Class | Case | Control | Case | Control | |
| ALA/GLA[b] | n-3/n-6 | 57.19 | 57.73 | 14.22, 385.6 | 8.07, 194.1 | 0.86 |
| SA | n-3 | 0.08 | 0.083 | 0.05, 0.20 | 0.06, 0.209 | 0.46 |
| DPA | n-3 | 6.13 | 5.76 | 0.16, 20.05 | 0.24, 25.22 | 0.31 |
| DHA | n-3 | 15.87 | 15.32 | 5.08, 59.14 | 2.47, 61.14 | 0.51 |
| LA | n-6 | 45.89 | 56.09 | 0.96, 868.5 | 1.92, 517.1 | 0.08 |
| DGLA | n-6 | 106.5 | 106.4 | 40.96, 377.1 | 26.81, 373.2 | 0.98 |
| AA | n-6 | 71.05 | 71.09 | 27.92, 234.9 | 12.42, 170.9 | 0.99 |
| Total n-3[c] | n-3 | 82.83 | 83.87 | 19.84–216.6 | 26.37–395.8 | 0.76 |
| Total n-6[d] | n-6 | 319.1 | 312.6 | 121.9–1069.4 | 139.3–1447.9 | 0.62 |
| Total PUFA[e] | n-3 and n-6 | 338.3 | 343.4 | 155.1, 1458.1 | 129.6, 1091.9 | 0.71 |

AA: arachidonic acid; ALA: alpha linolenic acid; ASD: autism spectrum disorder; DGLA: di-homo-gamma linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoci acid; GLA: gamma linolenic acid; LA: linoleic acid; PUFA: polyunsaturated fatty acid; SA: stearidonic acid.

[a]*p*-Value comparing geometric means between cases and controls.

[b]ALA and GLA could not be separated and were reported together.

[c]Total for n-3 includes SA, DPA, DHA, as well as the combined ALA/GLA.

[d]Total for n-6 includes LA, DGLA, AA, as well as the combined ALA/GLA.

[e]Total PUFA includes all listed PUFAs.

When considering potential nonlinear relationships between PUFA levels and ASD using cubic splines, we observed a significant nonlinear relationship with LA ($p = 0.009$), suggesting increased odds of ASD with lower to mid-range levels of linoleic acid, and decreased odds with higher levels. We also observed a significant nonlinear relationship with total n-3 levels ($p = 0.04$). However, in sensitivity analyses excluding those with imputed LA values (originally <LOQ, $n = 91$) for LA, or when removing ALA/GLA from the n-3 total, neither relationship remained (Table S6 and Fig. S1). In contrast, results of sensitivity analyses excluding those <LOQ from analyses of percentiles of LA levels yielded similar findings to primary analyses, albeit with wider CIs owing to the smaller sample size (for example, LA Q3 vs. Q1 AOR = 2.06, 95% CI 0.92, 4.61).

### *Correlation Between Maternal and Neonatal PUFAs*

Individual PUFAs within maternal or neonatal matrices were moderately or strongly correlated with each other. In particular, high abundance PUFAs (including arachidonic acid and docosapentaenoic acid, which make up one fifth of the brain's dry weight [Bentsen, 2017]), as well as linoleic acid with alpha-linolenic acid/gamma-linolenic acid and total PUFAs, had a strong positive correlations with one another within matrices. However, the correlation across maternal and neonatal samples was low for all PUFAs but DHA (Fig. 1). For analyses considering associations with PUFAs across maternal and neonatal time points (i.e., according to PUFAs levels in the highest quartiles for both maternal and neonatal samples, and in the lowest quartiles for both), no associations were observed (Table S7), though these estimates were based on relatively small numbers.

## Discussion

In this population-based case–control study examining neonatal PUFAs measured in newborn dried blood spots, overall, we did not find strong support for an association with ASD. We observed little correlation across maternal and neonatal time points. While we found decreased odds of ASD with some categories of higher levels of the n-6 PUFA linoleic acid, and some suggestion of nonlinearity, findings were not consistent and clear trends were not observed. Further considerations of our findings are described below.

In our primary analysis examining quartiles of neonatal PUFA levels, we found mostly null associations aside from a significantly elevated odds of ASD in the third quartile of linoleic acid. When exploring potential associations with extremes of the distribution according to high and low levels of PUFAs relative to more mid-range levels, some associations of increased odds with DGLA in low levels and AA in high levels, as well as decreased odds with LA in the lowest levels were found. However, no clear patterns emerged, and observed estimates did not uniformly fit the hypothesis of an increased risk with lower levels of PUFAs and decreased risk with higher levels.

