DR KOZETA MILIKU (Orcid ID : 0000-0002-9614-7191)
DR MALCOLM R SEARS (Orcid ID : 0000-0002-7113-4917)
DR PADMAJA SUBBARAO (Orcid ID : 0000-0003-0394-1933)
DR MEGHAN AZAD (Orcid ID : 0000-0002-5942-4444)

Article type : Original Article

Original Article

Sex-specific associations of human milk long-chain polyunsaturated fatty acids and infant allergic conditions

Kozeta Miliku MD PhD[1,2,3], Jacqueline Richelle MD[1], Allan B. Becker MD [1,2], Elinor Simons MD, PhD[1,2], Theo J. Moraes MD, PhD[4], Stuart E. Turvey MD, PhD [5], Piush J. Mandhane MD, PhD [6], Malcolm R. Sears MD[3], Padmaja Subbarao MD PhD[4], Catherine J. Field[7] PhD RD, and Meghan B. Azad PhD*[1,2]

Running title: Human milk fatty acids and infant allergies

1. Department of Pediatrics and Child Health, University of Manitoba, Canada
2. Manitoba Interdisciplinary Lactation Centre (MILC), Children's Hospital Research Institute of Manitoba, Canada
3. Department of Medicine, McMaster University
4. Department of Pediatrics, University of Toronto
5. Department of Pediatrics, University of British Columbia
6. Department of Pediatrics, University of Alberta
7. Department of Agricultural Food, and Nutritional Science, University of Alberta

*Corresponding author: Meghan Azad; Children's Hospital Research Institute of Manitoba
504C-715 McDermot Avenue, Winnipeg, Manitoba, Canada R3E 3P4.
+1-204-975-7754. meghan.azad@umanitoba.ca

Word count: Abstract: 242 Text: 2,756; Tables: 3; Figures: 4; Supplemental Material: 1

Financial Support

This research was funded by the Manitoba Medical Service Foundation and the Children's Hospital Research Institute of Manitoba. The Canadian Institutes of Health Research (CIHR) and the Allergy, Genes and Environment (AllerGen) Network provided core funding for the CHILD Cohort Study. KM received funding from the Canadian Lung Association. This research was supported in part by the Canada Research Chairs (CRC) Program. MBA holds a Canada Research Chair in the Developmental Origins of Chronic Disease, and is a CIFAR Fellow in the Humans and the Microbiome Program. MRS previously held the AstraZeneca chair in Respiratory Epidemiology at McMaster University. These entities had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication.

Conflicts of Interest

The authors report no conflicts of interest specifically related to the study. MBA receives research funding from the Canadian Institutes of Health Research, Research Manitoba, the Canada Foundation for Innovation, the Bill and Melinda Gates Foundation, the Manitoba Children's Hospital Foundation, Prolacta Biosciences, Mitacs, CIFAR, the Garfield G. Weston Foundation and the CRC Program. She regularly speaks at conferences and workshops on infant nutrition, some sponsored by Medela, the Institute for the Advancement of Breastfeeding & Lactation Education, and Prolacta Biosciences. She has contributed without remuneration to online courses on breast milk and the infant microbiome produced by Microbiome Courses. She serves in a volunteer capacity as Secretary to the International Society for Research on Human Milk and Lactation and as

a member of the National Academy of Sciences, Engineering and Medicine Committee on Scanning New Evidence on the Nutrient Content of Human Milk.

ABSTRACT

Background: Polyunsaturated fatty acids (PUFAs) may influence immune development. We examined the association of PUFAs in human milk with food sensitization and atopic dermatitis among breastfed infants.

Methods: Among a selected subgroup of 1,109 mother-infant dyads from the CHILD Cohort Study, human milk was analyzed by gas-liquid chromatography to quantify PUFAs including arachidonic acid (ARA) and docosahexaenoic acid (DHA). At 1 year of age, food sensitization was determined by skin-prick testing for egg, peanut, cow's milk, and soybean, and atopic dermatitis was diagnosed by pediatricians. Logistic regression analyses controlled for breastfeeding exclusivity, family history of atopy and other potential confounders.

Results: Overall, 184 infants (17%) were sensitized to one or more food allergens and 160 (14%) had atopic dermatitis. Sex-specific associations were observed between these conditions and milk PUFAs. Girls receiving human milk with lower proportions of DHA had lower odds of food sensitization (aOR 0.35; 95%CI 0.12, 0.99 for lowest vs. highest quintile) and a clear dose-dependent association was observed for the ARA/DHA ratio (aOR 2.98; 95%CI 1.10, 8.06 for lowest vs. highest quintile). These associations were not seen in boys. Similar sex-specific tendencies were observed for atopic dermatitis.

Conclusions: Human milk PUFA proportions and their ratios are associated with infant atopic conditions in a sex-specific manner. In female infants, a higher ratio of ARA/DHA may reduce the risk of food sensitization and atopic dermatitis. Further research is needed to determine the underlying mechanisms and clinical relevance of this sex-specific association.

Key Words: atopic dermatitis, breastfeeding, human milk, food sensitization, polyunsaturated fatty acids

Key Message and Clinical Implication:

This is the first study exploring the associations of human milk polyunsaturated fatty acids (PUFAs) and infant allergic conditions in boys and girls separately. The results show that the ratios of n-6 to n-3 human milk PUFAs (in particular, ARA to DHA) are associated with infant atopic outcomes in a dose-dependent manner, and are evident only in girls. These findings could have implications for maternal and infant nutrition guidelines, and allergy prevention strategies.

INTRODUCTION

Food allergy affects up to 7% of children in high-income countries (1, 2). The prevalence of atopic dermatitis is even higher at 10-20%, reflecting a 2-3 fold increase over the past 30 years (3). These conditions often arise during infancy, highlighting the importance of understanding their early origins (4-6).

Breastfeeding is an important early-life exposure, providing optimal infant nutrition (7) along with immunologic benefits and possible protection against allergic conditions (8-10). Polyunsaturated fatty acids (PUFA) are among the breast milk components that may contribute to immune system development (11). Human milk PUFAs vary greatly among mothers (12, 13). Essential long-chain PUFAs, linoleic acid (LA, 18:2n-6) and alpha-linoleic acid (ALA, 18:3n-3) are obtained from the diet (primarily found in vegetable oils), as are the n-3 PUFAs (e.g. docosahexaenoic acid (DHA, 22:6n-3), primarily found in fish). In contrast, n-6 PUFAs (e.g. arachidonic acid (ARA, 20:4n-6)) are mostly derived from maternal body stores, endogenous synthesis in the liver and mammary gland, and uptake from maternal plasma.These PUFAs are substrates in the biosynthetic pathway of eicosanoids, which are signaling molecules responsible for modulating the inflammatory response (10, 14). N-3 PUFAs in cell membranes may reduce allergic inflammation by decreasing inflammatory responses (15), while n-6 PUFAs may enhance inflammation by opposing the actions of n-3 PUFAs; suggesting that the ratio between n-6 to n-3 PUFAs is important. International authorities recommend supplementation of both arachidonic acid (ARA) and

docosahexaenoic acid (DHA) in formulas for infants who cannot be breastfed (16-18) to support growth (19) and immune development (20).

