

Human Milk Levels of 2'-Fucosyllactose and 6'-Sialyllactose are Positively Associated with Infant Neurodevelopment and are Not Impacted by Maternal BMI or Diabetic Status

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Abstract

Background and Aims: Human milk oligosaccharides are the third most abundant solid component of human milk and are associated with multiple health benefits. Whether or not certain maternal factors influence human milk oligosaccharide concentrations remain unclear.

Methods: In a pilot study, human milk samples from overweight, obese, normal weight and gestational diabetic mothers in the PROBE study were collected at 1 month postpartum. Eighty-two samples were analyzed by UHPLC-MS/MS to determine 2'-fucosyllactose (2'-FL) and 6'-sialyllactose (6'-SL) concentrations. Neurodevelopment of infants from mothers providing human milk samples was assessed by Bayley III scales at 6 and 18 months of age.

Results: No significant differences were found in 2'-FL or 6'-SL levels among study groups. A positive association was found between 6'-SL human milk concentration and cognitive and motor scale scores in infants at 18 months of age. An association between 2'-FL concentration and motor score at 6 months of age was found although was not significant in Low and High 2'-FL groups, separately.

Conclusions: 6'-SL and 2'-FL levels were not influenced by pre-conceptional maternal body mass index or the development of gestational diabetes mellitus. 6'-SL and 2'-FL levels in human milk were associated with infant cognitive development.

Keywords: 2'-fucosyllactose; 6'-sialyllactose; Brain; Gestational diabetes mellitus; Human milk oligosaccharides; Maternal pre-gestational weight

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total carbohydrate content of human milk do not differ between overweight/obese women compared to normal weight women [6,7]. However, there are inconsistencies regarding fat content. Some studies report caloric density and total fat are the same in human milk of overweight/obese and normal weight women [6-8] while others report significant differences with higher values in milk of overweight/obese women [9,10]. Several studies report differences in human milk fatty acid profiles of overweight versus normal weight mothers: overweight/obese mothers showed a lower content of n-3 Long Chain Polyunsaturated Fatty Acids (LCPUFAs), higher content of n-6 LCPUFAs [8,11-13], lower Monounsaturated Fatty Acids (MUFAs), higher amounts of Saturated Fatty Acids (SFAs) and lower PUFAs than normal weight mothers. Other molecules such as insulin, leptin [7,14,15], TNF- α , IL-6 [16,17] and glucose [10,16,17] have been reported to be higher in overweight/obese mother's milk. Human milk microbiome is also conditioned by maternal weight; a different microbiota profile and a lower diversity in human milk from obese women compared to human milk from normal-weight mothers have been observed [18-20]. Other maternal conditions such as diabetic status have been related to different human milk fat profiles compared to a reference group associated with changes in lipid metabolism [21]. Based on these macronutrient studies, the fat fraction of human milk is the most impacted by maternal variables such as overweight/obesity or diabetes.

Introduction

Human milk is the "Gold Standard" for term and preterm infant nutrition. Its unique composition is associated with multiple health benefits in human milk fed infants. Human milk composition is dynamic and changeable and depends on different factors such as preterm delivery [1], lactation stage, day, time [2] and some maternal variables such as parity, age, smoking habits, weight [3,4] or diet [5].

Substantial evidence suggests that maternal weight status may have an impact on human milk composition. Total protein and

Regarding carbohydrates, the total content is the same regardless of maternal weight [6,7]; however, little is known about the effect of those maternal conditions on the subset of human milk carbohydrates. The carbohydrate composition of human milk consists of lactose, as the main carbohydrate, with a concentration ranging from 67 to 78 g/L, and a diversity of oligosaccharides, which comprise approximately 10 g/L in human milk [2]. Human Milk Oligosaccharides (HMOs) significantly decrease through lactation: HMO concentrations have been reported to be 20–25 g/L in colostrum and reach 5–20 g/L in mature milk [22,23]. A recent research by Xu and coworkers showed that the concentration of HMOs at the 10th day of lactation was 19.3 ± 2.9 g/L and then decreased to the value 8.53 ± 1.18 g/L at the 120th day of lactation [24]. Not only lactation stage but also the delivery date has been noted as an important factor influencing HMO concentration. The concentration of total HMOs in preterm milk from days 10-23 of lactation was nearly two-fold lower than in term milk in the same period of lactation (3.6 g/L and 6.1 g/L, respectively). Recent research also showed important differences between preterm and term milk in mothers at the same postmenstrual age [25].

