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Maternal and Infant Factors Associated with Human Milk Oligosaccharides Concentrations According to Secretor and Lewis Phenotypes

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Abstract: Human milk oligosaccharides (HMOs) are multifunctional carbohydrates naturally present in human milk that act as prebiotics, prevent pathogen binding and infections, modulate the immune system and may support brain development in infants. HMOs composition is very individualized and differences in HMOs concentrations may affect the infant's health. HMOs variability can be partially explained by the activity of Secretor (*Se*) and Lewis (*Le*) genes in the mother, but non-genetic maternal factors may also be involved. In this cross-sectional, observational study, 78 single human milk samples ranging from 17 to 76 days postpartum (median: 32 days, IQR: 25–46 days) were collected from breastfeeding Brazilian women, analyzed for 16 representative HMOs by liquid chromatography coupled to mass spectrometry and associations between maternal and infant factors with HMOs concentrations were investigated. HMOs concentrations presented a high variability even in women with the same *SeLe* phenotype and associations with maternal allergic disease, time postpartum and with infant's weight, weight gain and sex. Overall, we present unprecedented data on HMOs concentrations from breastfeeding Brazilian women and novel associations of maternal allergic disease and infant's sex with HMOs concentrations. Differences in HMOs composition attributed to maternal *SeLe* phenotype do not impact infant growth, but higher concentrations of specific HMOs may protect against excessive weight gain.

Keywords: human milk oligosaccharides; human milk composition; bioactive components; exclusive breastfeeding; Secretor phenotype; Lewis phenotype

1. Introduction

Breastfeeding is the natural and best feeding type for infants, providing not only nutrition but improving the general health of the mother-infant dyad [1]. Besides the perfect balance of nutrients and water in an amount matching the infant's needs, human milk contains a myriad of bioactive components, including immunoglobulins, hormones, oligosaccharides, and others [2]. Human milk oligosaccharides (HMOs) are a complex group of free glycans synthesized by the lactating mammary gland composing the third major solid fraction of human milk, after lactose and lipids [3,4]. Emerging

evidence has shown that HMOs act as prebiotics [5–7], antimicrobials [8], prevent pathogen binding and infections [9–11], modulate the immune system [12] and also may support brain development [13,14].

HMOs are composed of the monosaccharides glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and *N*-acetylneuraminic acid (Neu5Ac). So far, about 150 individual HMOs have been identified [15], yet approximately 90% of the HMOs fraction is composed of less than 20 different structures [16,17]. Nearly all HMOs contain lactose in the reducing end, which can be elongated by the addition of GlcNAc and Gal to form type 1 (Gal β 1-3GlcNAc) or type 2 (Gal β 1-4GlcNAc) chains in β 1-3 or β 1-6 linkages, producing core structures. Additionally, Fuc and Neu5Ac can be attached to the HMO core or directly to the lactose reducing end [18]. According to the monosaccharides present in the structure, HMOs can be classified into three main groups: 1. neutral core, containing Glc, Gal and GlcNAc; 2. neutral fucosylated, containing one or more Fuc units; and 3. acidic, containing one or more Neu5Ac units.

The composition and concentrations of HMOs are unique in the milk of each woman and strongly depend on the activity of the Secretor (*Se*) and Lewis (*Le*) genes in the mammary glands. *Se* and *Le* genes encode the enzymes α 1-2-fucosyltransferase (FUT2) and α 1-3/4-fucosyltransferase (FUT3), respectively, involved in the biosynthesis of fucosylated HMOs [19,20]. Mutations on the *Se* gene inactivate FUT2, and consequently, milk from non-secretor (*Se*–) women contain no or only traces of α 1-2 fucosylated HMOs. Mutations on the *Le* gene inactivate FUT3, and consequently, milk from Lewis-negative (*Le*–) women contain no or only traces of α 1-4 fucosylated HMOs [21]. Based on the activity of the FUT2 (*Se*) and FUT 3 (*Le*) enzymes in the lactating woman, HMOs composition can be classified into four phenotypes: 1. *Se+Le+*, the most common, containing α 1-2 and α 1-4 fucosylated HMOs, such as 2'-fucosyllactose (2'-FL) and lacto-*N*-difuco-hexaose I (LNDFH I); 2. *Se–Le+*, which contain α 1-4 fucosylated HMOs, such as lacto-*N*-difuco-hexaose II (LNDFH II), but does not contain α 1-2 fucosylated HMOs, such as 2'-FL, lacto-*N*-fucopentaose I (LNFP I), difucosyllacto-*N*-hexaose c (DFLNH c) and LNDFH I; 3. *Se+Le–*, which contain α 1-2 fucosylated HMOs, such as 2'-FL and LNFP I, but does not contain α 1-4 fucosylated HMOs, such as DFLNH c, LNDFH I and II and; 4. *Se–Le–*, the least common phenotype, containing neither α 1-2 nor α 1-4, but only α 1-3 fucosylated HMOs, such as 3'-fucosyllactose (3'-FL) and difucosyl-para-lacto-*N*-neohexaose (DFpLNnH), which occur in all the four *SeLe* groups, since their synthesis apparently is not influenced by the *Se* and *Le* genes [22,23]. Besides the influence of the *Se* and *Le* genes in the composition of HMOs, great differences in HMOs concentrations occur in the milk of women with the same genetic background [24]. The variability within *SeLe* groups indicates that other factors besides the activity of *Se* and *Le* genes may be involved in HMOs biosynthesis influencing their concentrations in human milk. However, little is known about the influence of non-genetic factors on HMOs composition and concentrations and only a few studies have addressed this question. There is some conflicting evidence about the influence of gestational age and lactation time on HMOs concentrations [23,25,26]. Recently, maternal factors such as parity and body mass index (BMI), as well as environmental factors such as geographic location have been associated with HMOs concentrations [27–29].

The understanding of the influencing factors on HMOs composition and concentrations is important since some of the HMOs effects on infant health have been related to specific structures and usually in a dose-dependent manner. For example, infants whose mother's milk had low concentrations of α 1-2 fucosylated HMOs presented a higher incidence of *Campylobacter*, calicivirus and moderate-to-severe diarrhea than those whose mother's milk contained higher concentrations of α 1-2 fucosylated HMOs [11]. Higher concentrations of α 1-2 fucosylated HMOs also have been related to a lower risk of allergy at 2 and 5 years of age in infants with high hereditary allergy risk [30]. A lower total HMOs concentration and a higher proportion of 3'-sialyllactose (3'-SL) have been correlated with higher HIV transmission in Zambian infants [31]. Higher total HMOs concentrations were also associated with reduced mortality in HIV-exposed uninfected Zambian infants [32]. Furthermore, disialyllacto-*N*-tetraose (DSLNT), an acidic HMO, prevents necrotizing enterocolitis (NEC) in rats and lower amounts of DSLNT in human milk may predict the risk of NEC in preterm infants [33,34].

Furthermore, infant weight, body composition and nutritional status also have been associated with concentrations of specific HMOs [35,36].

Bearing this in mind, we developed this cross-sectional, observational study to measure absolute HMOs concentrations in a cohort of Brazilian mothers, characterize HMOs profiles and identify maternal and infant factors associated with HMOs concentrations.