Nonlinear associations were found for linoleic acid, alpha-linolenic acid/gamma-linolenic acid, and total n-3 (when including ALA/GLA) in cubic spline analyses. For the latter two, this may have been due to inclusion of combined alpha-linolenic acid and gamma-linolenic acid;

**Table 3. Associations Between Quartiles of Neonatal PUFA Levels and ASD**

| PUFA[a] | Quartile Median | Case/control n | Crude OR (95%CI) | Adjusted OR (95% CI)[b] | *p* for trend |
|---|---|---|---|---|---|
| ALA/GLA (18:3)[c] | | | | | |
| Q1[d] | 32.44 | 44/50 | 1.0 | 1.0 | 0.7945 |
| Q2 | 47.51 | 50/50 | 1.15 (0.65, 2.01) | 1.13 (0.62, 2.05) | |
| Q3 | 67.50 | 57/50 | 1.32 (0.74, 2.34) | 1.27 (0.69, 2.33) | |
| Q4 | 100.58 | 49/50 | 1.23 (0.64, 1.99) | 0.95 (0.52, 1.74) | |
| SA (18:4) | | | | | |
| Q1 | 0.067 | 40/50 | 1.0 | 1.0 | 0.9232 |
| Q2 | 0.077 | 61/50 | 1.54 (0.87, 2.71) | 1.66 (0.91, 3.03) | |
| Q3 | 0.086 | 50/50 | 1.26 (0.70, 2.25) | 1.42 (0.77, 2.64) | |
| Q4 | 0.099 | 49/50 | 1.24 (0.69, 2.21) | 1.16 (0.62, 2.15) | |
| DPA (22:5) | | | | | |
| Q1 | 3.30 | 58/50 | 1.0 | 1.0 | 0.7161 |
| Q2 | 5.69 | 52/50 | 0.90 (0.53, 1.53) | 1.08 (0.61, 1.92) | |
| Q3 | 7.77 | 41/50 | 0.70 (0.40, 1.23) | 0.81 (0.44, 1.51) | |
| Q4 | 10.60 | 49/50 | 0.84 (0.48, 1.46) | 0.95 (0.53, 1.73) | |
| DHA (22:6) | | | | | |
| Q1 | 8.50 | 58/50 | 1.0 | 1.0 | 0.8836 |
| Q2 | 13.68 | 51/50 | 0.88 (0.52, 1.50) | 0.85 (0.47, 1.52) | |
| Q3 | 18.99 | 35/50 | 0.59 (0.33, 1.06) | 0.53 (0.27, 1.03) | |
| Q4 | 27.46 | 56/50 | 0.97 (0.58, 1.64) | 0.96 (0.48, 1.91) | |
| LA (18:2) | | | | | |
| Q1 | 10.90 | 31/50 | 1.0 | 1.0 | 0.8154 |
| Q2 | 31.88 | 46/50 | 1.52 (0.84, 2.77) | 1.57 (0.82, 3.01) | |
| Q3 | 76.10 | 74/50 | 2.45 (1.37, 4.40) | **2.49 (1.31, 4.70)** | |
| Q4 | 180.98 | 49/50 | 1.62 (0.89, 2.98) | 1.34 (0.70, 2.57) | |
| DGLA (20:3) | | | | | |
| Q1 | 68.70 | 56/50 | 1.0 | 1.0 | 0.2586 |
| Q2 | 90.90 | 32/50 | 0.57 (0.32, 1.04) | 0.62 (0.33, 1.16) | |
| Q3 | 116.20 | 55/50 | 0.97 (0.55, 1.69) | 1.08 (0.60, 1.95) | |
| Q4 | 171.15 | 57/50 | 1.03 (0.59, 1.81) | 1.18 (0.65, 2.13) | |
| AA (20:4) | | | | | |
| Q1 | 49.67 | 59/50 | 1.0 | 1.0 | 0.4019 |
| Q2 | 64.36 | 34/50 | 0.59 (0.33, 1.04) | 0.55 (0.30, 1.02) | |
| Q3 | 76.72 | 43/50 | 0.73 (0.42, 1.28) | 0.65 (0.36, 1.18) | |
| Q4 | 106.71 | 64/50 | 1.09 (0.63, 1.86) | 1.07 (0.60, 1.91) | |
| Total n3 | | | | | |
| Q1 | 54.10 | 52/50 | 1.0 | 1.0 | 0.4632 |
| Q2 | 72.48 | 37/50 | 0.74 (0.41, 1.31) | 0.79 (0.43, 1.46) | |
| Q3 | 93.05 | 68/50 | 1.33 (0.77, 2.32) | 1.38 (0.76, 2.51) | |
| Q4 | 132.09 | 43/50 | 0.85 (0.49, 1.49) | 0.73 (0.40, 1.33) | |
| Total n6 | | | | | |
| Q1 | 191.91 | 41/50 | 1.0 | 1.0 | 0.6804 |
| Q2 | 258.60 | 50/50 | 1.22 (0.69, 2.15) | 1.04 (0.56, 1.91) | |
| Q3 | 352.00 | 55/50 | 1.34 (0.76, 2.37) | 1.18 (0.64, 2.16) | |
| Q4 | 512.76 | 54/50 | 1.31 (0.75, 2.29) | 1.12 (0.62, 2.02) | |
| Total PUFA | | | | | |
| Q1 | 216.41 | 42/50 | 1.0 | 1.0 | 0.6046 |
| Q2 | 280.83 | 46/50 | 1.09 (0.62, 1.93) | 0.92 (0.50, 1.71) | |
| Q3 | 372.89 | 59/50 | 1.41 (0.81, 2.47) | 1.29 (0.71, 2.35) | |
| Q4 | 544.48 | 43/50 | 1.25 (0.72, 2.19) | 1.09 (0.60, 1.97) | |

