Human milk oligosaccharide profiles and allergic disease up to 18 years

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Background: Human milk oligosaccharides (HMO) are a diverse range of sugars secreted in breast milk that have direct and indirect effects on immunity. The profiles of HMOs produced differ between mothers.

Objective: We sought to determine the relationship between maternal HMO profiles and offspring allergic diseases up to age 18 years.

Methods: Colostrum and early lactation milk samples were collected from 285 mothers enrolled in a high-allergy-risk birth cohort, the Melbourne Atopy Cohort Study. Nineteen HMOs were measured. Profiles/patterns of maternal HMOs were determined using LCA. Details of allergic disease outcomes including sensitization, wheeze, asthma, and eczema were collected at multiple follow-ups up to age 18 years. Adjusted logistic regression analyses and generalized estimating equations were used to determine the relationship between HMO profiles and allergy.

Results: The levels of several HMOs were highly correlated with each other. LCA determined 7 distinct maternal milk profiles with memberships of 10% and 20%. Compared with offspring exposed to the neutral Lewis HMO profile, exposure to acidic Lewis HMOs was associated with a higher risk of allergic disease and asthma over childhood (odds ratio asthma at 18 years, 5.82; 95% CI, 1.59-21.23), whereas exposure to the acidic-predominant profile was associated with a reduced risk of food sensitization (OR at 12 years, 0.08; 95% CI, 0.01-0.67).

Conclusions: In this high-allergy-risk birth cohort, some profiles of HMOs were associated with increased and some with decreased allergic disease risks over childhood. Further studies are needed to confirm these findings and realize the potential for intervention. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *Human milk oligosaccharides, allergy, childhood, latent class analysis*

Human milk oligosaccharides (HMOs) are the third largest solid component of breast milk after lactose and fat, amounting to between 5 and 15 g/L depending on the individual and lactation stage. They comprise a large family (>150 currently known[1]) of structurally diverse complex sugars that have a backbone of lactose (glucose + galactose) and additions of 3 other monosaccharides (*N*-acetylglucosamine, fucose, and *N*-acetylneuraminic [sialic] acid) in varying quantities and patterns.

HMOs are unique to human milk and have a diverse range of biological functions[2] including both direct and indirect influences on human immunity. Direct effects may occur at multiple body sites including the intestine, the systemic circulation, and the urinary system, where the small amount absorbed from the intestine is excreted. HMOs are largely nondigestible, but approximately 1% of the total volume is absorbed.[3,4] HMOs bind to cell surface receptors (lectins/glycan-binding proteins), which are present on intestinal and urinary tract epithelial cells and a large variety of immune cells (T cells, monocytes, macrophages, dendritic cells, neutrophils, eosinphils, basophils, and natural killer cells), thereby modulating immune responses.[5,6]

Even at low concentrations (1 μg/mL) there is evidence that HMOs are able to directly enhance maturation of T_H1 lymphocyte responses.[7] Another mechanism of direct immune defense in the intestine is through the ability of HMOs to act as decoy molecules for bacterial pathogens seeking to bind to epithelial surfaces. In this way, common pathogens causing infant gastroenteritis, including *Campylobacter jejunii* and *Escherichia coli*, are rendered nonpathogenic through binding to specific HMOs rather than intestinal epithelium.[8,9] There is also evidence of direct effects for viral gastroenteritis pathogens,[10] and for bacterial pathogens (urogenic *E coli*) in the urinary tract.[11]

HMOs also have indirect immune effects. Breast-feeding is a predominant factor in establishing the infant's gut microbiome,[12] through providing specific HMOs as an energy source for potentially benefical gut bacteria.[2,13] Establishing a healthy gut microbiome is vital for immune programming, educating and shaping the individual immune response, which in turn leads to protection

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This work was funded by the Asthma Australia and the National Health and Medical Research Council of Australia. None of the work was directed by or influenced by these institutions.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication November 18, 2019; revised June 18, 2020; accepted for publication June 24, 2020.

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0091-6749/$36.00

https://doi.org/10.1016/j.jaci.2020.06.027

Abbreviations used
CHILD: Canadian Healthy Infant Longitudinal Development
DSLNT: Disialyllacto-*N*-tetraose
HMO: Human milk oligosaccharide
LCA: Latent class analysis
LSTc: LS-tetrasaccharide c
MACS: Melbourne Atopy Cohort Study
6'SL: 6'-sialyllactose

from pathogenic organisms and tolerance of innocuous or commensal microorganisms. Specific maternal HMO profiles determine the type and quantity of gut bacteria forming the early-life gut microbiome.[2] Gut bacteria assist immune health through production of the short-chain fatty acids butyrate, acetate, and propionate. These short-chain fatty acids nourish the intestinal wall of the infant to maintain a healthy barrier between the gut and the internal body, lower the intestinal pH, and have anti-inflammatory properties.[5]