2. Materials and Methods

2.1. Study Design

A total of 78 mature human milk samples were analyzed in the study. Each mother provided a single human milk sample. Participants of the study were mothers and their infants who attended the Centro de Incentivo e Apoio ao Aleitamento Materno (Breastfeeding Incentive and Support Center) (CIAAM) of the Universidade Federal de São Paulo (UNIFESP) between December 2013 and October 2015. Healthy full-term (gestational age ≥ 37 weeks), singleton, exclusively breastfed infants with 17 to 76 days of life (median: 32 days, IQR: 25–46 days) were included in the study. Infants that received antibiotic treatment, probiotics, water or any other food besides human milk were not included.

Clinical and demographic data were obtained from the CIAAM medical records of the mother-infant pairs and through a face-to-face interview with the mother. Maternal weight and height and infant weight and length were measured at inclusion. Maternal overweight and obesity were defined as BMI (kg/m^2) > 25.0 and > 30.0 , respectively. The socioeconomic status of the families was obtained through a standardized questionnaire for the economic classification in Brazil developed by the Brazilian Association of Research Companies [37]. Considering that there is no validated instrument for the screening of allergic diseases in adults, the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire was used. This questionnaire was translated and validated in the Portuguese language for application in children and adolescents [38–40].

Human milk samples were obtained by manual expression of the breast opposite to the one previously emptied by the infant, as informed by the mother. A CIAAM nurse performed the milk expression when the mother could not do it by herself. With the use of gloves, after hand hygiene, massaging of the breast and the discard of the first drops, 5 to 15 mL of human milk were collected in a sterilized glass bottle and transferred to a Falcon polypropylene tube. All human milk samples were collected in the morning (8:30–12:00 a.m.) and stored at $-20\text{ }^\circ\text{C}$ until HMOs analysis. Data and human milk sample collection were carried out on the same day at CIAAM.

2.2. Ethical Considerations

This study was approved by the Ethics Committee of UNIFESP (protocol No. 419.162) and complied with the Declaration of Helsinki. All lactating women received detailed oral and written information about the study and voluntarily agreed to participate. Written informed consent was obtained from each participant prior to the data and sample collection.

2.3. HMOs Analysis

HMOs were extracted from human milk, identified and quantified by liquid chromatography-mass spectrometry (LC-MS) following a validated method previously reported in detail [41]. Analyses were performed in duplicate for each human milk sample and HMOs concentrations were reported as the mean value of the duplicates. Absolute quantification was carried out for 16 representative HMOs, including 4 neutral core (lacto-N-tetraose (LNT); lacto-N-neotetraose (LNnT); lacto-N-hexaose (LNH); lacto-N-neohexaose (LNnH)), 7 neutral fucosylated (2'-fucosyllactose (2'-FL); 3'-fucosyllactose (3'-FL); lacto-N-fucopentaose I (LNFP I); lacto-N-difuco-hexaose I (LNDFH I); lacto-N-difuco-hexaose II (LNDFH II); difucosyl-para-lacto-N-neohexaose (DFpLNnH); difucosyllacto-N-hexaose (c) (DFLNH c)) and 5 acidic HMOs (3'-sialyllactose (3'-SL); 6'-sialyllactose (6'-SL); LS-tetrasaccharide a (LSTa); LS-tetrasaccharide b (LSTb); LS-tetrasaccharide c (LSTc)). Analytical standards of the 16 HMOs

were purchased from Dextra Laboratories (Reading, UK). The 16 HMOs included in the analysis are within the most abundant HMOs [42] and are expected to represent about 90% of the total HMOs in human milk [27]. The criteria utilized to select the HMOs to be analyzed were: (a) availability of quantitative data in the literature; (b) structures reported among the most abundant in human milk; (c) structures that allowed the determination of the *SeLe* phenotype and (d) commercial availability of analytical standards.

2.3.1. HMOs Extraction

Aliquots of 100 μ L of the human milk samples were defatted via centrifugation at 5000 \times g for 15 min at room temperature; then 50 μ L was diluted with 950 μ L Milli-Q water and homogenized in vortex. Proteins were removed by ultrafiltration, transferring 500 μ L of the defatted diluted sample to an Amicon 10 kDa (Merck, Darmstadt, Germany) molecular weight cutoff filter and centrifuging at 12,000 g for 30 min. The filtrate, which contained the HMOs extract, was collected and 100 μ L was subjected to a reduction reaction with 100 μ L of 0.25 M sodium borohydride (Sigma-Aldrich, Saint Louis, USA) at room temperature. The reaction was terminated after 30 min by the addition of 100 μ L of 0.25 M acetic acid (JT Baker, Center Valley, USA). There was 20 μ L of reduced extracts injected into the LC-MS system for identification and quantification of the 16 HMOs.

2.3.2. HMOs Identification and Quantification by LC-MS

HMOs separation and quantification were performed on a Waters Alliance 2695 HPLC (Waters, Milford, USA) system equipped with a column heater set to 30 $^{\circ}$ C and a Hypercarb column (100 \times 2.1 mm, 3 μ m, Thermo Scientific, Waltham, USA) coupled to a Waters Micromass Quattro LC mass spectrometer equipped with an electrospray ionization (ESI) source. The software MassLynx 4.1 (Waters, Milford, USA) was used to control system components and for data acquisition and processing.

The mobile phases consisted of Milli-Q ultrapure water (A) and acetonitrile (B), both containing formic acid at 0.1% and delivered at a flow rate of 200 μ L/min. HMOs separation was carried out over 55 min with a gradient consisting of an initial increase from 0 to 12% B over 21 min, followed by a second increase from 12 to 40% B over 11 min and a third increase from 40 to 100% B over 5 min. A washing step was conducted at 100% B for 5 min. The gradient was then decreased to 0% B over 1 min and maintained at 0% B for 12 min for column equilibration.

The ionization parameters were optimized with individual standard solutions of the 16 HMOs at 100 μ g/mL in water injected directly into the MS. The optimized ionization conditions were as follows: capillary voltage at 3.0 kV, cone voltage at 30 V, cone gas flow at 86 L/h and the source, and desolvation temperatures at 150 $^{\circ}$ C and 250 $^{\circ}$ C, respectively. The dwell time was 0.055 min. The MS was operated with selected ion monitoring (SIM) in negative mode ($[M-H]^{-}$) for all HMOs. The deprotonated ions $[M-H]^{-}$ used for monitoring of neutral core HMOs were m/z 708.63 for LNT and LNnT and m/z 1073.96 for LNH and LNnH. The deprotonated ions $[M-H]^{-}$ used for fucosylated HMOs monitoring were m/z 489.44 for 2'-FL and 3'-FL, m/z 854.77 for LNFP I, m/z 1000.91 for LNDFH I and LNDFH II and m/z 1366.25 for DFpLNnH and DFLNHc. The deprotonated ions $[M-H]^{-}$ used for the monitoring of acidic HMOs were m/z 634.55 for 3'-SL and 6'-SL and m/z 999.88 for LSTa, LSTb, and LSTc.