*Note*. The bold values indicate statistical significance.

AA: arachidonic acid; ALA: alpha linolenic acid; ASD: autism spectrum disorder; DGLA: di-homo-gamma linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoci acid; GLA: gamma linolenic acid; LA: linoleic acid; PUFA: polyunsaturated fatty acid; SA: stearidonic acid.

[a]Fatty acid nomenclature listed in parenthesis after individual PUFAs; numbers indicate the number of carbons: number of double bonds in the fatty acid chain.

[b]Adjusted for: child's sex, month and year of birth, mother's education, age, BMI, race/ethnicity, insurance status at delivery, and indicator for short interpregnancy interval.

[c]ALA and GLA could not be separated and were reported together.

[d]Q1 represents the lowest quartile.

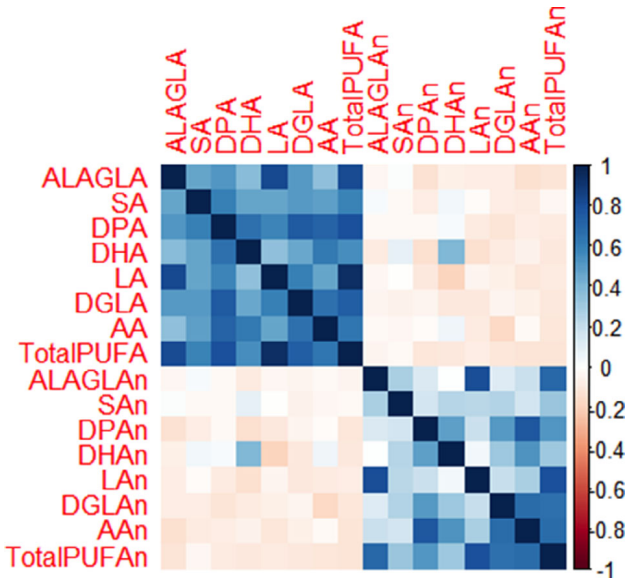


**Figure 1.** Correlation within and across neonatal and maternal PUFA levels. Pearson correlation between individual and total PUFA levels measured in newborn bloodspots (indicated by "n" at the end of the PUFA abbreviation) and maternal mid-pregnancy serum samples. Darker colors indicate stronger correlation, with values in blue indicating positive correlations and those in red negative correlations. AA: arachidonic acid; ALA: alpha linolenic acid; DGLA: di-homo-gamma linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoci acid; GLA: gamma linolenic acid; LA: linoleic acid; PUFA: polyunsaturated fatty acid; SA: stearidonic acid. ALA and GLA could not be separated and were reported together.