Maternal milk profiles are determined by several factors. Predominant among them is the genetic ability to make specific glycosyltransferases able to link the different monosaccharide building blocks to the HMO molecules in specific ways.[14] Two of these glycosyltransferases, which mimic blood group characteristics, are well known. Presence of a secretor gene coding for α-(1-2)-fucosyltransferase allows addition of fucose to HMO molecules using an α 1-2 link. The presence of the Lewis gene codes for α-(1-3/4)-fucosyltransferase, allowing fucose to be added using α 1-4/3 links. Similarly, there are transferases able to link other sugars to the HMO molecule including α-(2-3)-sialylyltransferases and α-(2,6)-sialylyltransferases; however, which of the different transferases catalyze which linkage is less well established. HMOs with the addition of 1 or more sialic acid are classed as acidic, whereas those without are considered to be neutral.

Only 3 studies have investigated the role of HMOs in allergic disease[15-17] and although the findings from these studies are valuable, their findings are inconsistent with respect to allergic disease outcomes, and more evidence is needed. All previous studies have been limited to outcomes in children younger than 6 years. This is not ideal because allergic manifestations in early childhood may be transient and not indicative of long-term allergic disease. One of the main difficulties in determining the relationship between HMOs and allergic outcomes stems from the large number of HMOs able to be accurately quantified in human breast milk and the likelihood that some may have similar or partially overlapping biological mechanisms. If their associations with allergic outcomes are similar and/or highly correlated, assessing the individual effects of each HMO is problematic. Furthermore, the pattern of HMOs rather than individual HMOs may be more important for establishment of healthy gut bacteria. It may, therefore, give a more accurate picture to investigate HMOs after categorizing them into representative groups or classes.

We aimed to investigate the association between HMOs and common allergic outcomes including asthma, eczema, and sensitization up to age 18 years in the Melbourne Atopy Cohort Study (MACS), a high-allergy-risk birth cohort. We aimed to investigate the association between these HMOs individually as well as characterize typical profiles or groups, using latent class analysis (LCA).

METHODS

The MACS was established in 1990, recruiting 620 babies, while *in utero*, at high risk of allergic disease. They had at least 1 immediate family member (mother, father, or sibling) with self-reported asthma, eczema, hay fever, or severe food allergy. The study commenced as a randomized controlled trial of the effects of infant formulas on weaning on allergic disease risk.[18] The study has continued as an observational birth cohort.[19] The children were followed up 18 times in the first 2 years, then yearly until age 7 years, and then at age 12 and 18 years with measurements of allergic disease outcomes. This study was approved by human research ethics committees of the Mercy Maternity Hospital, Royal Children's Hospital, and University of Melbourne.

Milk samples and HMO quantification

Milk samples were collected from 285 mothers as colostrum (within 3 days of birth; N = 181), day-5 breast milk (N = 121), and/or 3-month breast milk (N = 145). Milk samples were solicited from all mothers (there was no selection process). Of the 285 mothers, 9 mothers had breast milk only at 3 months. Mothers (or nurses) expressed milk into sterile containers with clean hands after wiping the areolar area with a clean face washer rinsed in water. They were asked to express in the morning, before the first feed of the day. If the sample was inadequate, they were asked to express before subsequent feeds in the same day. Most samples collected up to 5 days were completed in hospital with the help and supervision of MACS research nurses. Collected samples were placed directly in the freezer section of the fridge in either the hospital ward or the participants' home (−20°C). These samples were moved to a dedicated −20°C freezer in the Mercy Hospital after a few days (if mother was an inpatient) or were brought directly into the Mercy Hospital by the participants if the mother was an outpatient. Because mothers expressed milk before a hospital visit, the samples remained in participants' home freezers for a maximum time of 7 days. In 2008, these samples were transferred to a −80°C freezer. Only the first sample from each mother was used in this analysis.

Aliquoted samples had HMO measurement at the University of California, San Diego. The method used has been described in previous publications.[15] Raffinose was added to each sample before processing, providing an internal quantification standard. More detailed methods are outlined in this article's Online Repository at www.jacionline.org.

Allergic disease outcomes from questionnaires

Allergic outcomes up to age 2 years were defined by parental responses to telephone questionnaires conducted by a research nurse. These were performed every 4 weeks until 64 weeks, at 18 months, and at 24 months. Variables from 12- and 18-year follow-ups were defined by parental and personal responses to self-administered questionnaires at age 12 and 18 years.