HMOs absolute quantification was performed using a linear calibration curve (0.039–5 μ g/mL) constructed from a standard solution containing all the 16 HMOs analytical standards at 5 μ g/mL, in water, which was serially diluted to 0.039 μ g/mL. The calibration curve presented excellent linearity ($R^2 \geq 0.99$) for all the 16 HMOs. The limit of quantification (LOQ) was established as the lower concentration capable of generating a signal to noise ratio (s/n) ≥ 10 . LOQ was 0.039 μ g/mL for all HMOs except LNFP I, for which it was 0.156 μ g/mL.

2.4. Secretor and Lewis Phenotype Determination

The Secretor and Lewis phenotype of the mothers was determined based on the presence of indicative α 1-2 and α 1-4 fucosylated HMOs in the human milk sample, as previously done in

other studies [22,23,25,26,43,44]. Women whose human milk sample presented both α 1-2 and α 1-4 fucosylated structures were classified as Secretor positive/Lewis positive (Se+Le+) and attributed to Group 1. Women who presented α 1-4 but not α 1-2 fucosylated HMOs were classified as Secretor negative/Lewis positive (Se–Le+) and assigned to Group 2. Those who presented α 1-2 but not α 1-4 fucosylated HMOs in their sample were classified as Secretor positive/Lewis negative (Se+Le–) and attributed to Group 3. When the human milk sample did not present neither α 1-2 nor α 1-4 fucosylated HMOs, the woman was classified as Secretor negative/Lewis negative (Se–Le–) and assigned to Group 4. For the classification according to the Secretor phenotype, women from the Groups 1 and 3 were grouped as secretor (Se+) and women from Groups 2 and 4 were assigned to the non-secretor (Se–) group. Table 1 shows the fucosylated HMOs profile in the human milk utilized to assign the Secretor and Lewis phenotype of the lactating mothers participating in the study.

Table 1. Fucosylated Human milk oligosaccharides (HMOs) profile utilized to assign the Secretor and Lewis phenotype of the lactating mothers.

| HMO | Structure | Fucose Linkages | Group 1: Se+Le+ | Group 2: Se–Le+ | Group 3: Se+Le– | Group 4: Se–Le– |
|----------|--|------------------------------|-----------------|-----------------|-----------------|-----------------|
| 2'-FL | Fuc α 1-2Gal β 1-4Glc | α 1-2 | + | - | + | - |
| LNFP I | Fuc α 1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc | α 1-2 | + | - | + | - |
| LNDFH I | Fuc α 1-2Gal β 1-3(Fuc α 1-4)GlcNAc β 1-3Gal β 1-4Glc | α 1-2 α 1-4 | + | - | - | - |
| DFLNH c | $\begin{array}{c} \text{Gal}\beta 1-4\text{GlcNAc}\beta 1-6 \\ \diagdown \\ \text{Gal}\beta 1-4\text{Glc} \\ \diagup \\ \text{(Fuc}\alpha 1-2\text{)Gal}\beta 1-3\text{(Fuc}\alpha 1-4\text{)GlcNAc}\beta 1-3 \end{array}$ | α 1-2 α 1-4 | + | - | - | - |
| LNDFH II | Gal β 1-3(Fuc α 1-4)GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Glc | α 1-3 α 1-4 | + | + | - | - |
| DFpLNnH | Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4Glc | α 1-3 | + | + | + | + |
| 3'-FL | Gal β 1-4(Fuc α 1-3)Glc | α 1-3 | + | + | + | + |

Fuc: fucose; Gal: galactose; Glc: glucose; GlcNAc: N-acetylglucosamine.

2.5. Statistics

Descriptive statistics and statistical tests were performed with the use of the softwares Statistica 64, version 12 and R, version 3.4.4. As the normality assumption was not satisfied, a non-parametric Analysis of Covariance (Ranked ANCOVA or Quade test [45]) was employed to verify differences among the *SeLe* groups with postpartum days as a covariate and values of each of the HMOs as dependent variables. A free web-program [46] was used to perform the ranked ANCOVA (post-hoc Tukey-Kramer test). Analysis of Variance (ANOVA) and Kruskal-Wallis test were performed to verify associations between *SeLe* groups and quantitative clinical variables. Mann-Whitney, Student's *t* test and Pearson's chi-square were used to verify differences in HMOs concentrations, clinical and demographic characteristics between secretors and non-secretors. Fisher's exact test was conducted to verify associations between *SeLe* groups, secretor status, and allergic diseases. Spearman rank correlation was used to verify correlations between HMOs concentrations and quantitative clinical variables. The association of clinical and demographic characteristics with HMOs concentrations was analyzed separately for each *SeLe* and *Se* phenotypes. Since in our cohort we had only one participant assigned to Group 4 (Se–Le–), she was not included in the statistical analyses. Therefore, statistical tests performed to verify differences among the *SeLe* groups were conducted considering only Group 1, Group 2 and Group 3. For the statistical analyses considering only the *Se* phenotype, the woman from Group 4 was included, assigned to the non-secretor group. All statistical analyses were considered significant at $p < 0.05$.

3. Results

3.1. Study Population

Table 2 shows the *SeLe* phenotypes distribution and characteristics of the mothers participating in the study. There were no significant statistical differences among *SeLe* groups for the studied variables. However, when considering *Se* phenotype alone, the incidence of allergic disease was significantly higher (Fisher's exact test, $p = 0.028$) in women presenting *Se+* phenotype (23/68) than in *Se-* women (0/10).

Table 2. Descriptive characteristics of the mothers according to Secretor and Lewis phenotypes.

| Variables | Group 1 Se+ Le+ | Group 2 Se- Le+ | Group 3 Se+ Le- |
|------------------------------------|-----------------------|----------------------|----------------------|
| | <i>n</i> = 59 (75.6%) | <i>n</i> = 9 (11.5%) | <i>n</i> = 9 (11.5%) |
| | Mean ± SD | Mean ± SD | Mean ± SD |
| Age, years | 31 ± 7 | 29 ± 7 | 27 ± 4 |
| BMI, kg/m ² | | | |
| At inclusion | 27 ± 5 | 26 ± 4 | 28 ± 5 |
| Pre-gestational | 25 ± 5 | 24 ± 4 | 26 ± 6 |
| Parity, <i>n</i> | 2 ± 1 | 2 ± 1 | 1 ± 0 |
| Postpartum, days | 38 ± 14 | 32 ± 11 | 31 ± 13 |
| | <i>n</i> (%) | <i>n</i> (%) | <i>n</i> (%) |
| Cesarean delivery | 32 (54) | 6 (67) | 2 (22) |
| Allergic disease, yes ^a | 19 (32) | 0 (0) | 4 (50) |
| Pets, yes ^b | 18 (38) | 3 (38) | 3 (43) |
| Socioeconomic status | | | |
| Class A (<i>n</i> = 6) | 4 (7) | 1 (11) | 1 (11) |
| Class B (<i>n</i> = 45) | 34 (58) | 5 (56) | 6 (67) |
| Class C (<i>n</i> = 26) | 21 (36) | 3 (33) | 2 (22) |
| Education ^c | | | |
| Elementary school (<i>n</i> = 6) | 6 (12) | 0 (0) | 0 (0) |
| High school (<i>n</i> = 31) | 22 (42) | 5 (63) | 4 (50) |
| Graduate (<i>n</i> = 24) | 17 (33) | 3 (38) | 4 (50) |
| Postgraduate (<i>n</i> = 7) | 7 (13) | 0 (0) | 0 (0) |

^a Missing data from one mother of the group 3; ^b Missing data from 11 mothers of group 1; 1 mother from group 2 and 2 mothers from group 3; ^c Missing data from 7 mothers from group 1; 1 mother from group 2 and 1 mother from group 3.