no relationship with n-3 PUFA was observed in spline analyses when excluding this combined measure. For linoleic acid, results of the primary analyses including imputed values suggested an increased risk for ASD among those with the lowest neonatal levels and decreasing risk for those with higher levels, though these findings were not robust to sensitivity analyses excluding those <LOQ. A potential decrease in ASD with higher linoleic acid levels may be considered broadly consistent with our previous work investigating maternal fat intake in associated with ASD from the Nurses' Health Study II, which reported a significant reduced risk of ASD for those in the highest quartile and 95th percentile of levels of maternal linoleic acid according to reported diet [Lyall et al., 2013]. However, we did not observe significant associations with linoleic acid in analyses of deciles here, and as noted analyses of quartiles suggested an increased risk with the third quartile but not for others. It may be such categories did not best capture the true nature of the exposure-outcome relationship; alternatively, prior findings based on reported diet may have been influenced by exposure misclassification. We also cannot rule out the potential for chance findings resulting from multiple comparisons in the different parameterizations in our work.

In the few other studies that have measured levels of prenatal PUFAs in association with ASD, findings have not been consistent. In the Generation R cohort, higher mid-pregnancy linoleic acid levels, expressed as a percentage of total fatty acids, were associated with increasing ASD-related traits [J. Steenweg-de Graaff et al., 2016]; in the parent case–control study participants here were drawn from, no overall associations with maternal mid-pregnancy levels were found, though differences by ASD with and without ID were noted [Lyall et al., 2020]. Finally, in recent work conducted within the high familial risk cohort MARBLES [Huang et al., 2020], decreased risk of ASD was observed with higher n-3 intake during the second half of pregnancy according to reported diet, but significant associations were not seen according to measured plasma levels expressed as concentrations (though power may have been limited to detect effects). These conflicting findings, which may be related to dietary differences across countries/regions studied, differences in outcome definitions, sample size, exposure measurement and characterization, or other factors, suggest the need to clarify any potential relationship between linoleic acid and ASD in future work. A protective association with linoleic acid is biologically plausible given associations with inflammation and evidence that linoleic acid may reduce inflammatory markers in humans [Innes & Calder, 2018]. It has also been consistently demonstrated that linoleic acid and n-6 PUFAs have beneficial effects on cholesterol levels and reduce risk of cardiovascular disease [Harris et al., 2009]. Continued investigation as to the potential role of linoleic acid and other PUFAs in ASD is warranted.

Lack of strong associations with neonatal levels here may not rule out a potential role of PUFAs in ASD. As reviewed elsewhere [Agostoni et al., 2017; Gow & Hibbeln, 2014; Martins, Bandarra, & Figueiredo-Braga,-2019], low DHA has been linked with poorer neurodevelopmental outcomes, and has been suggested to be associated with increasing severity of attention deficit hyperactivity disorder. Associations between prenatal/early life PUFAs and outcomes such as IQ scores, language ability, and attention [Julvez et al., 2019; Steer, Lattka, Koletzko, Golding, & Hibbeln, 2013; Strain et al., 2012] suggest the need to consider associations with specific phenotypic aspects of ASD. Further, emerging work has reported interactive effects of PUFAs with pesticides on Parkinson's disease and child neurodevelopment scores [Kamel et al., 2014; Mérida-Ortega et al., 2019], suggesting future work might consider potential interactive effects with other ASD risk factors influencing suspected underlying pathways.

In addition to examining neonatal levels, we examined correlations with maternal mid-pregnancy levels and

assessed potential variability in PUFA levels across pregnancy. As noted, previous analyses within this study overall did not find strong associations between maternal PUFA levels and ASD [Lyall et al., 2020], though there was suggestion of potentially stronger inverse associations for ASD with co-occurring intellectual disability (ID), which we were not able to address due to sample size here. Given prior evidence that PUFAs measured in blood, including blood collected on filter paper, represent intake over the past several weeks to months [Hodson, Skeaff, & Fielding, 2008; Rapoport et al., 2001], and because the supply of PUFAs to the developing fetus is dependent on maternal diet [Hornstra, 2000], PUFAs measured in newborn blood spots are suspected to provide insight into late pregnancy levels versus earlier mid-pregnancy maternal serum samples [Hornstra, 2000]; thus these timepoints overlap with different neurodevelopmental fetal growth stages. Our finding of lack of correlation (aside from DHA) across maternal and neonatal PUFAs suggests the need to carefully time measurements for work examining associations in critical windows of neurodevelopment. It is also possible that cross-matrix comparisons resulted in the low correlation levels; however, existing evidence supports validity as compared to other measurements [Bell et al., 2011]. We did not have the ability to examine how downstream factors, such as breastfeeding and postnatal feeding, affected PUFA levels in the DBS. However, the positive correlation for DHA (the primary PUFA added to formulas and rich in breastmilk) across our two timepoints suggests feeding within the first several days of life was unlikely to have influenced our DBS levels.