Asthma/wheeze

Wheeze at age 6 months and 2 years was defined by parental report of cough, wheeze, or rattle in the past 6 months or 12 months, respectively. Asthma at age 12 and 18 years was defined as any positive response to the question "Have you had any episodes of asthma in the past 12 months" and/or the use of asthma medication during that time.

Eczema

Eczema at age 6 months and 2 years was defined by parental report of a doctor diagnosis of eczema or the use of steroid-containing creams for rash (except for the scalp and nappy area) in the past 6 months or 12 months, respectively. Eczema at age 12 and 18 years was defined by a positive answer to the question "Have you had eczema in the past 12 months or used any eczema medications in that time?"

Sensitization to food and aeroallergens

Clinical visits for skin prick test were performed at 0.5, 2, 12, and 18 years. All tests were performed by trained research staff with single-prick lancets. At

age 0.5, 2, and 12 years, children were tested with a panel of 3 food (egg white, cow's milk, and peanut) and 3 aeroallergens (house dust mite, rye grass, and cat dander) (Hollister-Stier). At age 18 years, along with the previous allergens, cashew, shrimp, *Penicillium notatum*, *Cladosporoides, Alternaria tenuis*, and mixed grass (providers included Hollister-Stier [Spokane, Wash], Alk-Abelló [Round Rock, Tex], and Stallergenes [Lyon, France]). Up to age 2 years, any wheal of size greater than or equal to 2 mm was considered positive. At age 12 and 18 years, 1 or more reactions with wheals greater than or equal to 3 mm were considered positive.

Covariates

We chose covariates to be included in the model with reference to existing literature and using a causal modeling approach using Directed Acyclic graphs.[20] After establishing that there was no good evidence that sex of the child or total length of breast-feeding was an effect modifier (interaction *P* between class and sex, or breast-feeding >.10), these 2 variables were included as confounders *a priori*. Variables that had the potential to be common causes of both exposure and outcome were considered for inclusion. We did not include variables believed to be common effects of exposure and outcomes or those considered to be on the causal path. Because most mothers were atopic, we did not include a variable for maternal atopy. Final confounders used in all models included sex, length of any breast-feeding, maternal age at birth of child, the presence of siblings, parental education at the child's birth (either parent tertiary educated vs neither), and parental smoking at the child's birth (either or both parents smoked).

Statistical analysis

A correlation analysis of the 19 measured HMOs found a high degree of correlation between several of the included HMOs (for heat map of correlation matrix, see Fig E1 in this article's Online Repository at www.jacionline.org). Therefore, we used LCA to determine latent groups of HMO profiles in the MACS mother cohort. The earliest milk samples from each mother were used. To facilitate the analysis, each HMO concentration was considered as a categorical variable and was coded 1 if above its median value and 0 if otherwise. The optimal number of classes expressing distinct profiles of maternal HMOs was determined using model fit statistics. As with other analyses of this kind, classes were chosen largely on the basis of Baysian information criterion. Once determined, these classes were used in multinomial regression analyses. We used both the most likely class for each mother and probability weighting for the chance of belonging to each class. We investigated the association between the classes identified from LCA and the respiratory outcomes measured at various time points. Logistic regression was used to investigate the association at each time point, whereas generalized estimating equations were used to investigate the association over time (see Table E5 in this article's Online Repository at www.jacionline.org). In both cases, the outcome was regressed against the classes identified from LCA. In line with ideas expressed by Rothman,[21] we have made no correction for multiple comparisons.

RESULTS

Characteristics

The MACS participants were predominantly of white ethnicity. Of the participants included in these analyses, 74% of families had at least 1 parent who had received tertiary education (Table I). The mothers were a highly allergic group, with 40% reporting asthma, 38% reporting eczema, and 38% reporting "severe" food allergy ever at the time of enrollment into the study. Only 57 mothers (19%) reported no allergic diseases. Relatively few of the mothers were smokers, with only 3% smoking within the first 4 weeks of the child's birth. The sex split of the children was roughly equal (48% boys), and they were breast-fed for an average of 48 weeks. The prevalence of eczema was high in early life, with 31% of children affected by age 6 months, but this reduced over time, with 19% of young adults reporting eczema at age 18 years. Wheeze was common in early life (59%), and at age 18 years, 18% of young adults had asthma. Food sensitization was most common at age 1 year (20%), a time when aeroallergen sensitization was least common (14%). This situation reversed over time and by age 18 years only 7% of young adults were sensitized to any food allergens whereas 46% were sensitized to at least 1 aero-allergen. The distributions of 19 HMOs found are presented in Table E1 in this article's Online Repository at www.jacionline.org.

LCA results

Using LCA, we found that a 7-class model was the best fit, with class membership varying from 10% to 20%. Table E2 in this article's Online Repository at www.jacionline.org presents model fit characteristics. Further inspection of the classes revealed distinct patterns of HMOs related to each class as illustrated by Fig 1 and Table E3 in this article's Online Repository at www.jacionline.org. We named these classes on the basis of not only our knowledge of secretor and Lewis status but also predominant or missing HMOs (Table II).