A recent systematic review reported some inverse associations between human milk n-3 PUFAs and the risk of atopic dermatitis and sensitization in breastfed children (21), although many studies found no association (22, 23) and one reported that n-3 PUFAs were *positively* associated with sensitization (24). Similarly, conflicting results were reported for n-6 PUFAs (21). Most studies have focused on DHA and ARA separately, with only a few investigating PUFA ratios, showing inconsistent results (22, 24, 25).

It is well known that allergic conditions develop differently in boys and girls (26), with a higher tendency in boys during early childhood (27). Emerging evidence suggests that milk composition may be different for male and female infants (28), however, it is unknown whether the potential associations between human milk PUFAs and infant atopic conditions are sex-specific.

To address these knowledge gaps, we examined the associations of breast milk PUFAs (and their ratios) with food sensitization and atopic dermatitis in the first year of life in the longitudinal CHILD Cohort Study.

METHODS

The CHILD Cohort Study is a general population birth cohort that recruited 3455 pregnant women between 2008-2012 across four Canadian sites (Vancouver, Edmonton, Manitoba, and Toronto) to study the development of allergic diseases (29). We studied a subsample of mother-infant dyads that breastfed for at least 3 months, had milk fatty acids analyzed (N=1,200) and had complete data on the outcomes of food sensitization and atopic dermatitis at 1 year (N=1,109) (**Supplementary Figure 1**). This subsample consisted of a representative sample of 417 dyads and an additional 692 dyads enriched for maternal and infant allergy outcomes, but with similar general characteristics with the representative sample **(Supplementary Table 1**) (13). Written informed parental consent

was obtained at enrolment and the study was approved by the Human Research Ethics Boards at McMaster University and the Universities of Manitoba, Alberta and British Columbia, and the Hospital for Sick Children.

Infant atopic sensitization

Allergic sensitization to food allergens at 1 year of age was determined by standardized skin-prick testing to egg white, peanut, cow's milk, and soybean (ALK Abello Pharmaceuticals Inc., Mississauga, ON, Canada) using Duotip-Test® II devices (Lincoln Diagnostics Inc., Decatur, IL, USA) (30, 31). Allergic sensitization was defined as a positive skin-prick test with a wheal measuring ≥ 2 mm in diameter compared to the negative control (glycerin). The diagnosis of atopic dermatitis in the first year of life was made by CHILD study pediatricians at 1 year of age (32) and defined as a recurrent or persistent pruritic skin rash and at least 3 of the following: history of itching in the skin creases or cheeks, history of hay fever or asthma or first-degree relative with a history of an atopic condition; dry skin within the past year; visible eczema on examination.

Human milk collection and fatty acid analysis

Analysis of breast milk fatty acids in the CHILD Cohort Study was described previously (13). Briefly, breast milk samples were collected at 3-4 months postpartum (median 15.1 weeks, 95% range 11.3, 28.1). In a sterile collection jar, mothers collected a mixture of foremilk and hindmilk from multiple feeds over a 24-hour period and kept the container refrigerated. Samples were stored at -80°C (33), and then analyzed by high-resolution capillary gas-liquid chromatography (34). For this study, human milk PUFAs including long-chain PUFAs (polyunsaturated fatty acids with chain lengths greater than 18 carbons) were analyzed, including n-3 PUFAs: alpha-linoleic acid (18:3n-3), eicosatetraenoic acid (20:4n-3), eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (22:5n-3), docosahexaenoic acid (DHA, 22:6n-3); and n-6 PUFAs: linoleic acid (18:2n-6), gamma-linolenic acid (18:3n-6), conjugated linoleic acid (CLA; 18:2c–9, t–11), dihomo-gamma-linolenic acid (DGLA, 20:3n-6), arachidonic acid (ARA, 20:4n-6). Each PUFA level was expressed as relative percentage of total fatty acids (including

SFAs, MUFAs and PUFAs) as described earlier (13). We also calculated total n-6 and n-3 PUFAs, their ratios, and the ARA/DHA ratio.

Statistical analyses

To facilitate comparability and interpretation of results for PUFAs present across a wide range, we converted measurements into a common scale using standard deviation (SD) scores and further categorized these SD scores into quintiles. We used t-tests to compare PUFA proportions between boys and girls, and the chi-square linear-by-linear association test for trend to compare the proportion of infants with each allergic outcome across PUFA quintiles. In addition, we used crude (unadjusted) and multivariable-adjusted logistic regression analyses to quantify these associations, expressed as odds ratios (OR) with 95% confidence intervals (CI) using the highest quintile as the reference group. The multivariable models were adjusted for study site, infant sex, age at milk collection, exclusivity of breastfeeding at the time of milk sample collection, and potential maternal confounders (maternal ethnicity, post-secondary education, food allergy, atopic dermatitis, and fish oil supplementation). These covariates were selected based on previous reports in the literature or because of a resultant change in the adjusted odds ratio (aOR) by >10% (21). In a sensitivity analysis, PUFA proportions (including; SFAs, MUFAs and PUFAs) were transformed using the centered log-ratio method (CLR) to control for constant-sum constraint (compositional nature of milk fatty acids), (35) and all of the above analyses were repeated using the CLR-transformed PUFA values. To assess whether associations differed by child sex or maternal allergies, we evaluated the statistical interaction by including the product term with PUFA variables in the models. Interactions with $p<0.10$ were further explored in stratified analyses. Analyses were performed using the Statistical Package for the Social Sciences version 21.0 for Windows (SPSS IBM, Chicago, IL, USA).

RESULTS

Among the 1,109 infants in this study, 184 (17%) were sensitized to food and 160 (14%) had atopic dermatitis at 1 year of age **(Table 1)**. About half (52%) of infants were

exclusively breastfed at the time of milk sample collection. The mean ± SD weight percentages of total n-3 and n-6 PUFAs were 2.42% ± 0.76% and 14.76% ± 3.13%, respectively **(Table 2)**, including the individual PUFAs ARA (0.38% ± 0.09%) and DHA (0.19% ± 0.14%). The mean ratio of total n-6/n-3 PUFA was 6.49 ± 1.74, and the mean ratio of ARA/DHA was 2.65 ± 1.45. There were no sex differences in the proportions or ratios of PUFAs (**Table 2**). The ranges of PUFA quintiles are given in **Table 3**.