Table 3 presents the clinical characteristics of the infants according to their mothers' *SeLe* phenotypes. From the total 78 infants, 37 (49%) were boys. Boys were 27 (47%) in Group 1; 4 (44%) in Group 2 and 5 (56%) in Group 3. The only one infant from Group 4 was a 37 days old boy. The range of the infants' age was 17 to 76 (IQR: 27–48) days in Group 1; 20 to 50 (IQR: 24–41) in Group 2 and 20 to 62 (IQR: 22–32) in Group 3. Infant's age corresponds to the day of milk sampling and presented a coefficient of variation (CV) of 37%, 35% and 42% in Groups 1, 2 and 3, respectively. There were no significant statistical differences among infants from *SeLe* groups for all the studied variables.

Table 3. Descriptive characteristics of the infants according to the Secretor and Lewis phenotypes of their mothers.

| Variables | Group 1 Se+ Le+ | Group 2 Se- Le+ | Group 3 Se+ Le- |
|---------------------------------|-----------------------|----------------------|----------------------|
| | <i>n</i> = 59 (75.6%) | <i>n</i> = 9 (11.5%) | <i>n</i> = 9 (11.5%) |
| | Mean ± SD | Mean ± SD | Mean ± SD |
| Age, days | 38 ± 14 | 32 ± 11 | 31 ± 13 |
| Gestational age, weeks | 39 ± 1 | 39 ± 1 | 39 ± 1 |
| Weight at birth, g | 3237 ± 392 | 3358 ± 579 | 3173 ± 434 |
| Weight at inclusion, g | 4262 ± 764 | 4448 ± 1052 | 4148 ± 730 |
| Length at inclusion, cm | 54 ± 3 | 54 ± 3 | 51 ± 6 |
| Weight gain, g/day ^a | 26 ± 16 | 24 ± 16 | 25 ± 6 |

^a Weight gain = (weight at inclusion – weight at birth)/age; $p > 0.05$ for all variables.

3.2. HMOs Composition and Concentrations

Table 4 shows the concentrations, expressed as mean, standard deviation (SD) and coefficient of variation of the HMOs concentrations according to the *SeLe* phenotypes and to the classification in fucosylated, neutral core, and acidic. HMOs concentrations were expressed as mean \pm SD to facilitate comparisons with other studies, however, for statistical analysis non-parametric tests were used. Complete descriptive statistics as well the results of the sole sample of the Group 4 (Se–Le–) are available as Supplementary Materials.

Table 4. HMOs concentrations in term mature human milk samples ($n = 77$) according to the *SeLe* phenotypes of the lactating women.

| HMOs | Concentration (g/L) | | | Statistical Analysis ^a | Multiple Comparison Analysis ^b |
|---------------------------|--|--|--|-----------------------------------|---|
| | Group 1 Se+ Le+ Mean \pm SD (CV, %) | Group 2 Se– Le+ Mean \pm SD (CV, %) | Group 3 Se+ Le– Mean \pm SD (CV, %) | | |
| <i>Fucosylated</i> | | | | | |
| 2'-FL | 2.20 \pm 0.98 (45) | – | 3.43 \pm 1.75 (51) | $p = 0.019$ | G3 < G1 < G2 |
| 3'-FL | 0.53 \pm 0.33 (63) | 1.62 \pm 0.42 (26) | 0.31 \pm 0.58 (189) | $p < 0.001$ | |
| LNFP I | 0.73 \pm 0.52 (71) | – | 2.03 \pm 1.51 (75) | $p = 0.015$ | |
| LNDFH I | 1.22 \pm 0.81 (66) | – | – | – | |
| LNDFH II | 0.02 \pm 0.05 (293) | 0.41 \pm 0.22 (55) | – | $p < 0.001$ | |
| DFLNHc | 0.08 \pm 0.05 (71) | – | – | – | |
| DFpLNnH | 0.04 \pm 0.07 (154) | 0.04 \pm 0.03 (72) | 0.05 \pm 0.10 (184) | $p = 0.702$ | |
| <i>Neutral core</i> | | | | | |
| LNH | 0.08 \pm 0.06 (75) | 0.05 \pm 0.06 (116) | 0.07 \pm 0.06 (94) | $p = 0.013$ | G1 = G3; G2 = G3; G2 < G1 |
| LNnH | 0.04 \pm 0.03 (85) | 0.02 \pm 0.03 (151) | 0.02 \pm 0.02 (87) | $p = 0.010$ | G2 = G3; G1 = G3; G2 < G1 |
| LNT + LNnT | 0.38 \pm 0.17 (46) | 0.54 \pm 0.25 (47) | 0.37 \pm 0.25 (67) | $p = 0.146$ | |
| <i>Acidic</i> | | | | | |
| 3'-SL | 0.18 \pm 0.04 (24) | 0.18 \pm 0.05 (28) | 0.17 \pm 0.03 (18) | $p = 0.736$ | |
| 6'-SL | 0.37 \pm 0.15 (40) | 0.39 \pm 0.23 (59) | 0.41 \pm 0.15 (37) | $p = 0.640$ | |
| LSTa | 0.01 \pm 0.01 (104) | 0.01 \pm 0.01 (90) | 0.01 \pm 0.00 (62) | $p = 0.074$ | |
| LSTb | 0.07 \pm 0.04 (54) | 0.10 \pm 0.06 (58) | 0.06 \pm 0.03 (53) | $p = 0.152$ | |
| LSTc | 0.16 \pm 0.09 (59) | 0.13 \pm 0.08 (64) | 0.14 \pm 0.06 (45) | $p = 0.005$ | G1 = G3; G2 = G3; G2 < G1 |
| <i>Total</i> | | | | | |
| Fucosylated ^c | 4.81 \pm 1.62 (34) | 2.07 \pm 0.61 (29) | 5.83 \pm 1.97 (34) | $p < 0.001$ | G1 = G3; G2 < G1; G2 < G3 |
| Neutral core ^d | 0.50 \pm 0.20 (41) | 0.61 \pm 0.26 (42) | 0.46 \pm 0.31 (67) | $p = 0.243$ | |
| Acidic ^e | 0.79 \pm 0.25 (31) | 0.81 \pm 0.35 (43) | 0.79 \pm 0.21 (27) | $p = 0.245$ | |
| Total HMOs ^f | 6.10 \pm 1.76 (29) | 3.50 \pm 0.84 (24) | 7.08 \pm 2.07 (29) | $p < 0.001$ | G1 = G3; G2 < G1; G2 < G3 |

^a ANCOVA (Quade test) adjusted for postpartum days; ^b Tukey-Kramer test; ^c Sum of all individual fucosylated HMOs; ^d Sum of all individual neutral core HMOs; ^e Sum of all individual acidic HMOs; ^f Sum of all individual HMOs; G1: Group 1; G2: Group 2; G3: Group 3.