Choosing a baseline class

Because we were interested in associations with allergic disease, we selected our baseline class as that least associated with allergic disease outcomes in our cohort based on a heat map exploring the relationship between the LCA classes and the allergic outcomes (see Fig E2 in this article's Online Repository at www.jacionline.org). We decided upon the neutral Lewis class (6) as baseline.

Association between latent classes of HMOs and allergic outcomes at different times

Where there was some evidence of association (defined by a *P* value < .1) between classes and allergic outcomes, the results are outlined in Table III. The other associations investigated are presented in Table E4 in this article's Online Repository at www.jacionline.org.

There was some evidence that infants exposed to HMOs in the acidic Lewis class (7) were at a higher risk of eczema and respiratory allergy over their childhood when compared with those exposed to HMOs in the neutral Lewis class (6). They had an increased risk of eczema (from age 6 months) and sensitization in early life (from age 2 years), with increased risk of wheeze at age 12 years and wheeze and asthma at age 18 years. Class 7 (acidic Lewis) differs from class 6 (neutral Lewis) by the presence of higher concentrations of sialylated (3'-sialyllactose, disialyllacto-*N*-tetraose [DSLNT], and LS-tetrasaccharide b) and some fucosylated (3-fucosyllactose and difucosyllactose) HMOs. There was also some evidence that this class may be associated with an increased risk of aero-allergen sensitization and asthma at age 18 years and eczema throughout childhood, with odds ratio point estimates ranging between 1.4 and 2.9 (Table E4).

Infants exposed to acidic-predominant (5) HMOs also appeared to have less risk of any and aero-allergen sensitization at age 6 months and food sensitization at age 2 and 12 years, when compared with those in the neutral Lewis class (6), although the risk of eczema was increased at age 2 and 12 years. The

TABLE I. Characteristics of parents and children

Parental characteristics at baseline (birth of child)	Value		
Parental			
Tertiary education, n (%)	222 (74)		
Maternal			
Age at child's birth (y) (average and SD)	31.4 (4.2)		
Reported asthma, n (%)	120 (40)		
Reported eczema, n (%)	115 (38)		
Reported severe food allergy, n (%)	113 (38)		
No. of cigarettes/day at birth (range and median) among smokers	2-30 (median, 4.5)		
Smoking first 4 wk	7 (3)		
Paternal			
No. of cigarettes/day at birth (average and SD)	1.9 (5.8)		
Smoking first 4 wk	39 (14)		
Child characteristics	**Value**		
Sex, boys, n (%)	145 (48)		
Breast-feeding duration (wk) (average and SD)	48 (25.5)		
No. of siblings at birth ≥1, n (%)	77 (26)		
Eczema, n (%)			
0.5 y	71 (24)		
2 y	80 (27)		
12 y	46 (24)		
18 y	35 (17)		
Wheeze, n (%)			
0.5 y	85 (28)		
2 y	35 (12)		
Asthma, n (%)			
12 y	41 (21)		
18 y	45 (21)		
Sensitization, n (%)	Food	Aero	Any allergen
0.5 y	56 (20)	24 (9)	66 (24)
2 y	36 (16)	64 (28)	75 (32)
12 y	34 (18)	94 (49)	96 (50)
18 y	22 (11)	138 (68)	140 (70)

acidic-predominant class (5) had high concentrations of the acidic HMOs (6'-sialyllactose [6'SL] and LS-tetrasaccharide c [LSTc]). Both 6'SL and LSTc have an α 2-6–linked sialic acid to the terminal galactose in common.

There was also some evidence that this class may be associated with a lower risk of several other allergic outcomes presented in Table E4. Notably, all point estimates for sensitization over childhood were less than 1 (range, 0.09-0.9). Also, there was some evidence of reduced asthma risk at age 18 years (OR, 0.25; 95% CI, 0.05-1.35).

DISCUSSION

Using the pattern recognition properties of LCA, we determined 7 discrete profiles of maternal HMOs in early breast milk. These profiles differed in biologically plausible ways according to the ability of the mother to add monosaccharide residues using fucosyl- and sialyltransferases. We found evidence of an association between exposure to acidic Lewis milk and increased risk of allergic respiratory disease over childhood, persisting up to age 18 years. We also found some evidence for reduced risk of food sensitization for those exposed to the predominantly acidic HMO profile.