HMOs are the third most abundant solid component of human milk after lactose and lipids [26]. Mammalian milk oligosaccharides include acidic oligosaccharides, mainly sialylated such as 6'-sialyllactose (6'-SL), or neutral oligosaccharides, mostly represented by fucosylated compounds, such as 2'-fucosyllactose (2'-FL). Fucosylated glycans are the most abundant form in human milk while sialylated structures represent 10–20% of total human milk oligosaccharides [27]. The fucosylation process of glycans in human milk depends on the status of the mother regarding Secretor and Lewis histo-blood group systems: the secretor gene encodes α 1-2 fucosyltransferase (FUT2), an enzyme that transfers a fucose residue to a terminal galactose in an α 1-2 linkage giving rise to α 1-2 fucosylated compounds, such as 2'-FL, present in maternal secretions such as saliva (H antigen), tears (H antigen), and milk (2'-FL). Lewis gene encodes the α 1-3/4fucosyltransferase (FUT3) which transfers a fucose residue to glucose or an N-acetylglucosamine molecule in α 1-3 or α 1-4 linkages, producing compounds such as 3'-fucosyllactose (3'-FL) [28]. Thus, 2'-FL is not present in all human milk and its existence depends on the secretor status of the mother. Non-secretor subjects lack 2'-FL among other HMOs in their milk; however, some studies have shown low concentrations of α 1-2 fucosylated compounds in milk from non-secretor mothers [29-31]. It has been suggested that although most non-secretor individuals have a non-functional FUT2 mutation, other FUT2 mutations may preserve certain enzyme activity [32]; another hypothesis could be the existence of alternative routes for the synthesis of 2'-FL [33].

In secretor milk, 2'-FL is typically the most abundant oligosaccharide [28] and 6'-SL; together with 3'-sialyllactose (3'-SL) are predominant sialylated compounds [34]. 2'-FL concentration in human milk ranges from 0.004 to 6 g/L and 6'-SL varies from 0.011 to 1.57 g/L [23,32,33,35-39]; both compounds levels decrease through the lactation period [23,34].

Aside from lactation stage, delivery date or genetic secretor/non-secretor status little is known about how other maternal factors or the environment may influence HMO composition. Azad and coauthors recently investigated the association between fixed and modifiable factors with HMO concentrations, and important variations within secretor groups were found indicating other factors may influence HMO composition; in fact, an association between HMO concentration and lactation stage, parity, ethnicity, city of residence,

season or exclusive human milk feeding was found. On the contrary, maternal diet seemed not to influence HMO composition [40].

Few studies have assessed the effect of maternal overweight/obesity or diabetic status on the distribution of carbohydrates in human milk, and specifically on human milk oligosaccharide profiles. Smilowitz et al. carried out a study in which milks from mothers with and without Gestational Diabetes Mellitus (GDM) were analyzed for HMO abundance including other variables; results showed there were no differences in HMO composition between the 2 groups [41]. Isganaitis et al. reported that maternal obesity was associated with differences in human milk oligosaccharide content and composition in human milk at 1 month postpartum [9]. In a recent study in which mature human milk samples from 78 mothers were analyzed, 2'-FL concentration was significantly correlated with Pre-gestational Body Mass Index (pBMI) in secretor mothers [42].

HMOs have been linked to multiple biological functions such as effects on immune system and gastrointestinal development [43,44], anti-infective and bifidogenic activity [45-47], modulation of inflammation [48], gut motility involving activation of enteric neurons [49] and enhancement of Central Nervous System (CNS) functions [50]. HMO effects on cognitive function have been demonstrated in several preclinical models in the last decade [27,51-55]; clinical data are still limited with recent evidences of a positive association between 2'-FL human milk levels and cognitive development in infants at 24 months of age [56] and positive associations of relative abundance of fucosylated and sialylated HMOs with language at 18 months [57].

The present pilot study aimed to determine if maternal PBMI or diabetic status during pregnancy are linked to HMO profiles, specifically 2'-FL and 6'-SL concentrations, and whether those HMOs impact neurodevelopment in the offspring. To that end, we evaluated 2'-FL and 6'-SL levels in 1-month human milk samples in a secondary analysis of an existing cohort, a clinical trial called "Role of nutrition and maternal genetics on the programming of development of fetal adipose tissue" (PREOBE Study); in the PREOBE study overweight, obese and diabetic pregnant women and their offspring were compared to healthy, normal weight mothers and their offspring. Subsequently, to assess the role of 2'-FL and 6'-SL on infant neurodevelopment, 2'-FL and 6'-SL concentrations in human milk were compared with results from neurodevelopment assessments of infants born to mothers from the PREOBE study at different ages.

Materials and Methods

Clinical study design and milk sampling

The PREOBE study is a case control prospective cohort trial coordinated by the Department of Pediatrics of the School of Medicine at the University of Granada (Spain). The clinical study protocol was approved by the medical ethics committees of the Clinical University Hospital San Cecilio and the Mother-Infant University Hospital in Granada, Spain. All participants signed an informed consent at the beginning of the study. The trial was registered at www.ClinicalTrials.gov, identifier (NCT01634464).

The study design was previously published [58]. The PREOBE study was aimed to obtain genetic and biochemical biomarkers for the programming of obesity in the early stages of life. Some results derived from this clinical trial have been published [58-61]. The target population of this study consisted of 18 to 45 years old pregnant women between 12- and 34-weeks' gestation. There were 4 study

groups, reaching a final sample size of 331 subjects recruited: Healthy normal weight women ($18.5 \leq \text{pBMI} < 25 \text{ kg/m}^2$; $n=132$), overweight women ($25 \leq \text{pBMI} < 30 \text{ kg/m}^2$; $n=56$), obese women ($\text{pBMI} \geq 30 \text{ kg/m}^2$; $n=64$), and women with GDM ($\text{pBMI} \geq 18.5 \text{ kg/m}^2$; $n=79$). GDM group included both mothers who had already been diagnosed with GDM and mothers from normal weight, overweight and obese groups who developed GDM between recruitment and 34 weeks. The study flow chart is showed in Figure 1.