HMOs concentrations demonstrated a great variability in the milk of women with the same *SeLe* phenotype, as expressed by the coefficient of variation (CV), which was higher than 20% for all the studied HMOs exceeding 100% in some cases. Fucosylated and neutral core HMOs presented the highest variation. Regarding individual HMOs, seven structures presented concentrations significantly different among the *SeLe* groups: 2'-FL, 3'-FL, LNFP I, LNDFH II, LNH, LNnH and LSTc.

Group 2 presented a significantly lower concentration of total and fucosylated HMOs when compared to Groups 1 and 3. Group 1 and Group 3 did not show differences in their concentrations either for total, fucosylated, acidic and neutral core HMOs. No differences were seen regarding total neutral core and total acidic HMOs among *SeLe* groups.

Figure 1 illustrates the HMOs profile in the milk according to the *SeLe* and *Se* phenotype of the mother. *Se* phenotype was responsible for the major differences in the HMOs profile and concentrations, as can be noted in Figure 1a that *Se+* groups (*Se+Le+* and *Se+Le-*) presented a clear different pattern—both in HMOs diversity and total concentration—from the *Se-* groups (*Se-Le+* and *Se-Le-*). Main differences occur in the fucosylated HMOs profile. *Se+* women had a significantly higher total fucosylated HMOs and total HMOs in the milk than *Se-* women (Mann-Whitney test, $p < 0.0001$). Neutral core and acidic HMOs profiles are quite similar among the *SeLe* and *Se* groups. Furthermore, there were no differences in total neutral core (Mann-Whitney test, $p = 0.095$) and total acidic HMOs (Mann-Whitney test, $p = 0.565$) between *Se+* and *Se-* milk.

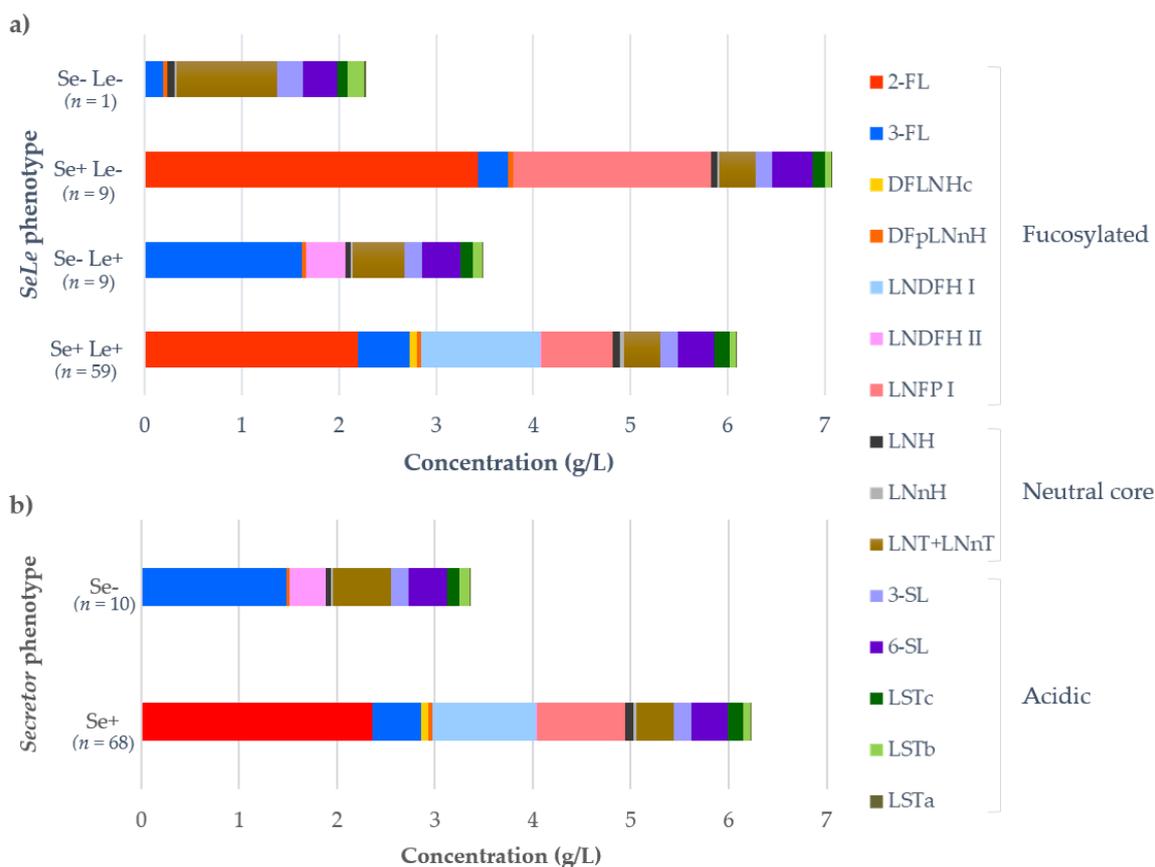


Figure 1. Human milk oligosaccharides (HMOs) profile and concentrations in human milk according to the *SeLe* (a) and *Se* (b) phenotype of the lactating mother.

3.3. Associations of Maternal and Infant Factors with HMOs Concentrations in *SeLe* Groups

Women presenting allergic disease had significantly higher DFpLNnH concentrations than women without allergic disease both in *Se+Le+* (0.05 and 0.04 g/L, respectively; Mann-Whitney test, $p = 0.023$) and *Se+Le-* (0.03 and 0.01 g/L, respectively; Mann-Whitney test, $p = 0.028$).

In *Se+Le-* milk, concentrations of some HMOs differed regarding the infant's sex. Mothers of girls had a significantly higher 2'-FL concentration in their milk than mothers of boys (4.84 and 2.31 g/L, respectively; Mann-Whitney test, $p = 0.016$). However, mothers of boys had higher concentrations of LNH in their milk than mothers of girls (0.10 and 0.02 g/L, respectively; Mann-Whitney test, $p = 0.016$), as well as LNT+LNnT (0.54 and 0.17 g/L, respectively; Mann-Whitney test, $p = 0.038$) and total neutral core HMOs (0.67 and 0.20 g/L, respectively; Mann-Whitney test, $p = 0.019$). Maternal nutritional status, type of delivery, socioeconomic status and having pets at home had no associations with HMOs concentrations in any of the *SeLe* groups.

Tables 5 and A1 and Table A2 (Appendix A) show the Spearman rank correlation coefficient (r) between HMOs concentrations and quantitative clinical variables by *SeLe* group. Several significant correlations ($p < 0.05$) occurred in all *SeLe* groups, especially negative correlations between acidic HMOs and postpartum days. 6'-SL and postpartum days presented a negative correlation in all three groups ($Se+Le+$, $r = -0.71$; $Se-Le+$, $r = -0.70$ and $Se+Le-$, $r = -0.83$). Postpartum days also had negative correlations with other acidic HMOs in $Se+Le+$ and $Se-Le+$ and with total acidic HMOs in $Se+Le+$ and $Se+Le-$ ($r = -0.79$ and $r = -0.74$, respectively). Weak but significant positive correlations occurred between postpartum days and 3'-FL ($r = 0.35$) and LNDFH II ($r = 0.27$) in $Se+Le+$ (Table 5).

