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LETTER TO THE EDITOR

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Human milk oligosaccharide profiles and food sensitization among infants in the CHILD Study

To the Editor,

Allergies originate early in life, and food sensitization is often the first manifestation of allergic disease.[1] Breastfeeding has been inconsistently associated with allergic conditions.[2] These inconsistencies could reflect differences in human milk composition, which varies across different settings and populations. However, it remains poorly understood which of the bioactive components in human milk contribute to the developmental programming of allergic disease.

Human milk oligosaccharides (HMOs) are the third most abundant component of human milk, yet they are absent from most infant formulas.[3] HMO composition is influenced by genetic fucosyltransferase-2 secretor status and also by lactation stage, gestational age, maternal health, ethnicity, geographic location, and breastfeeding exclusivity.[3] Among their many functions,[3] HMOs act as selective substrates to guide development of the infant gut microbiota.[4] We have previously reported that gut microbiota richness in early infancy is associated with subsequent food sensitization, suggesting that HMOs and other determinants of early gut colonization could influence the development of allergic disease.[5] This hypothesis is also supported by experimental research in rodents[6] and a small clinical study where low concentrations of the HMO lacto-N-fucopentaose III (LNFPIII) were associated with higher incidence of cow's milk allergy.[7] However, the potential impact of other individual HMOs on food sensitization is not known, and the impact of overall HMO composition has not been studied, yet this may be important because breastfed infants are naturally exposed to complex combinations of HMOs in human milk.

In this study, among 421 mother–infant dyads from the Canadian Healthy Infant Longitudinal Development (CHILD) cohort,[8] we examined the associations of 19 individual HMOs and overall HMO profiles with food sensitization at 1 year of age using Projection on Latent Structures-Discriminant Analysis (PLS-DA).[9] Detailed methods are provided in Supplementary Materials.

Overall, 59/421 infants (14.0%) were sensitized to 1 or more food allergens at 1 year of age (Table S1). We did not observe any significant associations for the 19 individual HMOs or total HMOs and food sensitization (Figure 1A); however, overall HMO profiles differed significantly in milk consumed by sensitized vs nonsensitized infants ($P < .001$; robust to leave-one-out cross-validation) (Figure 1B). The discrimination performance was "fair," with an area under the curve (AUC) of 0.73, 95% Confidence Interval (CI) 0.66-0.79 (robust to permutation testing with 100 replicates; $P = .02$) (Figure 1C). Similar results were observed in a sensitivity analysis excluding 22 infants with food allergy symptoms prior to milk sample collection (AUC 0.75, 95% CI: 0.69-0.81) (Figure S1).

Restricting our analysis to the top 10 most important HMOs contributing to the PLS-DA score resulted in similar discrimination (AUC 0.71; 95% CI: 0.64-0.78), indicating that these 10 HMOs are sufficient to explain the association of HMO profile and food sensitization. The rankings, PLS-DA scaled importance scores, and direction of association for these 10 HMOs are shown in Figure 1D. HMO profiles associated with lower risk of food sensitization were characterized by relatively higher concentrations of fucodisialyllacto-N-hexaose (FDSLNH), lacto-N-fucopentaose II (LNFPII), lacto-N-neotetraose (LNnT), lacto-N-fucopentaose I (LNFPI), sialyl-lacto-N-tetraose c (LSTc) and fucosyllacto-N-hexaose (FLNH), and relatively lower concentrations of lacto-N-hexaose (LNH), lacto-N-tetrose (LNT), 2′-fucosyllactose (2′FL), and disialyllacto-N-hexaose (DSLNH).