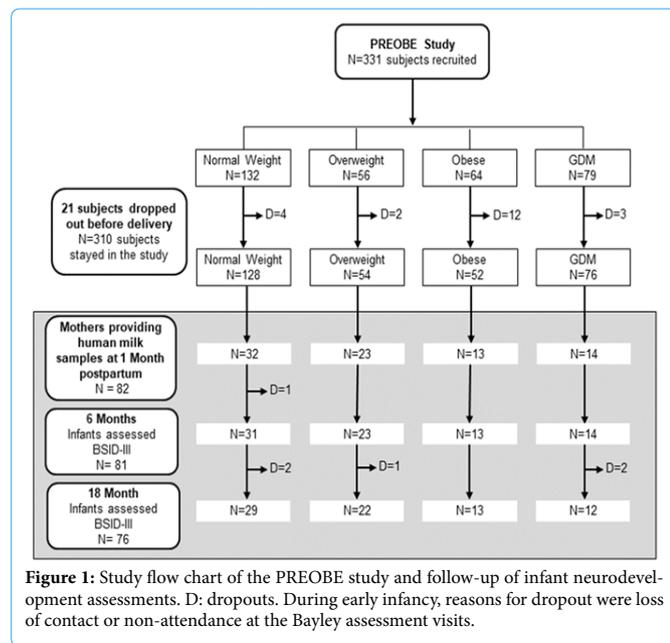


Figure 1: Study flow chart of the PREOBE study and follow-up of infant neurodevelopment assessments. D: dropouts. During early infancy, reasons for dropout were loss of contact or non-attendance at the Bayley assessment visits.

Subjects who participated in other research studies, received pharmacological treatment, used drugs, were on a vegan diet, or suffered from the following diseases: pre-gestational diabetes, hypertension or preeclampsia, fetal intrauterine growth retardation, maternal infection during pregnancy, hypo/hyperthyroidism, hepatic or renal diseases were excluded from the study.

The present neurodevelopment follow-up of infants born to mothers from the PREOBE study was conducted up to 18 months of age; infants' neurodevelopment was assessed at 6 and 18 months of age using the Bayley Scales of Infant Development, Third Edition (BSID-III). BSID-III has five domains: cognitive, language, motor, adaptive behavior and social-emotional development which are fully described in different publications [62,63]. The Bayley-III is appropriate for children between the ages of 1 month and 42 months. Generally, the evaluation of the adaptive and social-emotional domains is carried out using primary caregiver responses to a questionnaire.

Mothers were asked to collect human milk samples at 1 month postpartum (mature milk) in aliquots of 5 mL before and after each feed throughout 1 day, freeze aliquots at -20°C and bring them to the site within 1 week. A small cooling box was given to mothers to transport milk samples to the site. Once at the site, samples were mixed and then stored at -80°C and reserved for analysis.

UHPLC-MS/MS analysis

A total of 82 human milk samples collected at 1 month postpartum (mature milk) from the PREOBE study were analyzed by UHPLC-MS/MS as previously described [64] with some modifications. Briefly, human milk samples were thawed in a 37°C water bath for 10 minutes with smooth stirring to avoid separation of the fat fraction. A $25\mu\text{L}$ aliquot was placed in an Eppendorf tube with $500\mu\text{L}$ of MilliQ water,

vortexed for 1 minute and filtered through Amicon 10K filters 30 minutes at 8°C and 14,000 rpm.

Chromatographic analysis was performed using a UHPLC BEH Amide column ($2.1 \times 100 \text{ mm}$, $1.7 \mu\text{m}$ particle size) from Waters. The flow rate was $300 \mu\text{L}/\text{min}$. The column was maintained at 25°C , the sample at 20°C and the injection volume was $1 \mu\text{L}$. A gradient mobile phase consisting of 0.1% (v/v) ammoniac aqueous solution (solvent A) and 0.1% (v/v) ammonia in acetonitrile (solvent B) was used. Gradient conditions were: 0.0-3.0 min, 10-25% A; 3.0-8.0 min, 25-40% A; 8.0-8.1 min 40-10% A; and 8.1-10.0 min 10% A to stabilize the initial conditions. The total run time was 8.1 min, and the post-delay time for reconditioning the column with 10% A was 1.9 min. Weak solvent was a mixture of 25 mL of water and 75 mL of acetonitrile, and strong solvent was a mixture of 80 mL of water and 20 mL of acetonitrile. The quantification standard curves were prepared with 2'-FL and 6'-SL external standards at concentrations of 0.015, 0.1, 0.5, 2, 10, and 20 mg/L. Oligosaccharide standards were purchased from Inalco (>95% purity). The test samples were quantified in the appropriate dilution by interpolating within the linear dynamic range of the calibration equation. The concentration levels were obtained by applying its dilution factor afterwards.