Negative correlations occurred among several HMOs and clinical variables of the infant, mainly in $Se+Le+$, where infant's weight presented negative correlations with all the acidic HMOs, total acidic ($r = -0.61$), total neutral core ($r = -0.33$), total fucosylated ($r = -0.27$) and total HMOs ($r = -0.39$). Infants' length and weight gain also had negative correlations with several HMOs, including total neutral core (infants' length, $r = -0.32$), total fucosylated (weight gain, $r = -0.32$), total acidic (length, $r = -0.35$ and weight gain, $r = -0.42$) and total HMOs (weight gain, $r = -0.37$) in $Se+Le+$ (Table 5).

Associations Regarding *Se* Status Alone

Additionally, associations between clinical and demographic factors with HMOs concentrations were investigated considering *Se* phenotype alone, irrespective of the *Le* phenotype. Nutritional status was associated with concentrations of some HMOs in $Se+$ but not in $Se-$ women. $Se+$ mothers with overweight (BMI 25–29.9 kg/m²) had a significantly higher concentration of 2'-FL (Dunn test, $p = 0.030$) and lower 3'-FL concentration (Dunn test, $p = 0.011$) than $Se+$ mothers with adequate weight (BMI 18.5–24.9 kg/m²). $Se+$ mothers with obesity (BMI > 30.0 kg/m²) presented no difference in 2'-FL and 3'-FL concentrations from $Se+$ mothers with adequate weight or overweight (Dunn test, $p > 0.05$). Allergic disease of the mother, infant's sex, socioeconomic status and having pets at home were not associated with HMOs concentrations neither in $Se+$ nor in $Se-$ women.

Tables A3 and A4 (Appendix A) show the Spearman rank correlation coefficient (r) between HMOs concentrations and quantitative clinical variables by *Se* phenotype. Major significant correlations ($p < 0.05$) were observed in $Se+$ women, which presented mostly negative correlations of several HMOs with postpartum days and infants' clinical variables. In $Se+$ women, some positive correlations were also observed, for instance, between 2'-FL and maternal BMI ($r = 0.30$) and between 3'-FL and postpartum days ($r = 0.31$). In the $Se-$ group, significant correlations were mostly negative, involving both 6'-SL and LSTc with postpartum days and infants' clinical variables.

Table 5. Spearman rank correlation coefficient (r) between HMOs concentrations and quantitative clinical variables in Group 1 (Se+Le+; n = 59).

| HMOs Concentrations | Maternal Variables | | | | | | Infant Variables | | | | | |
|---------------------|--------------------|-------|-----------------|-------|--------|-----------|------------------|-----------------|-----------------|--------|--------|-------------|
| | Parity | Age | Postpartum Days | BMI | Weight | PG Weight | PG BMI | Gestational Age | Weight at Birth | Weight | Length | Weight Gain |
| <i>Fucosylated</i> | | | | | | | | | | | | |
| 2'-FL | -0.02 | 0.02 | -0.07 | 0.18 | 0.20 | 0.14 | 0.18 | -0.12 | 0.14 | -0.05 | 0.11 | -0.14 |
| 3'-FL | 0.09 | 0.06 | 0.35 | 0.06 | -0.09 | 0.04 | -0.09 | -0.07 | -0.10 | 0.23 | 0.11 | 0.17 |
| LNFP I | -0.07 | -0.15 | -0.47 | 0.05 | 0.11 | 0.01 | 0.05 | 0.09 | 0.08 | -0.37 | -0.14 | -0.28 |
| LNDFH I | -0.04 | -0.21 | -0.25 | 0.12 | 0.02 | 0.21 | 0.10 | 0.20 | 0.08 | -0.29 | 0.04 | -0.37 |
| LNDFH II | 0.07 | 0.04 | 0.27 | -0.02 | -0.13 | 0.01 | -0.08 | -0.14 | -0.14 | 0.08 | 0.01 | -0.02 |
| DFLNHc | -0.08 | 0.01 | -0.24 | 0.13 | 0.16 | 0.15 | 0.14 | 0.12 | 0.05 | -0.06 | 0.07 | 0.04 |
| DFpLNnH | 0.05 | -0.09 | 0.01 | 0.01 | -0.03 | 0.12 | 0.08 | -0.08 | -0.20 | -0.16 | -0.22 | -0.14 |
| <i>Neutral core</i> | | | | | | | | | | | | |
| LNH | -0.05 | 0.01 | -0.42 | 0.02 | 0.17 | -0.03 | 0.04 | 0.17 | 0.04 | -0.20 | -0.23 | 0.06 |
| LNnH | -0.12 | 0.05 | 0.10 | 0.11 | 0.23 | 0.14 | 0.25 | -0.04 | 0.06 | 0.09 | 0.02 | 0.07 |
| LNT+LNnT | 0.02 | -0.15 | -0.54 | -0.13 | -0.08 | -0.13 | -0.13 | 0.21 | 0.04 | -0.39 | -0.32 | -0.13 |
| <i>Acidic</i> | | | | | | | | | | | | |
| 3'-SL | -0.07 | 0.01 | -0.25 | -0.12 | -0.10 | -0.16 | -0.18 | -0.08 | -0.17 | -0.34 | -0.32 | -0.25 |
| 6'-SL | 0.11 | 0.05 | -0.71 | -0.02 | -0.07 | -0.01 | -0.09 | 0.22 | 0.07 | -0.49 | -0.25 | -0.30 |
| LSTa | -0.06 | 0.01 | -0.62 | -0.15 | -0.12 | -0.14 | -0.17 | 0.25 | 0.01 | -0.41 | -0.29 | -0.20 |
| LSTb | 0.05 | -0.17 | -0.26 | -0.08 | -0.07 | -0.12 | -0.11 | 0.01 | 0.03 | -0.30 | -0.22 | -0.21 |
| LSTc | 0.05 | -0.01 | -0.76 | -0.04 | -0.07 | 0.02 | -0.05 | 0.25 | 0.02 | -0.54 | -0.28 | -0.36 |
| <i>Total</i> | | | | | | | | | | | | |
| Fucosylated | -0.10 | -0.13 | -0.24 | 0.14 | 0.13 | 0.13 | 0.13 | 0.05 | 0.11 | -0.27 | 0.01 | -0.32 |
| Neutral core | -0.03 | -0.07 | -0.51 | -0.07 | 0.04 | -0.11 | -0.07 | 0.25 | 0.09 | -0.33 | -0.32 | -0.03 |
| Acidic | 0.05 | 0.02 | -0.79 | -0.07 | -0.10 | -0.05 | -0.13 | 0.26 | 0.01 | -0.61 | -0.35 | -0.42 |
| Total HMOs | -0.06 | -0.11 | -0.43 | 0.15 | 0.13 | 0.14 | 0.11 | 0.10 | 0.13 | -0.39 | -0.10 | -0.37 |

Significant correlations ($p < 0.05$) are highlighted in green (positive) and yellow (negative). PG: pre-gestational.