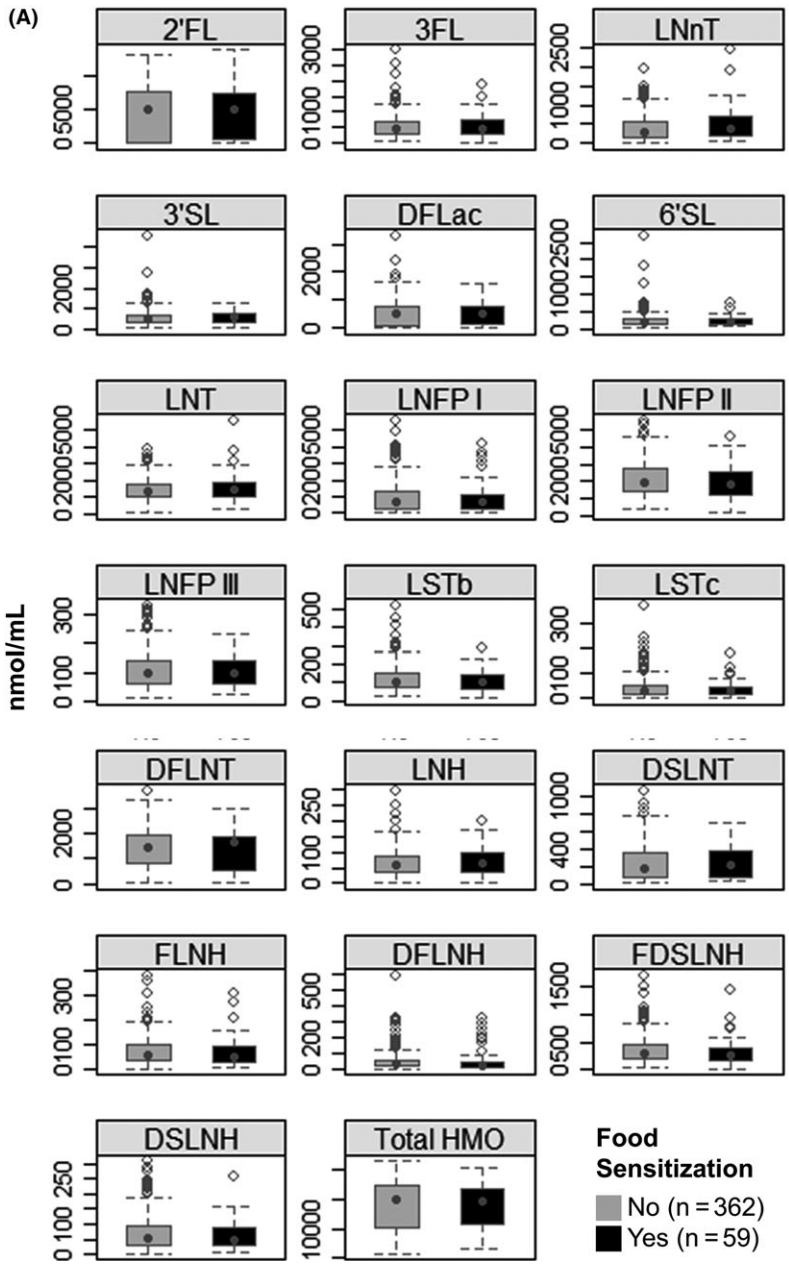
Finally, to account for potential confounders and adjust for known allergy risk factors, we evaluated the PLS-DA score in multivariable logistic regression models (Table 1). Compared to infants consuming milk with a discriminant score in the highest quintile, those in the lowest quintile had a 90% lower risk of food sensitization (Odds Ratio [OR] 0.10 [95% CI: 0.03, 0.34]).

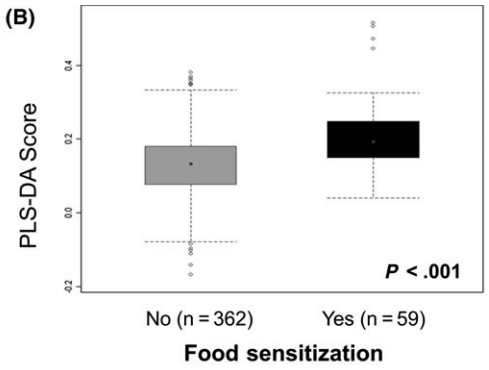
To our knowledge, only 1 previous study has explored the association of HMOs with food sensitization in children, where infants receiving milk with low LNFPIII concentrations were more likely to develop cow's milk allergy.[7] In contrast, we did not observe associations of any individual HMOs with food sensitization, and LNFPIII was not among the most discriminatory HMOs in our analysis. This may reflect differences in study populations (high-risk infants[7] vs our general population cohort), timing of milk collection (1 month vs 3-4 months), or outcomes assessed (confirmed milk allergy[7] vs sensitization to various food allergens).