Human milk N-3 PUFAs and atopic conditions in infancy

Sex-specific associations were observed between human milk PUFAs and infant food sensitization. In univariate analyses and unadjusted regression models, we observed that girls receiving milk with lower proportions of DHA had a lower prevalence of food sensitization (lowest vs highest quintile: 8% vs 24%, OR (95% Confidence Interval (CI)) 0.28 (0.12, 0.65); p-for-linear trend across quintiles <0.001) (**Figure 1** and **Supplementary Figure 2**) (p-for-sex-interaction =0.04). These differences persisted in the multivariable models adjusted for maternal food allergies and other risk factors aOR (95%CI) 0.35 (0.12, 0.99) (**Figure 2**). Similar associations were observed for total n-3 PUFA, EPA and DPA, but not for ALA and eicosatetraenoic acid. For example, girls who received human milk with n-3 PUFAs in the lowest quintile had 66% lower odds of food sensitization compared with those in the highest quintiles 0.34 (0.12, 0.95) (**Figure 2**). No associations were observed among boys (1.02 (0.47, 2.23)) **(Figures 1, 2** and **Supplementary Figure 2)**. Similar associations were observed using CLR-transformed fatty acid data (**Supplementary Figure 3**). Similar though non-significant tendencies were observed for atopic dermatitis (e.g. lowest vs highest quintile of milk n-3 PUFAs in girls: 0.51 (0.20, 1.28) (**Supplementary Figure 4**). Girls consuming milk with ALA proportions in the middle quintile (Q3 vs. Q5) had lower odds of atopic dermatitis. Opposite tendencies were observed in boys (**Supplementary Figure 4**).

Human milk N-6 PUFAs and atopic conditions in infancy

In girls only, a trend was observed among human milk ARA proportions and food

sensitizations (lowest vs. highest quintile: 12% vs 20%, OR 0.56: 95%CI 0.25, 1.24; p-for-trend =0.03, p-for-sex-interaction = 0.01) (**Figure 1** and **Supplementary Figure 5**). No clear associations were observed for total n-6 PUFAs, LA, or GLA **(Figure 3)**; however, girls consuming milk with CLA in the lowest vs. highest quintile had 65% lower odds of food sensitization (0.35 (0.14, 0.84)) (**Figure 3**). This association was not observed in boys. Results were generally similar using CLR-transformed fatty acid data), with one exception: a new association was observed for total n-6 PUFAs in girls (lowest vs. highest quintile: 0.39 (0.17, 0.92) (**Supplementary Figure 6**). Human milk n-6 PUFAs were not associated with infant atopic dermatitis in either sex (**Supplementary Figure 7**).

Human milk PUFA ratios and atopic conditions in infancy

Sex-specific and dose-dependent associations were observed between ARA/DHA ratio quintiles and food sensitization (p-for-trend <0.001 in girls only; p-for-sex-interaction =0.07). **(Figures 1** and **Supplementary Figure 8)**. Girls consuming milk with an ARA/DHA ratio in the lowest vs. highest quintile had a 3-fold higher odds of food sensitization (2.98 (1.10, 8.06)) (**Figure 4**). Total n-6/n-3 PUFA ratios were also associated with food sensitization in girls **(Figure 4)**. No associations were observed in boys. Similar sex-specific associations were observed for atopic dermatitis (**Supplementary Figure 9)**. There was no evidence of interaction between any PUFAs and maternal allergies.

DISCUSSION

Our research provides new evidence that human milk PUFAs may influence the development or prevention of atopic conditions in breastfed infants. Unlike prior studies on this topic, we considered PUFA ratios and evaluated sex differences, revealing two important nuances. First, the associations were only evident in girls. Second, when considered separately, it appeared that lower proportions of both n-3 and n-6 LC-PUFAs (in particular, DHA and ARA) were associated with lower odds of food sensitization and atopic dermatitis; however, when considered as a ratio, it became clear that the

proportion of these PUFA *in relation to each other* was associated with infant atopic outcomes in a dose-dependent manner. Specifically, we found that girls receiving human milk with a higher ratio of ARA/DHA at 3 months had lower odds of developing food sensitization and atopic dermatitis by 1 year of age. While further research is needed to replicate our findings and explore the potential role of endogenous fatty acid conversion pathways, our results suggest that a higher ARA to DHA ratio may be beneficial for infants who cannot be breastfed.

The role of n-3 and n-6 PUFAs in the inflammatory response is well-recognized (10, 14). N-3 PUFAs may reduce allergic inflammation by decreasing inflammatory responses (15), while n-6 PUFAs may enhance inflammation by opposing the actions of n-3 PUFAs; yet inconsistent results are reported on the associations of PUFAs and atopic conditions in children (21). Previous studies have not explored sex differences and many have not assessed total n-3 PUFA and n-6 PUFA and ARA/DHA ratios – which are key features of these associations in our study. Our findings on n-3 LC-PUFAs (including EPA, DPA, DHA) and sensitization are consistent with the findings of the Australian MACS study , where higher n-3 LC-PUFAs including DPA and DHA proportions were seen in non-sensitized children at six and 24 months of age (24). However, results from the Dutch PIAMA study showed no association of human milk n-3 PUFAs and sensitization at four years of age (22). Inconsistent results are also reported for n-6 PUFAs (including CLA, DGLA and ARA) and atopic conditions in children (22, 24, 36-38). In our study we observed that higher CLA proportions were associated with higher odds of food sensitizations in girls, which contradicts previous findings from the Dutch KOALA birth cohort study, where CLA appeared to be protective – although sex-stratified analyses were not performed (37).

There is a strong rationale for studying the ratio of total and individual n-3 and n-6 PUFA in the context of allergic disease because their biosynthesis pathways compete for the same enzymes, and their derivatives can have antagonistic pro- and anti-inflammatory effects. When we examined the proportions of individual and total PUFA in relation to each other, we found dose-dependent inverse association between n-6/n-3 PUFAs ratios and atopic conditions in 1-year-old girls. Altogether, these findings suggest

that when studying allergic conditions, the ratios of individual or total n-6/n-3 PUFAs may be more clinically relevant than the individual or total PUFA proportions.

Another novel aspect of our study is that that our results reveal sex differences, with associations observed in girls only. Sex differences in atopic disease are widely reported, and while the underlying pathogenesis is poorly understood, one hypothesis has attributed these differences to sex hormones (39). For example, females show higher antibody responses against infections due to the enhanced immune responses promoted by female sex hormones compared to the immunosuppressive effects of male hormones (40, 41). During infancy, specifically in the first 3-6 months of life, a rise in sex hormone levels occurs (42). Therefore, we speculate that the enhanced pro-inflammatory character of female sex hormones combined with an altered pro/anti-inflammatory balance of human milk n-6 and n-3 PUFAs could influence susceptibility for atopy in girls.

To our knowledge, this is one of the largest (N=1,109) prospective cohort studies of human milk PUFAs and infant atopic conditions. Other strengths of our study include the detailed assessments of atopic dermatitis and standardized skin testing to assess food sensitization, and the assessment of sex differences. The main limitation of our study is that we studied food sensitization (not clinical allergy) during infancy, which does not always persist into later childhood, although convincing evidence shows that food sensitization at 1 year predicts future atopic disease (31). In addition, breastmilk samples were collected only once during lactation (preventing us from analyzing the longitudinal variation of PUFAs during lactation) and were stored for up to 5-8 years at -80C before analysis. The storage time might have affected the total fat content but is unlikely to have altered the fatty acid composition. While we could reliably calculate the proportions of different human milk fatty acids, we could not quantify their absolute concentration because total milk fat content changes over the course of a feeding and diurnally, and we did not collect a full breast expression or standardize the timing of milk collection. We did not assess maternal PUFA status during pregnancy, therefore we could not address the impact of *in utero* exposure to PUFA, which is likely correlated with PUFA levels in human milk. Although we have captured n-3 and n-6 PUFAs in the breastmilk samples, there are other less common PUFAs (e.g. n-9 PUFAs) that may be of interest to study in

relation to human health. Finally, while we adjusted for many potential confounders, residual confounding is still possible in this observational study.