4. Discussion

There are currently a few studies reporting HMOs absolute concentrations obtained by state-of-the-art analytical methods—such as LC-MS—in well-defined human milk samples [47]. Knowing the exact amount of the most representative HMOs is important to access the daily intake of HMOs by the infants and the biological effects of these molecules, since some HMOs may act in a dose-dependent manner [31,32,34,48,49]. In this study, we presented absolute concentrations of 16 representative HMOs measured by LC-MS from 78 full-term, mature human milk samples classified according to the *SeLe* phenotype and investigated associations between maternal and infant characteristics and HMOs composition/concentrations.

The frequency of the *SeLe* phenotypes varies among different ethnic populations. In Caucasians and Asians, the *Se+Le+* phenotype (determined from human milk and blood samples) varies from 55 to 73%, *Se–Le+* varies from 20 to 31%, *Se+Le–* varies from 6 to 11% and *Se–Le–* varies from 3 to 5% [25,27,50,51]. In African population from Burkina Faso, the *SeLe* distribution (from saliva samples) was reported to be 54% (*Se+Le+*), 14% (*Se–Le+*), 25% (*Se+Le–*) and 7% (*Se–Le–*) [52]. A study in a semi-isolated Black community in Northern Brazil reported a very similar *SeLe* phenotypes (from saliva and blood samples) distribution to Burkina Faso [53]. A study conducted in the state of São Paulo, Brazil [54] with 827 participants regardless of ethnicity revealed a prevalence of *Se+Le+* phenotype (from blood samples) in 78% of the population, similarly with the *Se+Le+* prevalence in our cohort (76%; from human milk samples), which was also composed by different ethnicities. Our cohort presented a *SeLe* distribution closer to the Caucasian/Asian than the semi-isolated Black from Northern Brazil. In our cohort, only one lactating mother was assigned to Group 4 (*Se–Le–*), which agrees with the rare occurrence of this phenotype in Caucasians and Asians [22,27].

Our study demonstrated a difference in HMOs profile and concentrations among *SeLe* groups, supporting previously reported results [23,25–27,55]. Furthermore, our results showed that the different HMOs composition of the 3 *SeLe* groups had no influence on infant's weight gain and anthropometric parameters, which reinforces that human milk is nutritionally adequate, apart from the different HMOs composition. Similarly, Sprenger et al. (2017) did not observe differences in anthropometric parameters between infants who consumed breast milk with low or high 2'-FL content (*Se–* and *Se+* milk, respectively) [56]. However, as in the study of Sprenger et al. (2017), our cohort was composed of healthy term-born infants. Charbonneau et al. (2016) reported a difference in HMOs composition from *Se–* mothers having healthy or severely stunted infants, which was not observed in *Se+* mothers [36]. Therefore, the influence of *SeLe* milk groups on other populations, such as preterm or malnourished infants remains to be studied.

In a systematic review of 21 studies on HMOs concentrations, Thurl et al. (2017) reported TF-LNH, 2'-FL, DF-LNH II, and LNFP I to be the most abundant neutral HMOs and 6'-SL the most abundant acidic HMO in *Se+* term human milk. However, the authors warned about a possible bias on the high concentrations of TF-LNH and DF-LNH II, which needs to be confirmed [47]. The systematic review reported concentrations of 33 HMOs and the results corroborate that the 16 HMOs analyzed in our study are among the most representative in human milk, except for DFLNHc and DFpLNnH (in term milk), which were not reported in the systematic review [47]. Our results largely agree with the concentrations reported in the above-mentioned systematic review and with Kunz et al. (2017), not included in the review [26]. The most abundant HMO in *Se+Le+* e *Se+Le–* was 2'-FL, followed by LNDFH I in *Se+Le+* and by LNFP I in *Se+Le–*. Among the acidic HMOs, we also observed the highest concentration of 6'-SL not only in *Se+* but also in all *SeLe* groups. However, Kunz et al. (2017) reported a higher LNT concentration in *Se–Le+* than in *Se+Le+* and *Se+Le–*, which was not observed in our cohort.

Although our samples were collected in a single time point, we could observe some of the dynamics in HMOs concentrations during lactation reported in longitudinal studies, investigating correlations of HMOs concentrations and time postpartum. A significant increase in 3'-FL concentrations during the first months of lactation, as well as a significant decrease in LNFP I and LSTc have been previously

reported [23,29,47,57–60]. We observed a significant positive correlation between 3'-FL and time postpartum and significant negative correlations between several HMOs—including LNFP I and LSTc—and time postpartum in Se+Le+ milk (Table 5). As in the study of McGuire et al. (2017) [28], 6'-SL, LSTc and LNH negative correlations with time postpartum were also observed. Total acidic HMOs presented the highest negative Spearman rank correlation coefficient ($r = -0.79$) with time postpartum in Se+Le+, which is in accordance with a significant decrease of acidic HMOs in the first three months of lactation reported in the systematic review of HMOs concentrations [47]. However, although associated with the variability in the concentrations of some HMOs, time postpartum does not explain variability alone. For example, the HMO with the highest coefficient of variation in Se+Le+ milk was LNDFH II (CV 293%, Table 4), but the correlation of LNDFH II concentrations with time postpartum had a low correlation coefficient ($r = 0.27$, Table 5), although significant. This is even more evident with DFpLNnH in Se+Le+, with a CV of 154% (Table 4), but without significant correlation with time postpartum ($r = 0.01$, Table 5).

There are some hypotheses about the role of HMOs on infant allergy development, related to the establishment of the gut microbiota, but the influence of maternal allergic diseases on HMOs composition and concentrations have not been substantially studied [30,61–63]. In our study, we investigated whether maternal allergic disease (asthma, rhinitis or eczema) could explain, at least in part, the great variability in the concentrations of HMOs observed in each *SeLe* milk group. We observed significantly higher DFpLNnH concentrations in the milk from Se+Le+ and Se+Le- women with allergic disease than in those without allergic disease. When we considered secretor status alone, there were no differences in DFpLNnH concentrations or another HMO between the groups. Sjögren et al. (2007) [62] performed—in a smaller cohort—the only study previously published that investigated associations between maternal allergic diseases and HMOs concentrations and found no differences in the concentrations of nine neutral HMOs from colostrum of allergic and non-allergic mothers. However, DFpLNnH was not included in the analysis [62]. Furthermore, in our cohort, Se+ mothers presented a higher prevalence of allergic disease than Se- mothers, which agrees with a higher prevalence of asthma and a higher susceptibility to asthma exacerbation observed in Se+, particularly in blood group O/Se+ individuals [64,65]. On the other hand, a 1968 study reported no differences in the incidence of allergic diseases between Se+ and Se- individuals [66]. Similarly, Sprenger et al. (2017) did not observe differences in allergy prevalence on mothers presenting or not 2'-FL in the milk (Se+ and Se-, respectively) [30].