There is currently very little information on the associations between HMOs and allergic diseases,[22] and the published evidence is not consistent. Sjogren et al[23] performed a nested case-control study of 20 mother/child pairs from a birth cohort in Sweden, finding no association between individual concentrations of 9 neutral oligosaccharides and the risk of allergic disease up to age 18 months. There was a small amount of evidence to suggest that increasing total concentration of the 9 neutral HMOs might be associated with increased risk of allergic disease.[23]

In another Swedish study, Sprenger et al[17] found some evidence of an association between increased levels of FUT2-dependent HMOs and an increased risk of atopic eczema (43% [3 of 7] compared with 14% [6 of 44]) at age 2 years, but not at age 5 years in 51 high-risk infants born by cesarian section.

A case-contol study nested within a high-risk Finnish birth cohort investigated HMOs of the mothers of 39 infants with and 41 infants without cow's milk allergy confirmed by oral food challenge at age 6 months.[16] They found no evidence of an association between FUT2 secretor status and cow's milk allergy. However, they did find some evidence of an association between lower levels of 6' siayllactose (6'SL), lacto-*N*-fucopentaose I (LNFI), lacto-*N*-fucopentaose III (LNFIII), and disiayllacto-N-tetaose (DSLNT) in maternal milk and cow's milk allergy in the infants. They further investigated

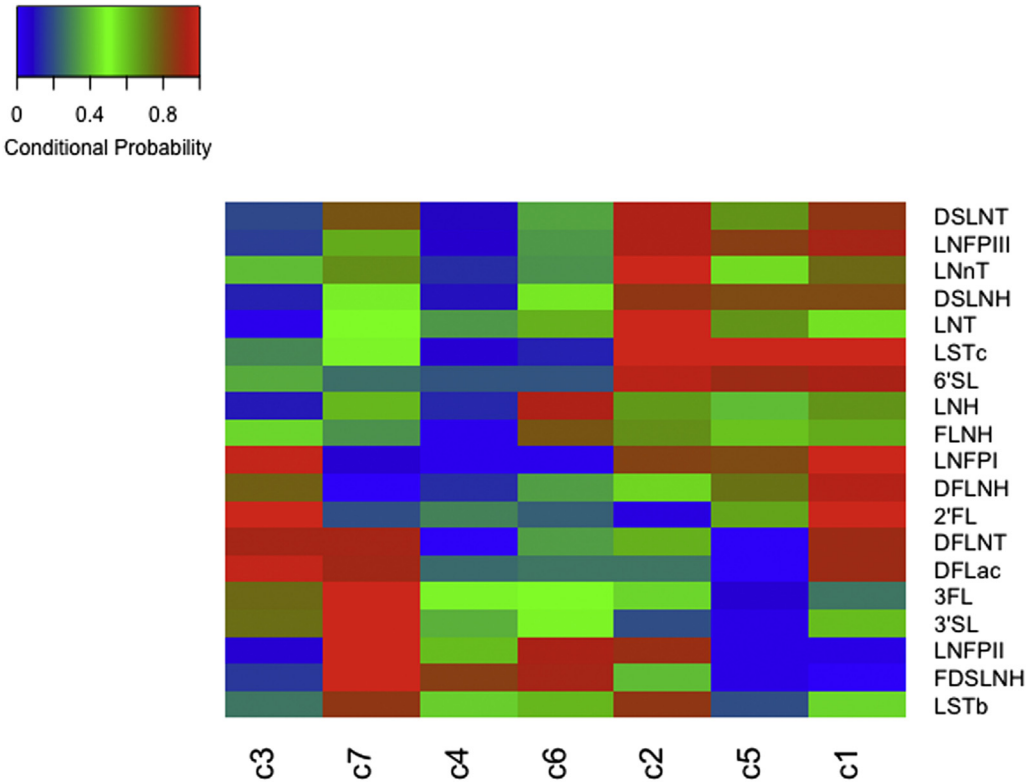

FIG 1. Heat map of the 19 HMOs (y-axis) for each of the 7 latent classes (x-axis). The colors represent the conditional probabilities of having HMO concentrations higher than the median, with blue being a low probability and red being a high probability. The classes are not in numerical order. *DFLac,* Difucosyllactose; *DFLNH,* difucosyllacto-*N*-hexaose; *DFLNT,* difucosyllacto-*N*-tetraose; *DSLNH,* disialyllacyo-*N*-hexaose; *FDSLNH,* fucosly-disiallacto-*N*-hexaose; *FLNH,* fucosyllacto-*N*-hexose; *LNFPI,* lacto-*N*-fucopentaose I; *LNFPII,* lacto-*N*-fucopentaose II; *LNFPIII,* lacto-*N*-fucopentaose III; *LNH,* lacto-*N*-hexasose; *LNnT,* lacto-*N*-neotetraose; *LNT,* lacto-*N*-tetraose; *LSTb,* LS-tetrasaccharide b; *2′FL,* 2′-fucosyllactose; *3FL,* 3-fucosyllactose; *3′SL,* 3′-sialyllactose.