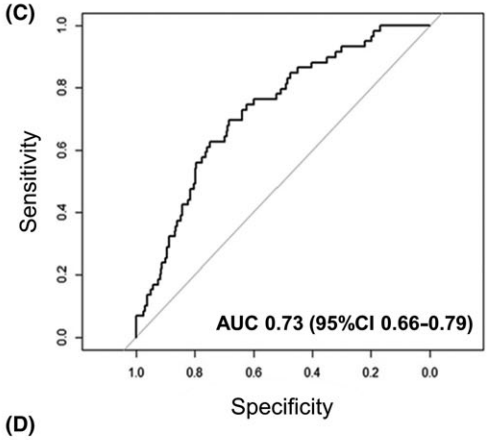
Recently, a randomized trial reported that infants receiving formula supplemented with 2′FL had more similar immune responses to breastfed controls, compared to infants receiving formula without 2′FL.[10] In addition, a rodent study showed that 2′FL and 6′SL can reduce symptoms of food allergy.[6] In contrast, we did not find an association of 2′FL or 6′SL or any other individual HMO with infant food sensitization. Instead, in our study, overall HMO composition was associated with food sensitization, reflecting the complexity of human milk and its evolution to supply the breastfed infant with many different HMOs.

While the causality of these associations remains to be determined, there are several plausible mechanisms by which HMO profiles could influence food sensitization. For example, HMOs modulate immune development through their prebiotic effects on gut bacteria, and by

[a]CHILD Study Investigators are listed in Appendix 1







(D)

HMO	Higher (+) or Lower (–) in milk consumed by healthy infants	PLS-DA Scaled Importance Score	Rank
FDSLNH	+	100	1
LNH	–	78	2
LNFPII	+	73	3
LNnT	+	73	4
LNT	–	67	5
LNFPI	+	67	6
LSTc	+	63	7
FLNH	+	60	8
2'FL	–	60	9
DSLNH	–	57	10

FIGURE 1 A-B, Association of individual and total HMOs (A) and overall HMO profile (B) at 3-4 months with food sensitization at 1 year in the CHILD cohort (N = 421). C, Receiver operating characteristic (ROC) curve. D, Scaled importance scores, ranking and direction of association for the top 10 HMOs contributing to the overall PLS-DA score. Boxes indicate interquartile range; white dots indicate median values; whiskers indicate range. Mann-Whitney *U* test ($P < .001$). Abbreviations: PLS-DA, Projection on Latent Structures-Discriminant Analysis, AUC, area under the curve. 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; LNnT, lacto-N-neotetraose; 3'SL, 3'-sialyllactose; DFLac, difucosyllactose; 6'SL, 6'-sialyllactose; LNT, lacto-N-tetrose; LNFP I, lacto-N-fucopentaose-I; LNFP II, lacto-N-fucopentaose-II; LNFP III, lacto-N-fucopentaose-III; LSTb, sialyl-lacto-N-tetraose b; LSTc, sialyl-lacto-N-tetraose c; DFLNT, difucosyllacto-N-tetrose; LNH, lacto-N-hexaose; DSLNT, disialyllacto-N-tetraose; FLNH, fucosyllacto-N-hexaose; DFLNH, difucosyllacto-N-hexaose; FDSLNH, fucodisialyllacto-N-hexaose; DSLNH, disialyllacto-N-hexaose.

influencing lymphocyte maturation.[3] Further research is needed to determine whether the "beneficial" HMO profile we have identified can optimally stimulate these developmental processes, and to identify the maternal and environmental factors that promote a "beneficial" HMO profile.

To our knowledge, this is the largest study to examine the association of HMOs and allergy development in infants, and the first to evaluate overall HMO profiles. Key strengths include the prospective design within a large population-based cohort, and standardized skin testing to assess food sensitization. Our methods allowed absolute quantification of HMOs, and we applied a novel multivariate approach to account for the natural occurrence of HMOs in complex combinations within human milk. The main limitation of our study is the lack of external validation; however, our PLS-DA results were robust to cross-validation. Finally, we

TABLE 1 Association of HMO profiles at 3-4 mo with food sensitization at 1 y in the CHILD cohort (N = 421)

	Food sensitization at 1 y	
HMO profile: PLS-DA score quintile (range)	Basic model OR (95% CI) N = 421	Adjusted model OR (95% CI) N = 369
Quintile 1 (−1.69, 0.63)	**0.12 (0.04, 0.37)****	**0.10 (0.03, 0.34)****
Quintile 2 (0.63, 1.18)	**0.12 (0.04, 0.37)****	**0.10 (0.03, 0.32)****
Quintile 3 (1.18, 1.57)	**0.32 (0.14, 0.72)***	**0.26 (0.10, 0.67)***
Quintile 4 (1.57, 2.17)	0.59 (0.29, 1.22)	0.62 (0.26, 1.46)
Quintile 5 (2.17, 5.16)	*Reference*	*Reference*
P for trend	<.001	<.001