CONCLUSIONS

This study provides evidence that PUFAs and their ratios are associated with infant atopic conditions in a sex-specific manner. Our research suggests that a higher ratio of ARA/DHA in human milk may reduce the risk of food sensitization and atopic dermatitis in female infants. This suggests it is important to consider the amount of n-6 relative to n-3 PUFAs (ARA to DHA) consumed or supplemented during infancy. Further research is needed to validate these findings and determine the optimal ratio, explain the sex-specific associations, and investigate how the maternal-infant transfer and balance of PUFAs may influence immunity, inflammation and allergy development.

ACKNOWLEDGMENTS

We are grateful to all the families who took part in this study and the entire CHILD team, which includes interviewers, nurses, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, and receptionists at the following institutions: McMaster University, University of Manitoba, University of Alberta, University of Toronto, and University of British Columbia. We thank Ms. Susan Goruck (University of Alberta) for performing the fatty acid assays, and Ms. Stephanie Goguen (University of Manitoba) for assistance with generating the figures.

Author contributions

The authors' responsibilities were as follows—KM and MBA designed and managed the project; TJM, ABB, PJM, SET, MRS, and PS conceived the CHILD cohort design, managed study recruitment, and oversaw clinical assessments of study participants; ES contributed expertise on clinical allergy phenotypes; CJF oversaw and performed FA

analysis of human milk samples; KM and JR conducted all the statistical analyses; KM, JR and MBA interpreted the data and wrote the manuscript; and all authors provided feedback and read and approved the final manuscript. KM and MBA have full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

TABLES & FIGURE LEGENDS

Table 1. Descriptive characteristics of mother-infant dyads in this subset of the CHILD Cohort (N=1,109)

Maternal characteristics	
Age, years	33.0 ±4.3
Primiparous, n (%)	633 (57.1)
Completed post-secondary education, n (%)	888 (80.1)
Ethnicity, n (%)	
Asian	190 (17.1)
Caucasian	803 (72.4)
First Nations	41 (3.7)
Other	73 (6.6)
Study site, n (%)	
Edmonton	237 (21.4)
Toronto	282 (25.4)
Vancouver	296 (26.7)
Winnipeg	294 (26.5)
BMI, kg/m^2	23.1 (18.3, 37.8)
Diet quality, HEI 2010 score	73.9 ±7.9
Prenatal fish oil supplement use, n (%)	256 (23.1)
Prenatal multivitamin intake, n (%)	777 (70.1)
Prenatal smoking, n (%)	53 (4.8)
Maternal food allergy, n (%)	306 (27.6)
Maternal atopy, n (%)	807 (72.8)
Breast milk characteristics	
Age at breast milk collection, weeks	15.1 (11.3, 28.1)

Exclusive breastfeeding at sample collection, n (%)	580 (52.3)
Season at breast milk sample collection, n (%)	
Winter: Dec-Feb	266 (24.0)
Spring: Mar-May	304 (27.4)
Summer: Jun-Aug	267 (24.1)
Fall: Sept-Nov	270 (24.3)
Duration of exclusive breastfeeding, months	4.5 (0.0, 6.0)
Duration of breastfeeding, months	12.0 (3.8, 24.0)
Infant characteristics	
Sex, male, n (%)	606 (54.6)
Gestational age, weeks	39.1 ±1.4
Birth weight, g	3450 ±497
Atopic dermatitis at 1 year, n (%)	160 (14.4)
Allergic sensitization to food at 1 year, n (%)*	
Any	184 (16.6)
Egg	113 (10.2)
Peanut	83 (7.5)
Milk	34 (3.1)
Soy	14 (1.3)
Both atopic dermatitis and food sensitization, n (%)*	67 (6.0)

Values reflect percentages of non-missing data for categorical variables, means ±SD for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution. *Positive SPT indicated by ≥ 2 mm wheal. Maternal atopy and food allergy are self-reported.

Table 2. Human milk PUFAs at 3 months postpartum in the CHILD Cohort Study, stratified by child sex (N=1,109)

LC-PUFA		All Children	Milk for Girls	Milk for Boys
Total n-3 PUFA (%)		**2.42 ± 0.76**	**2.42 ± 0.73**	**2.41 ± 0.80**
α-linolenic acid (ALA)	18:3n-3	1.92 ± 0.66	1.92 ± 0.63	1.93 ± 0.68
Eicosatetraenoic acid	20:4n-3	0.08 ± 0.03	0.08 ± 0.03	0.08 ± 0.03
Eicosapentaenoic acid (EPA)	20:5n-3	0.08 ± 0.07	0.08 ± 0.07	0.08 ± 0.07
Docosapentaenoic acid (DPA)	22:5n-3	0.13 ± 0.06	0.13 ± 0.06	0.13 ± 0.05
Docosahexaenoic acid (DHA)	22:6n-3	0.19 ± 0.14	0.19 ± 0.14	0.19 ± 0.14
Total n-6 PUFA (%)		**14.76 ± 3.13**	**14.66 ± 3.17**	**14.84 ± 3.11**
Linoleic acid (LA)	18:2n-6	13.60 ± 3.05	13.51 ± 3.08	13.68 ± 3.03
Conjugated linoleic acid (CLA)	18:2c–9, t–11	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

γ-linolenic acid (GLA)	18:3n-6	0.10 ± 0.06	0.10 ± 0.06	0.10 ± 0.06
Dimomo-γ-linolenic acid (DGLA)	20:3n-6	0.35 ± 0.11	0.34 ± 0.11	0.35 ± 0.11
Arachidonic acid (ARA)	20:4n-6	0.38 ± 0.09	0.38 ± 0.09	0.38 ± 0.10
Ratio of PUFAs				
Total n-6/ Total n-3 PUFA		6.49 ± 1.74	6.39 ± 1.67	6.56 ± 1.79
ARA/DHA		2.65 ± 1.45	2.63 ± 1.55	2.67 ± 1.36

Values are mean ± SD. Individual PUFAs are reported as percentages of total fatty acids (g/100 g of fatty acids). No sex differences were observed when compared by t-test or the non-parametric Mann-Whitney test. Abbreviations: polyunsaturated fatty acid (PUFA).