Statistical analysis

All statistical analyses were carried out using the R statistical software package for Windows (version 3.4). To compare continuous variables, analysis of variance (One-way ANOVA) was performed for normally distributed data and Kruskal-Wallis rank-sum test for non-normal variables; Chi-square test was used for proportions. Data was expressed as Mean \pm Standard Deviation or frequencies depending on the outcome. Bonferroni or Dunn post hoc comparisons were used to identify significant pairwise group differences.

Pearson correlation and multivariate linear regression analyses were performed to assess the relationship between 2'-FL and 6'-SL concentrations and Bayley scales scores. Multivariate regression is defined by a β -coefficient being the degree of change in the outcome variable for every 1-unit of change in the predictor variable. Pearson correlation is defined by r coefficient that measures the strength and direction of association between two variables. Coefficient values may be negative or positive. To determine which confounding effects should be considered on the Bayley outcomes in the multivariate regression analyses, baseline or background factors were compared between the groups and those variables which were statistically different between groups were used as confounders.

Results

Demographic data, baseline and background characteristics

Demographic information, baseline conditions and other pertinent information obtained during the study period are shown in Table 1. As expected, a significant difference among groups in the pre-conceptual maternal weight and BMI was found with the lowest value for the normal weight group and the highest one for the obese group. Similar observations were found with maternal weight by the end of gestation (week 40). Groups were also different in Gestational Weight Gain (GWG): diabetic subjects reached an average of around 6 kg gained during pregnancy while normal weight subjects approximately doubled that value. Paternal IQ scores and maternal educational level were also different when comparing study groups. Regarding anthropometric infant variables at birth, waist circumference showed statistically significant differences with the highest average for infants born to obese mothers.

VARIABLES		Normal weight n=32	Overweight n=23	Obese n=13	GDM n=14	P value
PARENTS						
Maternal age (Years)		31.47 ± 3.84	32.39 ± 3.76	31.69 ± 4.09	33.36 ± 3.03	0.429
Pre-conceptional weight (Kg)		58.78 ± 5.16 ^a	72.83 ± 5.95 ^b	85.13 ± 8.73 ^b	75.05 ± 26.6 ^b	<0.001*
Pre-conceptional BMI (Kg/m ²)		21.98 ± 1.53 ^a	27.46 ± 1.39 ^{ab}	33.64 ± 2.86 ^b	28.42 ± 9.19 ^{ab}	<0.001*
Gestational Weight Gain (GWG) (Kg)		12.59 ± 3.48 ^a	11.52 ± 5.65 ^a	9.28 ± 5.51 ^a	5.7 ± 11.35 ^b	0.013*
Smoking	No	26	22	13	14	0.062
	Yes	6	1	0	0	
Alcohol consumption	No	32	22	12	13	0.507
	Yes	0	1	1	1	
Placenta weight (g)		498.71 ± 134.18	504 ± 122.71	536.15 ± 136.23	465.38 ± 90.06	0.556
Type of delivery	Vaginal: Eutocic	21	13	8	7	0.521
	Vaginal: Dystocic	5	3	1	3	
	C-section	6	7	4	4	
Maternal IQ (points)		112.92 ± 11.99	103.5 ± 15.37	111.27 ± 13.18	106.17 ± 13.35	0.123
Paternal IQ (points)		109.08 ± 11.2 ^{ab}	102.78 ± 11.21 ^{ab}	92.67 ± 6.35 ^a	111.89 ± 6.25 ^b	0.025*
Maternal educational level	Primary/Secondary	11 ^a	13 ^{ab}	12 ^b	8 ^{ab}	0.005*
	University	21 ^a	10 ^{ab}	1 ^b	6 ^{ab}	
Family status	Single/Separated	0	0	1	0	N/A
	Married/Co-habiting	32	23	12	14	N/A
INFANTS						
Gestational age (weeks)		39.5 ± 1.14	39.64 ± 1.18	40.31 ± 1.11	39.23 ± 1.42	0.119
Birth weight (Kg)		3.33 ± 0.39	3.31 ± 0.49	3.49 ± 0.42	3.24 ± 0.41	0.481
Birth Length (cm)		50.47 ± 1.77	50.2 ± 2	51.35 ± 1.89	50.58 ± 2.11	0.383
Birth head circumference (cm)		34.13 ± 1.28	34.36 ± 1.34	35.04 ± 1.16	34.88 ± 0.92	0.106
Birth waist circumference (cm)		32.19 ± 2.14 ^a	32.24 ± 2.35 ^a	34.55 ± 2.24 ^b	33.41 ± 2.01 ^{ab}	0.029*
Sex	Boy	14	10	7	8	0.786
	Girl	18	13	6	6	
Type of feeding at 1 month	Breastfed	30	21	10	13	0.733
	Infant formula	0	0	0	0	
	Mixed	2	2	2	1	
Type of feeding at 3 months	Breastfed	19	14	7	7	0.901
	Infant formula	5	4	4	4	
	Mixed	8	5	2	3	
Type of feeding at 6 months	Breastfed	6	6	2	4	0.724
	Infant formula	12	11	4	6	
	Mixed	13	6	7	4	

GDM: Gestational diabetes mellitus. P-values for overall differences between PREOBE-groups. Data was expressed as Mean ± Standard Deviation or frequencies depending on the outcome. Analysis of variance for normally distributed variables, Kruskal-Wallis rank-sum tests for non-normal continuous variables and Chi-square test for proportions.