TABLE II. Naming the classes identified using LCA

Class	HMO Characteristics	Name
1	High 2'FL, DFLac, 6'SL, LNFPI, LSTc, DFLNT, DFDLNH Low LNFPII, FDSLNH	Acidic secretor
2	High LNnT, 6'SL, LNT, LNFPIII, LSTc, DSLNT Low 2'FL	Generally high, acidic nonsecretor
3	High 2'FL, DFLac, LNFPI, DFLNT Low LNT, LNFPII	Neutral secretor
4	Highest is FDSLNH Low LNFPI, LNFPIII, LSTc, DFLNT, FLNH, DSLNH	Generally low
5	High 6'SL, LSTc Low 3FL, 3'SL, DFLac, FLNPII, DFLNT, FDSLNH	Acidic predominant
6	High LNFPII, LNH, FDSLNH Low LNFPI	Neutral Lewis
7	High 3FL, 3'SL, DFLac, LNFPII, DFLN, FDSLNH Low LNFPI, DFLNH	Acidic Lewis

DFLac, Difucosyllactose; *DFLNH*, difucosyllacto-*N*-hexaose; *DFLNT*, difucosyllacto-*N*-tetraose; *DSLNH*, disialyllacyo-*N*-hexaose; *FDSLNH*, fucosly-disiallacto-*N*-hexaose; *FLNH*, fucosyllacto-*N*-hexose; *LNFPI*, lacto-*N*-fucopentaose I; *LNFPII*, lacto-*N*-fucopentaose II; *LNFPIII*, lacto-*N*-fucopentaose III; *LNH*, lacto-*N*-hexasose; *LNnT*, lacto-*N*-neotetraose; *LNT*, lacto-*N*-tetraose; *2'FL*, 2'-fucosyllactose; *3FL*, 3-fucosyllactose; *3'SL*, 3'-sialyllactose.

HMO patterns, finding a coexpressed cluster (6'SL, LSTc, and LNFIII) that was associated with an increased risk of cow's milk allergy.

More recently, Miliku et al[15] investigated 421 infants in the Canadian Healthy Infant Longitudinal Development cohort for the relationship between HMOs and food sensitization at age 1 year using a projection on latent structures discriminant analysis. They found, in a general population sample, that HMO profiles characterized by higher concentrations of fucosly-disial-lacto-*N*-hexaose, lacto-*N*-fucopentaose II, lacto-*N*-neotetraose,

TABLE III. Evidence of associations between latent classes of HMOs and allergic outcomes (compared with baseline class 6) (associations presented if $P < .10$)

Latent class	Allergic outcome	n/N (%)	Reference class 6, n/N (%)	Age of outcome	Odds ratio	95% CI	*P* value
(1) Acidic secretor	Aero-allergen +	1/37 (3%)	9/51 (18%)	6 mo	0.11	0.01-1.12	.062
(1) Acidic secretor	Food allergen +	6/37 (16%)	14/51 (27%)	12 y	0.23	0.04-1.31	.098
(2) Acidic nonsecretor	Eczema	9/40 (23%)	8/59 (14%)	6 mo	2.59	0.85-7.93	.095
(2) Acidic nonsecretor	Any allergen +	5/18 (28%)	23/40 (58%)	12 y	0.34	0.1-1.2	.094
(3) Neutral secretor	Aero-allergen +	3/47 (6%)	9/51 (18%)	6 mo	0.27	0.07-1.02	.054
(3) Neutral secretor	Wheeze	17/36 (47%)	11/40 (28%)	18 y	2.99	1.11-8.09	.031*
(4) Generally low	Eczema	14/50 (28%)	8/59 (14%)	6 mo	2.59	0.96-7.0	.061
(4) Generally low	Aero-allergen +	3/40 (8%)	9/51 (18%)	6 mo	0.3	0.08-1.12	.072
(5) Acidic predominant	Eczema	11/40 (28%)	8/59 (14%)	6 mo	2.5	0.87-7.12	.088
(5) Acidic predominant	Eczema	11/28 (39%)	7/39 (18%)	12 y	3.14	0.96-10.27	.058
(5) Acidic predominant	Any allergen +	5/39 (13%)	17/51 (33%)	6 mo	0.31	0.1-0.99	.048*
(5) Acidic predominant	Aero-allergen +	2/39 (5%)	9/51 (18%)	6 mo	0.23	0.04-1.2	.081
(5) Acidic predominant	Food allergen +	1/30 (3%)	7/42 (17%)	2 y	0.11	0.01-0.97	.047*
(5) Acidic predominant	Food allergen +	1/29 (3%)	8/40 (20%)	12 y	0.09	0.01-0.69	.021*
(7) Acidic Lewis	Eczema	12/32 (38%)	8/59 (14%)	6 mo	4.24	1.36-13.24	.013*
(7) Acidic Lewis	Eczema	7/19 (37%)	7/39 (18%)	12 y	3.68	0.95-14.27	.06
(7) Acidic Lewis	Aero-allergen +	12/23 (52%)	10/42 (24%)	2 y	3.75	1.2-11.69	.023*
(7) Acidic Lewis	Any allergen +	12/23 (52%)	10/42 (24%)	2 y	3.73	1.21-11.53	.022*
(7) Acidic Lewis	Wheeze	8/20 (40%)	7/42 (17%)	12 y	3.8	1.06-3.6	.040*
(7) Acidic Lewis	Wheeze	11/18 (61%)	11/40 (28%)	18 y	6.1	1.57-23.74	.009*
(7) Acidic Lewis	Asthma	9/20 (45%)	7/40 (18%)	18 y	5.11	1.42-18.36	.012*