Table 3. Human milk PUFAs at 3 months postpartum in the CHILD Cohort Study (N=1,109): median and quintile ranges

LC-PUFA	Median 95%CI	Q1	Q2	Q3	Q4	Q5
n-3 PUFA (%)	2.32 (1.14, 4.12)	0.90-1.80	1.80-2.18	2.18-2.51	2.51-2.96	2.96-6.62
ALA	1.86 (0.84, 3.40)	0.44-1.40	1.40-1.71	1.71-2.00	2.00-2.38	2.38-5.82
Eicosatetraenoic acid	0.08 (0.03, 0.17)	0.01-0.06	0.06-0.07	0.07-0.08	0.08-0.10	0.10-0.27
EPA	0.06 (0.03, 0.30)	0.01-0.04	0.04-0.05	0.05-0.07	0.07-0.11	0.11-0.54
DPA	0.12 (0.06, 0.28)	0.04-0.09	0.09-0.11	0.11-0.13	0.13-0.16	0.16-0.48
DHA	0.15 (0.06, 0.59)	0.02-0.09	0.09-0.13	0.13-0.18	0.18-0.26	0.26-1.09
n-6 PUFA (%)	14.48 (9.44, 21.90)	7.13-12.00	12.00-13.70	13.70-15.10	15.10-16.92	16.92-27.35
LA	13.33 (8.52, 20.64)	6.17-11.00	11.00-12.54	12.54-14.00	14.00-15.74	15.74-26.20
CLA	0.02 (0.00, 0.04)	0.00-0.01	0.01-0.02	0.02-0.02	0.02-0.03	0.03-0.18
GLA	0.09 (0.03, 0.26)	0.17-0.06	0.06-0.08	0.08-0.10	0.10-0.14	0.14-0.40
DGLA	0.33 (0.16, 0.59)	0.06-0.24	0.24-0.30	0.30-0.36	0.36-0.44	0.44-0.83
ARA	0.37 (0.22, 0.59)	0.15-0.30	0.30-0.35	0.35-0.39	0.39-0.45	0.45-0.85
Total n-6/n-3 PUFA	6.24 (3.91, 10.72)	2.35-5.10	5.10-6.00	6.00-6.60	6.60-7.60	7.60-17.28
ARA/DHA	2.47 (0.66, 5.42)	0.28-1.41	1.41-2.10	2.10-2.90	2.90-3.70	3.70-17.60

Values are medians and 95%CI and quintiles ranges of PUFAs reported as percentages of total fatty acids, calculated by weight (g/100 g of fatty acids).
Abbreviations: polyunsaturated fatty acid (PUFA), α-linolenic acid (ALA), Eicosapentaenoic acid (EPA), Docosapentaenoic acid (DPA), Docosahexaenoic acid (DHA), Linoleic acid (LA), Conjugated linoleic acid (CLA), γ-linolenic acid (GLA), Dimomo-γ-linolenic acid (DGLA), Arachidonic acid (ARA).

Figure 1. Prevalence of food sensitization at 1 year according to human milk DHA, ARA and ARA/DHA ratio quintiles (Q1=lowest, Q5=highest) in breastfed boys and girls in the CHILD Cohort Study.
P-trend are p-values from chi-square linear-by-linear association test for trend.

Figure 2. Sex-stratified associations of total and individual human milk n-3 PUFAs with infant food sensitization (n/N=184/1,109) in the CHILD Cohort Study

Values are Odds Ratios (95% confidence interval) based on multivariable adjusted logistic regression models. The models are adjusted for study site, age at milk collection, exclusivity of breastfeeding at the time of milk sample collection, maternal ethnicity, post-secondary education, food allergy, and fish oil supplementation. Abbreviations: ALA, alpha-linoleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. (Quintile 1=lowest, Quintile 5=highest) ***P-values < 0.05**

Figure 3. Sex-stratified associations of total and individual human milk n-6 PUFAs with infant food sensitization (n/N=184/1,109) in the CHILD Cohort Study

Values are Odds Ratios (95% confidence interval) based on multivariable adjusted logistic regression models. The models are adjusted for study site, age at milk collection, exclusivity of breastfeeding at the time of milk sample collection, maternal ethnicity, post-secondary education, food allergy, and fish oil supplementation. Abbreviations: LA, linoleic acid; GLA, gamma-linolenic acid; CLA, conjugated linoleic acid; DGLA, dihomo-gamma-linolenic acid; ARA, arachidonic acid. (Quintile 1=lowest, Quintile 5=highest) ***P-values < 0.05**

Figure 4. Sex-stratified associations of human milk PUFA ratios with infant food sensitization (n/N=184/1,109) in the CHILD Cohort Study

Values are odds ratios and 95% Confidence Intervals. Models are adjusted for study site, age at milk collection, breastfeeding exclusivity at the time of milk sample collection, and maternal ethnicity, post-secondary education, food allergy, and fish oil supplementation. Abbreviations: ARA/DHA, arachidonic/ docosahexaenoic acid ratio. (Quintile 1=lowest, Quintile 5=highest) ***P-values < 0.05**

REFERENCES

1. Chafen JJ, Newberry SJ, Riedl MA, Bravata DM, Maglione M, Suttorp MJ, et al. Diagnosing and managing common food allergies: a systematic review. *JAMA* 2010;**303**(18):1848-1856.
2. Clarke AE, Elliott SJ, St. Pierre Y, Soller L, La Vieille S, Ben-Shoshan M. Temporal trends in prevalence of food allergy in Canada. *The Journal of Allergy and Clinical Immunology: In Practice* 2020;**8**(4):1428-1430.e1425.
3. Eichenfield LF, Hanifin JM, Beck LA, Lemanske RF, Jr., Sampson HA, Weiss ST, et al. Atopic dermatitis and asthma: parallels in the evolution of treatment. *Pediatrics* 2003;**111**(3):608-616.
4. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 2006;**368**(9537):733-743.
5. Flohr C, Mann J. New insights into the epidemiology of childhood atopic dermatitis. *Allergy* 2014;**69**(1):3-16.
6. Sicherer SH, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol* 2014;**133**(2):291-307.
7. American Academy of Pediatrics. Breastfeeding and the use of human milk. *Pediatrics* 2012;**129**(3):e827-841.
8. Critch JN, Canadian Paediatric Society N, Gastroenterology C. Nutrition for healthy term infants, birth to six months: An overview. *Paediatr Child Health* 2013;**18**(4):206-209.
9. Chan ES, Cummings C, Canadian Paediatric Society CPC, Allergy S. Dietary exposures and allergy prevention in high-risk infants: A joint statement with the Canadian Society of Allergy and Clinical Immunology. *Paediatr Child Health* 2013;**18**(10):545-554.
10. Calder PC, Krauss-Etschmann S, de Jong EC, Dupont C, Frick JS, Frokiaer H, et al. Early nutrition and immunity - progress and perspectives. *Br J Nutr* 2006;**96**(4):774-790.
11. Richard C, Lewis ED, Field CJ. Evidence for the essentiality of arachidonic and docosahexaenoic acid in the postnatal maternal and infant diet for the development of the infant's immune system early in life. *Appl Physiol Nutr Metab* 2016;**41**(5):461-475.