To our knowledge, this is the first study to measure PUFAs from newborn dried blood spots in association with ASD. This technique of measuring biomolecules that may influence neurodevelopment from blood spots holds strong potential, given near-universal collection of newborn blood spots [Ostler, Porter, & Buxton, 2014]. Analytical techniques can be applied across a variety of other projects as a method of studying the late pregnancy/neonatal time period. Blood spots are also less invasive than other collection methods and have minimal field storage requirements [Ostler et al., 2014]. Here, consistent with only a handful of prior studies [Hewawasam et al., 2017; Metherel et al., 2019] we demonstrated ability to measure most PUFAs in such samples, stored for a period of up to 5 years.

This study has several strengths, including the noted novel measurement of PUFAs in newborn dried blood spots, consideration of consistency of PUFAs across two matrices/time points, and use of population-based cases and controls with prospectively collected biospecimens in relevant windows of neurodevelopment. However, several limitations should be noted. We had limited power to detect more modest associations, and small numbers to powerfully examine associations with very high or low levels of these PUFAs. Suggestions of potential nonlinear associations with certain PUFAs here were not robust to sensitivity analyses but suggest it may be worthwhile to consider nonlinear patterns and associations with the highest and lowest levels further in larger studies. We were not able to analytically separate alpha-linolenic acid and gamma-linolenic acid, limiting our ability to investigate total n-3 PUFAs and n-6 PUFAs as distinct groups. Nonetheless, evidence from human studies supports beneficial effects of both groups of PUFAs on inflammatory markers and overall health [Harris et al., 2009; Innes & Calder, 2018]. As noted above, we were not able to account for nutritional status and cannot rule out the influence of recent feeding, though our overall null findings suggest any such bias would have been toward the null. Given background evident in the filter paper, as well as potential differential degradation across PUFAs [Powers, Chen, Sternberg, Momin, & Schleicher, 2011], we could not assess total fatty acid content, and therefore did not examine PUFAs as a percent of total fatty acids. There is some debate regarding the use of this percent total approach versus a concentration-based approach (as used here), though work has suggested the latter may be preferable for examining associations with disease outcomes and that associations in the opposite direction may be found if based on the former [Schwertner & Mosser, 1994; Sergeant et al., 2016]. While one study examining maternal serum PUFAs according to both concentrations and percent totals in association with neurodevelopment maintained consistent conclusions with either approach [Valent et al., 2013], future work may consider comparative analyses of these approaches for associations with ASD. Due to our sample size, we also were not able to study phenotypic subgroups of ASD, including most notably ASD with and without intellectual disability.

With a prevalence of 1 in 59 children [Baio et al., 2018], there is a growing public health drive to better understand ASD and what factors influence its development. PUFAs have known importance in neurodevelopment and pathways relevant to ASD [Agostoni et al., 2017; Gottrand, 2008; Liu et al., 2015; Schuchardt et al., 2010]. Continued investigation in well-powered studies is needed to further our understanding of the role of these PUFAs in ASD-related outcomes. Given differences suggested here between maternal and neonatal levels, this future work should also carefully consider timing of measurements in order to accurately capture important developmental time windows. As noted, future directions include continued study of nonlinear patterns, consideration of interactions with other risk factors, and examination of associations with specific phenotypic aspects of ASD.

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## Conflict of Interest

The authors do not have any conflicts of interest to disclose.

## Author Contributions

Ms. Bostwick drafted and edited the manuscript and assisted with data analyses. Mr. Whitman conducted data analyses and edited the manuscript. Dr. Lyall obtained funding for the study, assisted with manuscript drafting, and edited the manuscript. Drs. Pearl and Robinson edited the manuscript. Dr. Snyder edited the manuscript and conducted laboratory assessments of PUFAs. Drs. Newschaffer and Windham edited the manuscript and were involved with study design.

## Ethics Statement

All study procedures were approved by the IRB of Drexel University, as well as of the Committee for Protection of Human Subjects of California.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Data S1 Table S1**. Polyunsaturated fatty acids measured in newborn dried blood spots, their detection rates in the study samples, and Coefficient of Variation (CV) (%) of Quality Controls (QCs)*

**Table S2**. Basic characteristics of the study population by highest (Q4) and lowest (Q1) total neonatal PUFA levels