Table 1: Baseline and background characteristics of mother-child pairs who provided 1-month milk samples (n=82), including group comparisons among the four PREOBE-groups.

HMO concentrations in human milk

6'-SL concentrations: Content of 6'-SL in 1-month postpartum human milk samples was analyzed and results are shown in Table 2. No significant differences (p=0.641) were found when comparing the overall study groups.

2'-FL concentrations in human milk: 2'-FL is not present in all mother's milk and its presence in human milk depends on the status of the mother regarding the secretor histo-blood group system: non-secretors typically lack 2'-FL among other α1-2 fucosylated oligosaccharides due to the absence of the pertinent enzyme [28]. In some studies secretor or non-secretor status was determined

through serological tests, as mentioned in a systematic review recently published [65]; however, these tests were not included in this clinical trial protocol. Since we were able to detect 2'-FL in all milk samples we refer to subjects as mothers with low or high 2'-FL concentrations in their milk as opposed to non-secretor and secretor subjects. 2'-FL concentrations for the 82 mothers were graphically represented (Figure 2, Table 3). The graph showed a clear distribution of samples in two different groups depending on 2'-FL concentration: Low and High 2'-FL milk concentration groups. 2'-FL was detected in all milk samples. Out of a total of 82 mothers, 35 fell into the Low 2'-FL group and 47 mothers fell into the High 2'-FL group representing approximately 43% and 57% of total subjects, respectively.

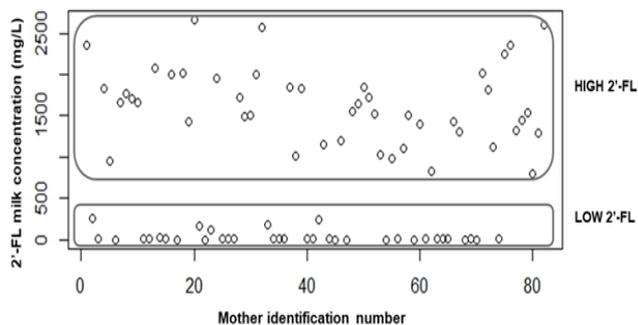


Figure 2: Distribution of subjects in Low 2'-FL and High 2'-FL milk concentration groups.

Content of 2'-FL in 1-month postpartum human milk samples is shown in Table 3. No significant differences were found comparing 2'-FL concentrations of the overall study groups (normal weight, overweight, obese and GDM) in the Low 2'-FL ($p=0.092$) or High 2'-FL sets ($p=0.215$). The study groups with low 2'-FL showed the median concentrations ranged between 3.89 to 10.2 mg/L in GDM and normal weight mothers respectively; high 2'-FL groups had median values ranging from 1153.03 mg/L to 1712.80 mg/L for GDM and normal weight groups respectively. For each 2'-FL concentration group, the lowest median values were those of GDM group and higher values were those for the normal weight group, but they were not statistically different.

STUDY GROUP	6'-SL						P value
	n	Mean	SD	Median	Min	Max	
Normal weight	32	394.10	156.02	393.12	133.56	782.21	0.641
Overweight	23	381.54	213.08	317.94	93.74	1096.37	
Obese	13	457.85	220.32	507.36	164.64	930.89	
GDM	14	397.34	215.11	393.58	97.86	955.25	

Table 2: 6'-SL Concentrations (mg/L) in 1-month postpartum human milk from mothers of the different groups participating in the PREOBE study.

n: number of cases; SD: Standard Deviation; Min: minimum; Max: maximum; P-value: level of significance. GDM: Gestational Diabetes Mellitus; Kruskal-Wallis rank sum test (non-parametric).

STUDY GROUP	LOW 2'-FL GROUP						HIGH 2'-FL GROUP					
	n	Mean	SD	Median	Min	Max	n	Mean	SD	Median	Min	Max
Normal weight	10	51.19	71.60	10.20	2.99	173.49	22	1754.36	381.88	1712.80	945.65	2597.28
Overweight	13	23.70	71.57	4.35	1.34	261.83	10	1560.17	507.46	1491.44	794.93	2352.63
Obese	5	10.37	12.19	4.61	2.71	31.73	8	1641.45	519.25	1492.55	1025.01	2662.59
GDM	7	39.12	92.79	3.89	0.65	249.47	7	1366.73	548.26	1153.03	827.48	2357.25
P value	0.092						0.215					

Table 3: 2'-FL Concentrations (mg/L) in 1-month postpartum human milk from mothers of the different groups participating in the PREOBE study.

n: number of cases; SD: Standard Deviation; Min: minimum; Max: maximum; GDM: Gestational Diabetes Mellitus; P-value: level of significance. Kruskal-Wallis rank sum test (non-parametric).