12. Jia X, Pakseresht M, Wattar N, Wildgrube J, Sontag S, Andrews M, et al. Women who take n-3 long-chain polyunsaturated fatty acid supplements during pregnancy and lactation meet the recommended intake. *Appl Physiol Nutr Metab* 2015;**40**(5):474-481.
13. Miliku K, Duan QL, Moraes TJ, Becker AB, Mandhane PJ, Turvey SE, et al. Human milk fatty acid composition is associated with dietary, genetic, sociodemographic, and environmental factors in the CHILD Cohort Study. *Am J Clin Nutr* 2019;**110**(6):1370-1383.
14. Calder PC. Early life programming of immune and lung function: can we now exclude a role of arachidonic acid exposure? *Br J Nutr* 2009;**102**(3):331-333.
15. Prescott SL, Calder PC. N-3 polyunsaturated fatty acids and allergic disease. *Curr Opin Clin Nutr Metab Care* 2004;**7**(2):123-129.
16. Koletzko B, Bergmann K, Brenna JT, Calder PC, Campoy C, Clandinin MT, et al. Should formula for infants provide arachidonic acid along with DHA? A position paper of the European Academy of Paediatrics and the Child Health Foundation. *Am J Clin Nutr* 2020;**111**(1):10-16.
17. Agostoni C, Braegger C, Decsi T, Kolacek S, Koletzko B, Michaelsen KF, et al. Breast-feeding: A commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr* 2009;**49**(1):112-125.
18. Food and Agriculture Organization of the United Nations. Fats and Fatty Acids in Human Nutrition: Report of a Joint FAO/WHO Expert Consultation. Rome, FAO. 2010.
19. Innis SM. Polyunsaturated fatty acids in human milk: an essential role in infant development. *Adv Exp Med Biol* 2004;**554**:27-43.
20. Field CJ, Van Aerde JE, Robinson LE, Clandinin MT. Effect of providing a formula supplemented with long-chain polyunsaturated fatty acids on immunity in full-term neonates. *Br J Nutr* 2008;**99**(1):91-99.
21. Waidyatillake NT, Dharmage SC, Allen KJ, Lodge CJ, Simpson JA, Bowatte G, et al. Association of Breast Milk Fatty Acids and Allergic Disease Outcomes - a Systematic Review. *Allergy* 2017;**10.1111/all.13300**.
22. Wijga AH, van Houwelingen AC, Kerkhof M, Tabak C, de Jongste JC, Gerritsen J, et al. Breast milk fatty acids and allergic disease in preschool children: the Prevention and Incidence of Asthma and Mite Allergy birth cohort study. *J Allergy Clin Immunol* 2006;**117**(2):440-447.
23. van Elten TM, van Rossem L, Wijga AH, Brunekreef B, de Jongste JC, Koppelman GH, et al. Breast milk fatty acid composition has a long-term effect on the risk of asthma, eczema, and sensitization. *Allergy* 2015;**70**(11):1468-1476.

24. Stoney RM, Woods RK, Hosking CS, Hill DJ, Abramson MJ, Thien FC. Maternal breast milk long-chain n-3 fatty acids are associated with increased risk of atopy in breastfed infants. *Clin Exp Allergy* 2004;**34**(2):194-200.
25. Kankaanpaa P, Nurmela K, Erkkila A, Kalliomaki M, Holmberg-Marttila D, Salminen S, et al. Polyunsaturated fatty acids in maternal diet, breast milk, and serum lipid fatty acids of infants in relation to atopy. *Allergy* 2001;**56**(7):633-638.
26. Mohrenschlager M, Schafer T, Huss-Marp J, Eberlein-Konig B, Weidinger S, Ring J, et al. The course of eczema in children aged 5-7 years and its relation to atopy: differences between boys and girls. *Br J Dermatol* 2006;**154**(3):505-513.
27. Gerada E, Agius Muscat H, Camilleri L, Montefort S. Gender differences in the prevalence and severity of wheezing, rhinitis and eczema in 5- to 8- year old and 12- to 15- year old Maltese children (ISAAC-Malta). *European Respiratory Journal* 2015;**46**(suppl 59):PA1337.
28. Galante L, Milan AM, Reynolds CM, Cameron-Smith D, Vickers MH, Pundir S. Sex-Specific Human Milk Composition: The Role of Infant Sex in Determining Early Life Nutrition. *Nutrients* 2018;**10**(9).
29. Subbarao P, Anand SS, Becker AB, Befus AD, Brauer M, Brook JR, et al. The Canadian Healthy Infant Longitudinal Development (CHILD) Study: examining developmental origins of allergy and asthma. *Thorax* 2015;**70**(10):998-1000.
30. Tran MM, Lefebvre DL, Dai D, Dharma C, Subbarao P, Lou W, et al. Timing of food introduction and development of food sensitization in a prospective birth cohort. *Pediatr Allergy Immunol* 2017;**28**(5):471-477.
31. Tran MM, Lefebvre DL, Dharma C, Dai D, Lou W, Subbarao P, et al. Predicting the atopic march: results from the Canadian Healthy Infant Longitudinal Development Study. *J Allergy Clin Immunol* 2017;**32**(6):556-567.
32. Williams HC, Burney PG, Pembroke AC, Hay RJ. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. *Br J Dermatol* 1994;**131**(3):406-416.
33. Moraes TJ, Lefebvre DL, Chooniedass R, Becker AB, Brook JR, Denburg J, et al. The Canadian healthy infant longitudinal development birth cohort study: biological samples and biobanking. *Paediatr Perinat Epidemiol* 2015;**29**(1):84-92.
34. Cruz-Hernandez C, Goeuriot S, Giuffrida F, Thakkar SK, Destaillats F. Direct quantification of fatty acids in human milk by gas chromatography. *J Chromatogr A* 2013;**1284**:174-179.

35. Logan CA, Brandt S, Wabitsch M, Brenner H, Wiens F, Stahl B, et al. New approach shows no association between maternal milk fatty acid composition and childhood wheeze or asthma. *Allergy* 2017;**72**(9):1374-1383.
36. Oddy WH, Pal S, Kusel MM, Vine D, de Klerk NH, Hartmann P, et al. Atopy, eczema and breast milk fatty acids in a high-risk cohort of children followed from birth to 5 yr. *Pediatr Allergy Immunol* 2006;**17**(1):4-10.
37. Thijs C, Muller A, Rist L, Kummeling I, Snijders BE, Huber M, et al. Fatty acids in breast milk and development of atopic eczema and allergic sensitisation in infancy. *Allergy* 2011;**66**(1):58-67.
38. Soto-Ramirez N, Karmaus W, Zhang H, Liu J, Billings D, Gangur V, et al. Fatty acids in breast milk associated with asthma-like symptoms and atopy in infancy: a longitudinal study. *J Asthma* 2012;**49**(9):926-934.
39. Afify SM, Pali-Scholl I. Adverse reactions to food: the female dominance - A secondary publication and update. *World Allergy Organ J* 2017;**10**(1):43.
40. Da Silva JA. Sex hormones and glucocorticoids: interactions with the immune system. *Ann N Y Acad Sci* 1999;**876**:102-117.
41. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol* 2008;**8**(9):737-744.
42. Lanciotti L, Cofini M, Leonardi A, Penta L, Esposito S. Up-To-Date Review About Minipuberty and Overview on Hypothalamic-Pituitary-Gonadal Axis Activation in Fetal and Neonatal Life. *Front Endocrinol* 2018;**9**:410.