Values are odds ratios (OR) and 95% confidence interval (CI). Basic models are adjusted for child's sex and age. Multivariable-adjusted models are basic models additionally adjusted for maternal ethnicity, education, self-reported maternal food allergy, lactation stage (weeks postpartum), infant birthweight and gestational age at birth, breastfeeding duration, breastfeeding exclusivity at 6 mo, timing of introduction of solid food, household pets, and study site.
CHILD, Canadian Healthy Infant Longitudinal Development; HMOs, human milk oligosaccharides; PLS-DA Projection on Latent Structures-Discriminant Analysis.
P for trend is obtained using HMOs discriminant score as an ordinal variable in the regression models.
P-value <.001** <.05*.

acknowledge that food sensitization during infancy does not always persist into later childhood; however, it is an important clinical outcome and a strong predictor of future atopic disease.[1]

In conclusion, our results demonstrate that HMO composition is associated with the development of food sensitization in the first year of life, and emphasize that overall profiles should be considered when examining the health effects of HMOs or considering their utility for therapeutic interventions. Further research is warranted to confirm our findings in other populations, explore the underlying biological mechanisms, and establish the long-term consequences of HMO composition on confirmed allergic disease later in childhood.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

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

APPENDIX 1

CHILD investigators include the following: Subbarao P (Director), The Hospital for Sick Children & University of Toronto; Turvey SE, University of British Columbia (co-Director), Anand SS, McMaster University; Azad M, University of Manitoba; Becker AB, University of Manitoba; Befus AD, University of Alberta; Brauer M, University of British Columbia; Brook JR, University of Toronto; Chen E, Northwestern University, Chicago; Cyr M, McMaster University; Daley D, University of British Columbia; Dell SD, The Hospital for Sick Children & University of Toronto; Denburg JA, McMaster University; Duan Q, Queen's University; Eiwegger T, The Hospital for Sick Children & University of Toronto; Grasemann H, The Hospital for Sick Children & University of Toronto; K HayGlass, University of Manitoba; Hegele RG , The Hospital for Sick Children & University of Toronto; Holness DL, University of Toronto; Hystad P, Oregon State University; Kobor M, University of British Columbia; Kollman TR, University of British Columbia; Kozyrskyj AL, University of Alberta; Laprise C, Université du Québec à Chicoutimi; Lou WYW, University of Toronto; Macri J, McMaster University; Mandhane PJ, University of Alberta; Miller G, Northwestern University, Chicago; Moraes TJ, The Hospital for Sick Children & University of Toronto; Paré, University of British Columbia; Ramsey C, University of Manitoba; Ratjen F, The Hospital for Sick Children & University of Toronto; Sandford A, University of British Columbia; JA Scott, University of Toronto; Scott J, University of Toronto; Sears MR, (Founding Director), McMaster University; Silverman F, University of Toronto; Simons E, University of Manitoba; Takaro T, Simon Fraser University; Tebbutt S, University of British Columbia; To T, The Hospital for Sick Children & University of Toronto.

SUPPLEMENTARY MATERIALS

Human milk oligosaccharide profiles and food sensitization among infants in the CHILD Study

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Supplementary Methods

Design and study population

This study was embedded in the CHILD Study, a population-based birth cohort recruited from four sites in Canada (1). Women with singleton pregnancies from Edmonton, Manitoba, Toronto and Vancouver were enrolled between 2009 and 2012 and remained eligible if they delivered a healthy infant >35 weeks gestation. This study was performed in a representative subsample of breastfed infants of the CHILD study (N=421). Mother-infant dyads with available breast milk samples and 1 year food sensitization data were randomly selected with equal weighting from the 4 study sites. This study was approved by the Human Research Ethics Boards at McMaster University and the Universities of Manitoba, Alberta, Toronto and British Columbia.