Adjusted for sex, smoking (at least 1 parent smoked), mother's and father's education, mother's age centered at mean, and presence of siblings.
*$P < .05$.

lacto-*N*-fucopentaose I, LSTc, and fucosyllacto-*N*-hexose and lower concentrations of lacto-*N*-hexasose, lacto-*N*-tetraose, 2'-fucosyllactose, and disialyllacyo-*N*-hexaose were associated with less risk of food sensitization at age 1 year.

In analyses of HMOs and allergic disease in breast-feeding mothers, Seppo et al[16] found an association between maternal atopy and increased concentrations of DSLNT. There is also some evidence of immunologic effects from trials of HMO supplementation. Goehring et al[24] investigated allergic disease markers in a substudy (n = 165) from a randomized controlled trial of formula feeding with or without supplementation of 2'-fucosyllactose compared with a breast-fed group. They found evidence of a relationship between 2'-fucosyllactose in formula or human milk and lower inflammatory cytokines compared with formula feeding without supplementation.[24]

There are several strong reasons why there may be poor agreement between different studies. There is evidence that the HMO profiles of women from different ethnic backgrounds, in differing environments, and with differing allergic predispositions may be quite distinct.[25,26] At this stage it is not known how much of these differences stem from genetics or environment. However, the existing differences may make the findings between studies nonconcordant. Another compelling reason is the emerging science for measuring HMOs. Because this is a relatively new field, methods of assessment and standardization may differ between laboratories, making between-study comparisons difficult. However, one of the most important reasons is the differences in analytical methods between studies. Some studies have investigated these associations using individual HMOs,[16,23] whereas others have used neutral HMOs[23] or FUT 2–dependent HMOs,[17] and finally some have used pattern recognition techniques[16] to define clusters or classes.

Our findings show some agreement with the associations found for FUT 2–dependent HMOs and neutral HMOs as investigated by Sprenger et al[17] and Sjogren et al,[23] respectively. In our analysis, pure secreters were represented by acidic secreters (1) and neutral secreters (3). As the names suggest, these classes differ from each other in terms of the levels of acidic HMOs. We found some evidence of an association between exposure to neutral secreter milk and increased risk of eczema at age 2 years and increased risk of wheeze over time when compared with neutral Lewis (6) milk.

Our research also shows some agreement with the findings of Seppo et al.[16] The coexpressed cluster of 6'SL, LSTc, and LNFIII is very similar to our acidic-predominant (5) class. This is the class for which we also found some evidence for a reduced risk of sensitization at age 1 year and food sensitization at age 18 years. Finding this protective pattern in 2 different cohorts using different methods adds an independent confirmation to the classes determined by our analysis. In terms of the more recent and larger analysis on the Canadian general population in the CHILD study, there are also some similarities, but the HMOs mentioned do not fit any one discrete class identified by our analysis. The CHILD study analysis[15] differs from ours in that, similar to a principal-components analysis, it determined the 10 most important HMOs for explaining the association of HMO exposure and food sensitization. Then, the association with food allergy was interpreted with reference to higher or lower values of all these 10 HMOs. The CHILD study did not determine classes or clusters, and it is perhaps not surprising that there were few similarities with these very different methods.

Allergic diseases are known to have a genetic component and maternal atopy is known to be related to HMO profiles.[26] Maternal atopy may be confounding the relationship between HMO profiles and allergic disease in the child, or HMO profiles

may mediate the relationship between maternal atopy and allergic disease. Because our cohort of mothers was almost entirely atopic, with only 57 nonatopic mothers, we were unable to investigate this relationship due to lack of power when these mothers were divided between 7 classes. However, finding distinct HMO profiles with varying associations with allergic disease suggests that factors other than atopy are influencing HMO profiles and allergic disease risk.