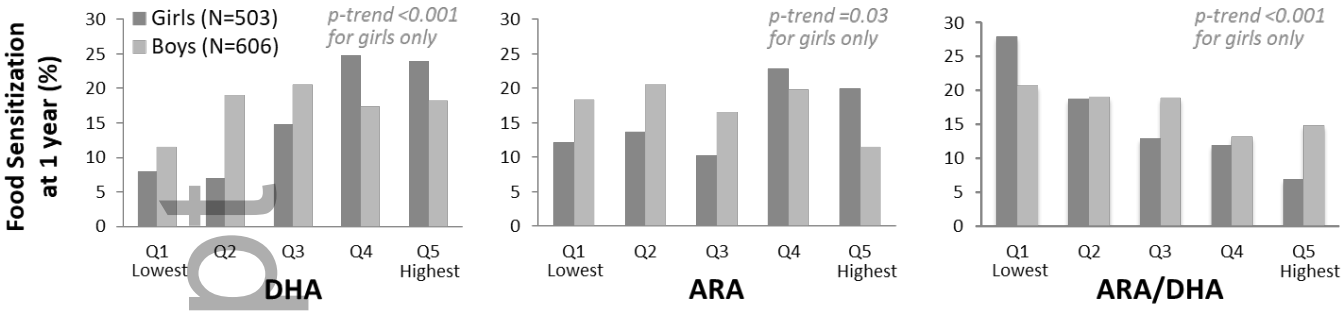

Figure 1. Prevalence of food sensitization at 1 year according to human milk DHA, ARA and ARA/DHA ratio quintiles (Q1=lowest, Q5=highest) in breastfed boys and girls in the CHILD Cohort Study.
P-trend are p-values from chi-square linear-by-linear association test for trend.

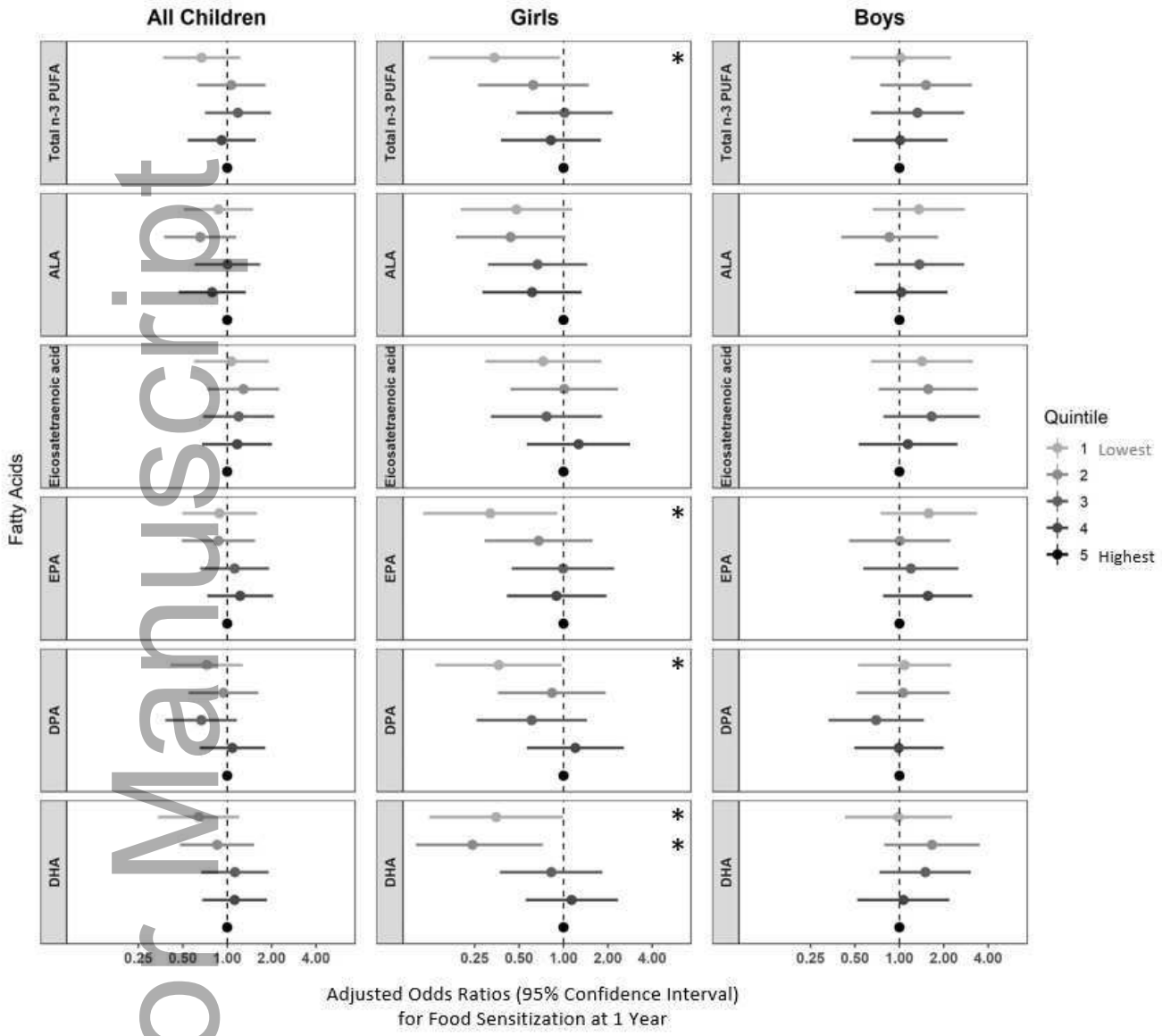

Figure 2. Sex-stratified associations of total and individual human milk n-3 PUFAs with infant food sensitization (n/N=184/1,109) in the CHILD Cohort Study

Values are Odds Ratios (95% confidence interval) based on multivariable adjusted logistic regression models. The models are adjusted for study site, age at milk collection, exclusivity of breastfeeding at the time of milk sample collection, maternal ethnicity, post-secondary education, food allergy, and fish oil supplementation. Abbreviations: ALA, alpha-linoleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. (Quintile 1=lowest, Quintile 5=highest) ***P-values < 0.05**