Neurodevelopment assessment: BSID-III

Of the 82 mothers that provided milk samples at 1 month postpartum, 81 consented to have their babies examined by BSID-III at 6 months of age and 76 consented at 18 months of age.

Pregestational BMI or diabetic status did not influence 2'-FL or 6'-SL concentrations in mature milk. Therefore, we pooled the milk samples results of the 82 mothers from the different PREOBE study groups to determine if there was an association between 2'-FL and 6'-SL concentrations in human milk and BSID-III scores obtained in their infants at 6 and 18 months of age.

Unadjusted and adjusted multivariate regression analyses are shown in Tables 4 and 5. Statistical adjustment was carried out based on two different approaches: (1) possible confounders of GWG, paternal IQ and maternal educational level since those factors were significantly different between the PREOBE study groups (Table 1); (2) a similar baseline and background characteristics evaluation as shown in Table 1 was performed for High 2'-FL and Low 2'-FL groups and

any significantly different variable was used as a confounder. Results from this last statistical analysis (data not shown) indicated that only maternal pre-conceptional weight was different between Low 2'-FL and High 2'-FL groups.

Regarding 6'-SL, a positive association ($p=0.023$) was observed between 6'-SL concentrations and composite cognitive scores at 18 months of age (See also correlation graph in Figure 3a) whilst a trend ($p=0.082$) was detected for 6'-SL concentrations and composite scores for motor skills at the same age (Table 4). In the multivariate regression adjusted for GWG, paternal IQ, maternal educational level and PREOBE study group, the positive association between 6'-SL concentrations and the cognitive scores at 18 months of age was maintained ($p=0.041$). For the second statistical approach in which maternal pre-conceptional weight and the PREOBE study groups were taken as confounders, a positive association between 6'-SL concentrations and composite cognitive scores at 18 months of age ($p=0.019$) was found as well as for 6'-SL concentration and composite motor scores at the same age ($p=0.043$) (Table 4).

	6'-SL Concentration					
	Unadjusted		Adjusted**		Adjusted***	
	β coefficient	P value	β coefficient	P value	β coefficient	P value
Bayley scores at 6 months						
Compositecognitive	0.007	0.831	0.006	0.741	-0.001	0.954
Compositelanguage	-0.007	0.241	-0.007	0.914	-0.007	0.214
Composite motor	0.001	0.791	-0.005	0.551	-0.002	0.851
Composite socio- emotional	-0.003	0.321	0.120	0.251	-0.004	0.214
Bayley scores at 18 months						
Compositecognitive	0.012	0.023*	0.051	0.041*	0.016	0.019*
Compositelanguage	0.001	0.521	0.002	0.751	0.004	0.417
Composite motor	0.012	0.082 [†]	-0.011	0.124	0.019	0.043*
Composite socio-emotional	-0.110	0.177	-0.080	0.145	-0.011	0.201

*: denotes a significant difference; †: denotes a trend.
 **: Adjusted model for 6'-SL concentrations considering as confounders: GWG+PaternalIQ+Cultural level Mother + PREOBE Study group.
 ***: Adjusted model for 6'-SL concentrations considering as confounders: Maternal Pre-conceptionalweight+PREOBE Study group.

Table 4: Multivariate linear regression analysis for 6'-SL human milk concentration and the different Bayley scale scores at 6 and 18 months of age.

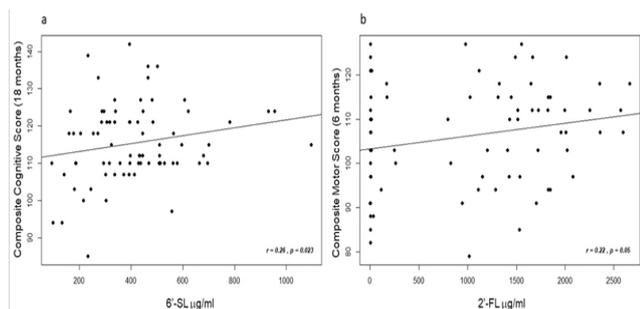


Figure 3: Pearson correlation graphs between 6'-SL concentrations and composite cognitive score at 18 months of age (a) and 2'-FL concentration and composite motor score at 6 months of age (b).

As for 2'-FL, the unadjusted multivariate regression showed a trend (p=0.0502) for a positive association between 2'-FL concentration and motor scores at 6 months of age that reached significance (p=0.041) when adjusting for maternal pre-conceptional weight and PREOBE groups (Table 5 and correlation graph in Figure 3b). Similar multivariate regression analyses (unadjusted and adjusted) were also

conducted considering 2'-FL levels for High 2'-FL and Low 2'-FL groups separately, however no significant results were obtained likely due to the low number of subjects in each group and/or to the influence of the subjects with low 2'-FL concentration (data not shown).