HMO measurements

In advance of the 3-4 months postpartum CHILD home visit (median 15.6 weeks, 95% range 11.6, 30.6), mothers were provided a sterile collection jar and instructed to collect, mix and refrigerate breastmilk from multiple feeds during a 24 hour period (2). Breastmilk samples were aliquoted aseptically according to a standardized protocol, and then stored at -80°C. Samples were analyzed at the University of California San Diego as previously described (3). Raffinose was added to each milk sample as an internal standard for absolute quantification. HMOs were isolated by high-throughput solid-phase extraction, fluorescently labeled with 2-aminobenzamide, and analyzed by High Performance Liquid Chromatography (HPLC) with fluorescence detection. The following 19 HMOs were detected and quantified based on standard retention times and mass spectrometric analysis: 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), difucosyllactose (DFLac), difucosyllacto-N-hexaose (DFLNH), difucosyllacto-N-tetrose (DFLNT), disialyllacto-N-hexaose (DSLNH), disialyllacto-N-tetraose (DSLNT), fucodisialyllacto-N-hexaose (FDSLNH), fucosyllacto-N-hexaose (FLNH), lacto-N-fucopentaose (LNFP) I, LNFP II, LNFP III, lacto-N-hexaose (LNH), lacto-N-neotetraose (LNnT), lacto-N-tetrose (LNT), sialyl-lacto-N-tetraose b (LSTb), and sialyl-lacto-N-tetraose c (LSTc). The total HMO concentration (nmol/L) was calculated as the sum of the annotated oligosaccharides. In addition to absolute concentrations we created an overall HMO discriminant score using a multivariable classifier (Projection on Latent Structures Discriminant Analysis, PLS-DA).

Food sensitization

Allergy skin tests were performed using the Duotip-Test II (Lincoln Diagnostics Inc, Mississauga, ON, Canada) with the following food allergens (ALKAbello, Mississauga, ON, Canada): milk, egg, soy and peanut. Histamine (1 mg/mL) was the positive control, and glycerine was the negative control. Food sensitization was defined as a positive skin test response to one or more food allergen. A wheal size of 2 mm or greater than that elicited by the negative control was considered positive (4). Detailed information on food sensitization assessment has been previously reported (5, 6).

Covariates

We used questionnaires at enrollment in the study (second or third trimester of pregnancy) to collect information about maternal age, ethnicity, educational level (completion of postsecondary degree) and study site (2). Maternal food allergies were self-reported and lactation stage (infant age when milk samples were collected) was documented (2). Infant sex, gestational age and weight at birth were obtained from medical records. Information on child age at food sensitization assessment, breastfeeding duration, breastfeeding exclusivity at 6 months, timing of introduction to solid foods and pet ownership was collected using postnatal questionnaires (2).

Statistical analysis

We examined the univariate associations between food sensitization and HMOs using Mann-Whitney tests. We used a multivariable classifier (Projection on Latent Structures-Discriminant Analysis, also known as Partial Least Squares-Discriminant Analysis, PLS-DA) to evaluate associations with overall HMO profiles (7-9). Employing the *pls* package version 2.5-0 in R (9), we fitted a PLS model to combine and reduce the HMO data from each sample into a single PLS-DA score for food sensitization (7-9). The weighted sum of absolute regression coefficients provides a single measure for the importance of each HMO measure based on the reduction in the error sum of squares and was used to order the relative importance of the various metabolites. Thereafter we explored the association of the discriminant score with food sensitization at 1 year using a receiver operating curve (ROC) function and standard multivariable logistic regression analyses. The discriminant score was analyzed both continuously and using quintiles. The regression models were first adjusted for child's sex and exact age at food sensitization assessment (basic models), and subsequently additionally for maternal education, ethnicity, lactation time postpartum, maternal food allergies, infant birthweight and gestational age at birth, breastfeeding duration, breastfeeding exclusivity at 6 months, timing of introduction to solid foods, household pets and study site (adjusted model). These covariates were included in the models based on previous literature or a change in discriminant score effect estimates of >10% (10).

To assess whether the associations were different by infant sex, maternal atopy or secretor status, or breastfeeding exclusivity (11), we evaluated the potential statistical interaction by adding the product term of the covariate and discriminant score to the models. However, we did not stratified the analyses in any of these factors because no

significant interaction terms were observed (p-values >0.3). Lastly, we performed a sensitivity analysis excluding infants with parent-reported food allergy symptoms before the age of milk sampling (n=399) (**Figure S1**). Analyses were performed using the Statistical Package for the Social Sciences version 21.0 for Windows (SPSS IBM, Chicago, IL, USA) and RStudio (Version 0.99.896, R Foundation for Statistical Computing,Vienna).