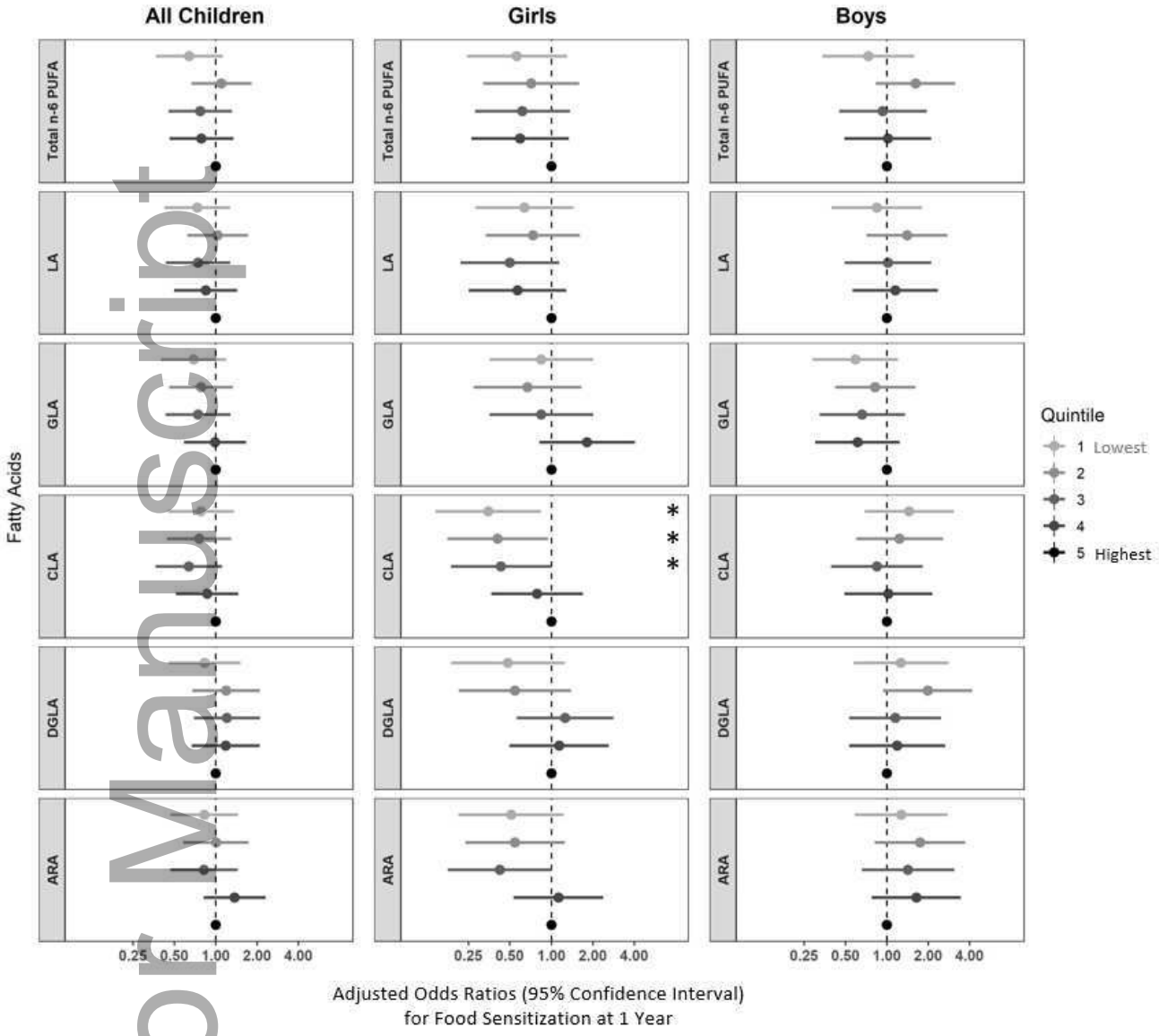

Figure 3. Sex-stratified associations of total and individual human milk n-6 PUFAs with infant food sensitization (n/N=184/1,109) in the CHILD Cohort Study

Values are Odds Ratios (95% confidence interval) based on multivariable adjusted logistic regression models. The models are adjusted for study site, age at milk collection, exclusivity of breastfeeding at the time of milk sample collection, maternal ethnicity, post-secondary education, food allergy, and fish oil supplementation. Abbreviations: LA, linoleic acid; GLA, gamma-linolenic acid; CLA, conjugated linoleic acid; DGLA, dihomo-gamma-linolenic acid; ARA, arachidonic acid. (Quintile 1=lowest, Quintile 5=highest) ***P-values < 0.05**

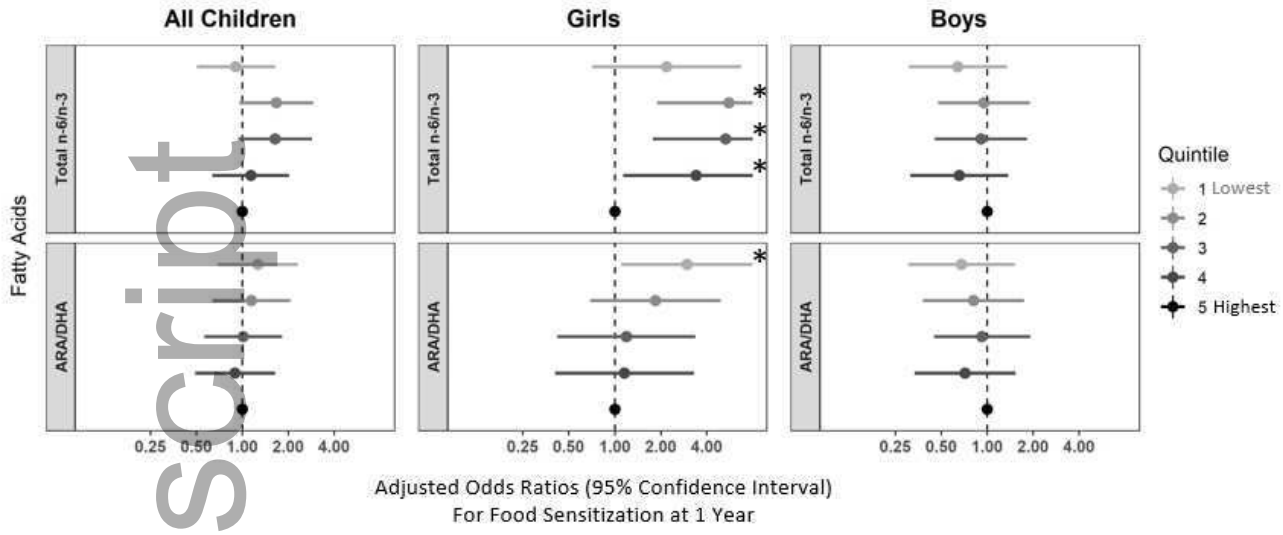

Figure 4. Sex-stratified associations of human milk PUFA ratios with infant food sensitization (n/N=184/1,109) in the CHILD Cohort Study

Values are odds ratios and 95% Confidence Intervals. Models are adjusted for study site, age at milk collection, breastfeeding exclusivity at the time of milk sample collection, and maternal ethnicity, post-secondary education, food allergy, and fish oil supplementation. Abbreviations: ARA/DHA, arachidonic/ docosahexaenoic acid ratio. (Quintile 1=lowest, Quintile 5=highest) ***P-values < 0.05**