Discussion

In the present pilot study, mature human milk samples were analyzed from the PREOBE clinical study to evaluate the effects of maternal factors such as overweight/obesity and gestational diabetes on human milk concentrations of two abundant HMOs, 2'-FL and 6'-SL. Subsequently, we assessed the association between those human milk HMO concentrations and the BSDI-III scores obtained from infants born to mothers who provided respective milk samples. The results showed that 2'-FL and 6'-SL levels in human milk are not influenced by pre-gestational maternal weight or gestational diabetic status. In terms of infant neurodevelopment, a positive association was found between 6'-SL content in human milk and BSDI-III cognitive scale scores of human milk fed infants at 18 months of age; 2'-FL and 6'-SL milk concentrations were also positively associated with composite motor scores at 6 and 18 months of age, respectively.

	2'-FL Concentration					
	Unadjusted		Adjusted**		Adjusted***	
	β coefficient	P value	β coefficient	P value	β coefficient	P value
Bayley scores at 6 months						
Compositecognitive	0.004	0.667	0.004	0.551	0.004	0.421
Compositelanguage	0.006	0.592	0.005	0.214	0.001	0.285
Composite motor	0.002	0.0502 [†]	-0.002	0.732	0.003	0.041*
Composite socio- emotional	0.003	0.852	-0.001	0.124	-0.004	0.974
Bayley scores at 18 months						
Compositecognitive	0.002	0.521	0.002	0.321	0.002	0.551
Compositelanguage	0.002	0.839	0.001	0.558	-0.001	0.821
Composite motor	-0.001	0.721	0.001	0.421	0.002	0.695
Composite socio-emotional	0.007	0.686	0.002	0.686	0.002	0.688

*: denotes a significant difference; †: denotes a trend.
 **: Adjusted model for 2'-FL concentrations considering as confounders: GWG + Paternal IQ + Cultural level Mother + PREOBE Study group.
 ***: Adjusted model for 2'-FL concentrations considering as confounders: Maternal Pre-conceptional weight + PREOBE Study group.

Table 5: Multivariate linear regression analysis for 2'-FL human milk concentration and the different Bayley scale scores at 6 and 18 months of age.

HMO composition of human milk is determined by genetics. Certain genes encode for enzymes involved in essential steps of HMO biosynthesis such as elongation, branching, fucosylation or sialylation and thereby, variation in enzyme expression among lactating mothers give rise to different HMO profiles in human milk. The impact of other maternal factors such as, diet, health status, age or medication intake on HMO synthesis in the human mammary gland remains mostly unknown [66].

In the case of pBMI and gestational diabetic status, a few studies evaluated the influence of these factors on HMO profiles. Smilowitz et al. analyzed milk samples collected at 2 weeks postpartum from 24 mothers that delivered at full term; 8 of those 24 mothers suffered from GDM. The results showed no difference in the absolute quantitation or relative percentage of HMO composition between milk from women with or without GDM [41]. Even though the current study focused on the concentration of 2 specific HMOs, 2'-FL and 6'-SL, as opposed to the total amount of HMOs measured in the Smilowitz's work, our data also suggest GDM would not have a major impact on HMO profiles of human milk. Regarding maternal pBMI, Isganaitis et al. reported significant differences in 3 HMOs analyzed through untargeted LC-GC-MS in 1-month postpartum human milk samples from 35 mothers assigned to 2 different groups: those with a pBMI < 25 (n=16, normal weight) or ≥ 25 kg/m² (n=15, overweight/obese); 2'-FL, lacto-N-fucopentanose II/III and lacto-N-fucopentanose I levels were significantly associated with maternal pBMI after adjustment for potential confounders [9]. Those results differ from the data obtained for 2'-FL concentrations in our study where differences were not found among groups. The PROBE study distinguished 3 different study groups in terms of pBMI, normal weight, overweight and obese subjects, while Isganaitis' study included 2 pBMI ranges, normal weight and overweight/obese subjects. Noteworthy within the PROBE study a statistical analysis was also conducted between normal and obese/overweight subjects after pooling obese and overweight mothers into one group (pBMI ≥ 25 kg/m²) but a significant difference between groups was not found. In a recent cross-sectional, observational study [42], human milk samples from 17 to 76 days postpartum were analyzed for different HMOs and a positive correlation between pBMI and 2'-FL concentration was found in secretor mothers; however, this difference was not maintained when considering the mother's Lewis phenotype and the number of subjects with overweight or obesity was not clearly stated in that work which makes it difficult to compare these results with the present study.