Our analysis used exploratory methods. We investigated 7 latent classes and several different outcomes (sensitization, eczema, wheeze, asthma) at various outcome ages between birth and age 18 years. Because of multiple comparisons, some findings may be due to chance alone. However, the consistent findings for our risk exposure class, acidic Lewis (7), and the similar protective findings from another study with respect to the acidic-predominant (5) class add weight to our findings. The confirmation of this lower food allergy risk acidic-predominant class in a separate study using different population and methods suggests that this finding is reasonably robust. There are as yet no confirmatory literature demonstrating similar findings for our acidic Lewis (7) class.

The classes found using LCA may be related to common linking structures for the sugar monosaccharides and, furthermore, may be related to the ability to express specific transferases. For example, the acidic Lewis class (7) is characterized by higher concentrations of sialylated (3'-sialyllactose, DSLNT, and LS-tetrasaccharide b) and some fucosylated (3-fucosyllactose and difucosyllactose) HMOs. Two of the 3 sialylated HMOs in this class, 3'-sialyllactose and DSLNT, carry the same α 2-3–linked sialic acid on the terminal galactose, and both LS-tetrasaccharide b and DSLNT share the α 2-6–linked sialic acid on the internal *N*-acetylglucosamine. Both 3-fucosyllactose and difucosyllactose are α 1-3–fucosylated on the reducing-end glucose. The acidic-predominant class (5) on the other hand has high concentrations of 6'SL and LSTc, and both of these acidic HMOs carry an α 2-6–linked sialic acid on the terminal galactose.

It is surprising that total duration of breast-feeding was found not to modify our relationships with allergic outcomes. If total dosage of HMOs was related to risk of allergic outcomes, then one would expect that greater or lesser exposure to HMOs would influence these relationships. However, we speculate that our finding that duration of breast-feeding did not modify the associations could suggest that there are critical windows of exposure. We might not have the ability to tease out this relationship in the MACS because all included women breast-fed for at least 2 weeks and more than 90% for greater than 29 weeks. Furthermore, the sample size might not be large enough to determine these relationships. In addition, because our samples are 25 years old, general degradation may limit our ability to determine these relationships.

Self-reported wheeze and asthma in adolescents may have a degree of misclassification for true asthma; however, there is evidence that self-reported asthma is valid when compared with doctor's diagnosis[27] and self-reported wheeze is a good proxy for asthma in adolescents.[28] At the 18-year follow-up in MACS, only 14 of 168 participants who answered yes to "asthma ever" did not have asthma confirmed by a doctor. Nevertheless, 7 of these 14 were using short- acting bronchodilators. Any misclassification of these variables was likely to be nondifferential so that the found associations may be attenuated.

We lacked information on maternal diet during breast-feeding. A recent systematic review found no consistent evidence of a relationship between maternal diet and childhood allergy, and so maternal diet is unlikely to be a strong confounder in our analysis.[29] However, maternal diet is known to influence HMO concentrations,[30] and it is unknown whether changes in diet influence membership of the 7 milk profiles described in our study. This would be important knowledge, because we may be able to promote beneficial HMO profiles through maternal dietary supplements or adjustments.

Recently, there has been an increase in randomized controlled trials investigating HMOs and non-HMO oligosaccharides and their supplementation to formula feeding. The stated aims of these studies are to "normalize" the infant gut microbiome to resemble that of breast-fed babies and to reduce the risks of necrotizing enterocolitis and infectious diseases. Although, from the body of evidence, it is likely that HMOs play a very important role in human immunity, the evidence from observational studies for a relationship between breast-feeding and allergic disease is still not clear,[31] and we do not yet have strong evidence concerning HMOs, or their profiles and allergic disease risk, to inform which supplements to be trialed.

Our analysis found some evidence that children exposed to acidic Lewis HMOs were at increased risk of allergic respiratory disease up to age 18 years, whereas those exposed to acidic-predominant HMOs may be protected from food sensitization. Because of multiple associations tested, it is possible that some findings may have arisen by chance. However, we believe that there is enough evidence of association shown by the consistent trends found for these objectively defined classes to warrant further research into this potentially important area. Further investigations are needed on the relationship between HMOs and allergic disease outcomes in large long-running birth cohorts, including on different populations and in different cultures. Furthermore, we need to understand through direct investigation of both HMOs and the microbiome, how the HMOs may modify the microbiome composition to influence the risk of allergic disease.

We thank John Thorburn for administration and recruitment, Anne Balloch for data management, and MACS parents and participants.

Clinical implications: Allergy risk in children may be linked to complex maternal HMO profiles and further research is needed on this potentially modifiable exposure.

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