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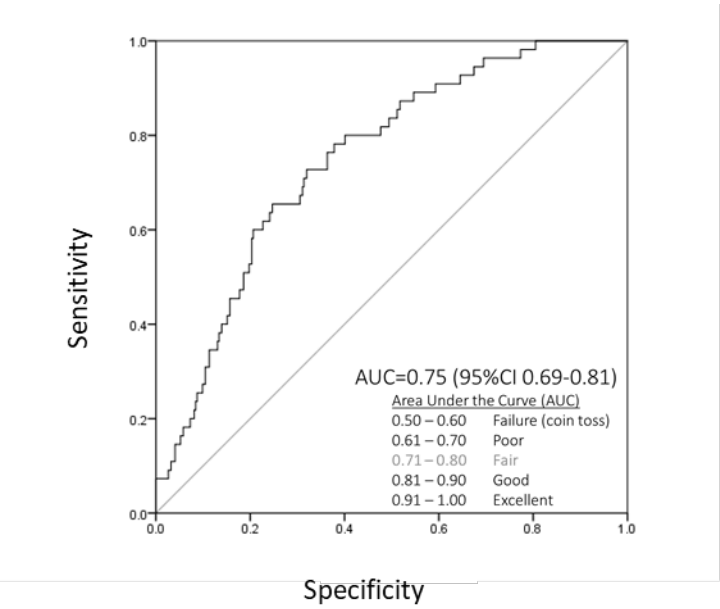
Table S1. Subjects characteristics in the CHILD cohort (N=421)

Maternal characteristics	
Age, y	33.0 (4.2)
Ethnicity, n (%)	
Asian	76 (18.1)
Caucasian	310 (73.6)
First Nations	17 (4.0)
Other	18 (4.3)
Completed post-secondary education, n (%)	347 (83.0)
Maternal food allergies, n (%)	123 (29.2)
Infant characteristics	
Sex, boys, n (%)	218 (51.8)
Age at food sensitization assessment, months	12.2 (11.0, 16.8)
Gestational age at birth, weeks	39.0 (36.0, 41.0)
Birthweight, g	3467.6 (479.9)
Furry pets, n (%)	221 (52.6)
Food sensitization at 1 year, n (%)*	59 (14.1)
Egg	34 (8.1)
Milk	12 (2.9)
Peanut	25 (6.0)
Soy	5 (1.2)
Feeding characteristics	
Lactation time at milk sample collection, weeks	15.6 (11.6, 30.6)
Breastfeeding duration, months	12.9 (5.7)
Exclusive breastfeeding for 6 months, n (%)	105 (25.1)
Timing of introduction to solid foods, months	5.0 (1.0)
HMOs, (nmol/mL)	
2'FL	4997.0 (0.5, 11695.5)
3FL	485.8 (101.4, 1306.6)
LNnT	284.2 (37.7, 1254.3)
3'SL	516.2 (153.3, 1299.2)
DFLac	516.0 (14.2, 1374.4)
6'SL	208.0 (77.6, 678.3)
LNT	1364.9 (444.1, 3225.6)
LNFP I	652.3 (68.1, 3497.7)
LNFP II	1904.2 (691.4, 4490.1)
LNFP III	97.6 (27.9, 260.0)
LSTb	102.1 (28.8, 290.1)
LSTc	30.7 (3.5, 167.7)
DFLNT	1461.4 (75.2, 2862.8)
LNH	60.9 (6.3, 164.7)
DSLNT	181.5 (29.9, 677.4)
FLNH	59.3 (9.1, 15.8)
DFLNH	26.6 (6.6, 228.3)
FDSLNH	295.7 (83.1, 935,6)
DSLNH	50.3 (7.9, 219.6)
Total	14861.7 (6518.8, 20801.7)

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. *Sensitized to one or more of: egg, milk, soy or peanut (mean wheal diameter ≥2 mm). Abbreviations of HMOs are given in the S**upplementary Methods.**

Figure S1. The receiver operating characteristics (ROC) curve on overall HMO profile and infant food sensitization, excluding infants with food allergies prior to milk sample collection, in the CHILD Study (N=399)



Sensitivity analysis on the overall HMO profile and infant food sensitization in the CHILD Study, excluding infants with food allergies prior to milk sample collection (N=399). Abbreviations: AUC, area-under-the-curve.