Clinical trials have been conducted to determine HMO effects on growth and tolerance in infants and to evaluate immune outcomes [43,67,68] however, cognitive outcomes have been scarcely evaluated in clinical studies to date. HMO effects on cognitive function have been reported in preclinical animal models. Different forms of sialic acid (Sia) supplementation, including 6'-SL, have been tested using piglet [69] and rodent models [27,52] showing a positive impact on cognitive performance. In some studies, supplementation with an exogenous source of Sia resulted in increased sialic acid in certain areas of the brain [51,52,69-73]. Fucosylated HMOs such as 2'-FL have also been evaluated in rodent cognition models. These studies demonstrated a role for 2'-FL in brain functionality, administered with the diet in adult animals [53,54] or through an oral supplementation during the lactation period in rat pups [55]. Regarding clinical data on HMO levels in human milk and cognitive development, a recent trial studied the relationship between the levels of nineteen HMOs in 1 and 6 months human milk samples and infant cognitive development at 24 months of age relative to maternal obesity and human milk feeding

frequency [56]; results obtained show a positive association between 2'-FL human milk concentration at 1 month postpartum and cognitive function at 24 months of age using BSID-III. In the present study, an association between 2'-FL and 6'-SL human milk concentrations and infant cognitive function were also assessed using BSID-III but at earlier ages (6 and 18 months) than in the Berger study; moreover the results were evaluated considering different Bayley scales of infant development (cognitive, motor, language and socio-emotional scales) while in the Berger study analyses were limited to the cognitive scale.

In relation to the results obtained in the present study, a positive association between 6'-SL content in human milk and cognitive scale scores was found in infants assessed at 18 months of age. Cognitive scale in Bayley's assessment consists of 91 items which evaluate children's sensorimotor development, exploration and manipulation of objects, concept formation, and memory. Hence, infants complete tasks that measure their interest in novelty, attention to familiar and unfamiliar stimuli, and problem-solving [74]. These clinical observations align with preclinical research showing supplementation with 6'-SL during the lactation period in rat pups improved cognitive outcomes related to memory and long-term potentiation measurements at 1 year of age [27]. Additionally, data in the present study also suggest 6'-SL and 2'-FL concentrations may be associated with infants' motor skills after adjusting for potential confounders. Nevertheless, 2'-FL association with motor scores was not significant when samples were separated into low and high 2'-FL levels, probably due to the low number of subjects in each group and/or to the influence of the mother-infant pairs with low 2'-FL concentration.

Aside from the confounders considered in the statistical analysis of the present study, children's health is determined by the interaction of biological, behavioral, and environmental (physical and social) influences that may also influence child neurodevelopment and thereby, the scores obtained in Bayley's assessment [75].

Although BSID-III was initially designed as a diagnostic instrument to identify suspected developmental delays in children identified with possible disorders and disabilities [76], in the last decade it has been widely used to assess the developmental functioning of healthy children without developmental disorders, as is the case in the present study. A potential relationship between cognitive outcomes and 6'-SL levels was detected even though children evaluated did not suffer from a developmental disorder. However, it may also explain why a significant association was not detected between 2'-FL concentration and Bayley cognitive scale score: slight differences in behavior in children without disabilities may have been undetected.

One of the limitations of the present study is the low number of mother-infant pairs evaluated due to the reduced number of human milk samples available. Consequently, the pilot nature of this work should be noted; nevertheless, it may be useful as a starting point to calculate the size of future large-scale trials aiming to determine the influence of HMOs on Bayley III scores.

The mechanism(s) of action by which HMOs exert their cognitive effects remains unclear although hypotheses have been proposed. The improved cognitive performance derived from HMO supplementation in different preclinical models may be related to its prebiotic function due to metabolites that reach the brain producing structural changes responsible for enhanced cognitive skills. Preclinical research showed administration of an exogenous source of sialylated compounds increased the sialic acid concentration of key areas of the brain [51,52,69-73] suggesting sialic acid reached the brain; it is unlikely

that intact 2'-FL goes to the brain directly [77] but may act via the Gut-Brain Axis (GBA) and through the vagus nerve [54]. GBA is a complex bidirectional network that sends signals between these two organs controlling their function [78]. Microbiota may also have an important role in the interaction [79,80], but this remains unclear. Interestingly, a recent study derived from the PREOBE study has shown an association between gut microbiota and infant cognitive performance at 6 months of age [81].

In summary, in this preliminary study 6'-SL and 2'-FL levels were not impacted by pBMI or the development of GDM. 6'-SL level in human milk was associated with infant neurodevelopment. 2'-FL concentrations were associated with motor scores at 6 months of age when considering Low and High 2'-FL milk samples together; this association was lost when Low and High 2'-FL groups were treated separately. Further research and full-scale clinical studies are needed to elucidate the effects of HMOs in pediatric neurodevelopment as well as the mechanism(s) of action involved and the influence of Low and High 2'-FL populations.

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Author Contributions

Investigation, E.O., C.C., and M.J.M.; Present methodology, E.O., C.C. and M.J.M.; Coordinator of the PREOBE study, C.C.; Present Supervision, C.C. and M.J.M.; HMO analysis, A.S. Mothers' milk recruitment and storage, F.J.T.E. and M.T.S.; PREOBE follow-up: F.J.T.E., M.T.S. and M.E.; Neuropsychological assessment and interpretation: F.J.T.E. and A.C.; Statistical analysis, H.A.; Writing, original draft, E.O. Writing, review and editing, C.C., R.B., M.R., R.R. and M.J.M.

Conflicts of Interest

E.O., R.B., M.R., A.S., R.R. and M.J.M. are employees of Abbott Laboratories, a company that manufactures infant formulas. C.C., F.J.T.E., M.T.S., M.E., A.C. and H.A., have no conflicts of interest to declare.

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