We observed differences in 2'-FL and 3'-FL concentrations between overweight and eutrophic Se+ mothers, as well as a positive correlation between 2'-FL concentrations in Se+ milk and maternal BMI. However, the difference in 2'-FL and 3'-FL concentrations from mothers with distinct nutritional statuses was no longer significant when considering the Lewis phenotype together. Positive correlations between maternal BMI or weight and total HMOs or 2'-FL concentrations have been previously reported [27,28]. It has been hypothesized that maternal diet may influence HMOs concentrations, yet Azad et al. (2018) found no associations between the overall diet quality and HMOs concentrations [29]. More studies are needed to verify dietary and nutritional effects on HMOs composition and concentrations.

To date, few studies have investigated associations between parity and HMOs concentrations, without agreement. While Elwakiel et al. (2018) found no associations [27], Azad et al. (2018) observed higher LNT and LNnT and lower 3'-FL concentrations in multiparous mothers [29]. Interestingly, we observed a significant positive correlation between parity and some HMOs—including LNT+LNnT—and a negative correlation between parity and 3'-FL, similarly to Azad et al. (2018), but only in Se+Le- mothers (Table A2). However, Se+Le- was a small group, which may limit conclusions. We found no correlations between HMOs concentrations and parity in other *SeLe* groups.

We observed unprecedented significant differences ($p < 0.04$) in some HMOs concentrations between Se+Le- milk from mothers of boys and girls, even with a small number of mother-infant pairs in Se+Le- group. As previously reported, we found no associations between HMOs concentrations and socioeconomic status as well as with the type of delivery in any *SeLe* group [27,29,59].

Exclusively breastfed infants present lower weight gain—although adequate—during the first year of life than formula-fed infants [67,68]. There is a possible effect of breastfeeding on obesity prevention, related to the lower weight gain in the first year of life, but the mechanisms involved in this protection are not yet understood [69]. Importantly, the intricate composition and high amounts of HMOs are responsible for crucial differences between human milk and infant formula, which may contribute to the differences in health outcomes between breastfed and formula-fed infants [4,70]. HMOs may influence infant growth and the development of obesity directly since they are absorbed into the circulation and can induce systemic effects [71,72]. A study demonstrated that the HMO lacto-N-fucopentaose III modulates metabolic functions by improving glucose tolerance, insulin sensitivity and suppressing lipogenesis in the liver of diet-induced obese mice [73]. Additionally, HMOs may also modulate infant growth and obesity development indirectly by altering the gut microbiome [36,74]. Inverse associations between concentrations of individual HMOs and infant body composition have been previously reported. Alderete et al. (2015) observed that each 1 µg/mL increase in LNFP I concentration was associated with a 0.4 g lower infant weight at 1 month postpartum. At 6 months, inverse associations were also observed between LNFP I and body weight, lean and fat mass, as well as between LNnT and fat mass [35]. A higher proportion of LSTc in milk was associated with a lower infant's weight-for-age (WAZ) score in the study conducted by Davis et al. (2016), who also observed that a higher proportion of 3'-SL, LNFP I + III and DFLNH_a contributed positively to WAZ and height-for-age scores [75]. In our study, although the infants—all exclusively breastfed—presented adequate weight gain, we demonstrated inverse associations between concentrations of specific HMOs in breast milk—including LNFP I, LNT+LNnT and LSTc—and infant weight and weight gain at 1 month after birth (Table 5). The observed inverse associations between HMOs concentrations and infant weight gain suggest a potential role of HMOs on infant growth and metabolism, which deserves future investigations.

Among the major strengths of our study are the accurate, extensively validated method utilized for HMOs quantification and the standardized human milk collection procedure, which occurred in short time intervals to minimize natural diurnal variations and biases caused by milk sampling. Another important strength of our study is that our population was composed by exclusively breastfed infants, since the consumption of solid foods and water besides breastfeeding can impact infant growth, adding some bias on the results about the influence of human milk components on infant's health. However, our study has some limitations. Although we selected 16 representative HMOs from the ~150 different structures occurring in human milk, it is possible that maternal and infant factors are associated with other HMOs not included in our analyses. The utilization of the ISAAC questionnaire for the screening of maternal allergy is also considered a limitation, however, there is no validated instrument available to be applied in the adult population. Furthermore, the small number of Se-Le+, Se+Le-, and Se-Le- mothers impaired more robust conclusions regarding these groups.

5. Conclusions

Our study reveals unprecedented data on HMOs composition from breastfeeding Brazilian women, as well as novel associations of maternal allergic disease and infant's sex with concentrations of specific HMOs. Our results also showed that maternal and infant factors might influence HMOs concentrations in some *SeLe* group but not in others. For instance, the infant's sex was associated with specific HMOs only in Se+Le-. Ultimately, the results showed that different HMOs composition does not impact infant growth, but higher concentrations of specific HMOs may protect against excessive weight gain.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6643/11/6/1358/s1>, Spreadsheet1: complete descriptive statistics of HMOs concentrations according to *SeLe* and *Se* phenotype.

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Appendix A

Table A1. Spearman rank correlation coefficient (r) between HMOs concentrations and quantitative clinical variables in Group 2 (Se–Le+; n = 9).

| HMOs Concentrations | Maternal Variables | | | | | | | Infant Variables | | | | |
|---------------------|--------------------|-------|-----------------|-------|--------|-----------|---------|------------------|-----------------|--------|--------|-------------|
| | Parity | Age | Postpartum Days | BMI | Weight | PG Weight | PPG BMI | Gestational Age | Weight at Birth | Weight | Length | Weight Gain |
| <i>Fucosylated</i> | | | | | | | | | | | | |
| 3'-FL | 0.28 | -0.06 | 0.35 | -0.58 | -0.52 | -0.41 | -0.14 | -0.26 | -0.14 | 0.46 | 0.49 | 0.77 |
| LNDFH II | 0.39 | 0.42 | -0.08 | -0.30 | 0.00 | -0.32 | 0.11 | -0.04 | -0.29 | -0.04 | 0.05 | 0.14 |
| DFpLNnH | -0.06 | -0.28 | 0.25 | -0.02 | -0.19 | -0.28 | -0.08 | -0.18 | -0.10 | 0.36 | 0.40 | 0.09 |
| <i>Neutral core</i> | | | | | | | | | | | | |
| LNH | -0.17 | 0.01 | -0.50 | 0.08 | -0.21 | -0.08 | -0.42 | -0.54 | -0.26 | -0.56 | -0.75 | -0.06 |
| LNnH | -0.17 | -0.44 | 0.11 | -0.32 | -0.60 | -0.32 | -0.61 | -0.49 | -0.21 | 0.11 | -0.02 | 0.26 |
| LNT+LNnT | 0.17 | 0.73 | -0.35 | 0.00 | 0.19 | -0.18 | -0.16 | 0.22 | -0.19 | -0.43 | -0.45 | -0.49 |
| <i>Acidic</i> | | | | | | | | | | | | |
| 3'-SL | 0.62 | 0.38 | -0.34 | -0.08 | 0.05 | -0.22 | 0.19 | -0.22 | -0.36 | -0.46 | -0.52 | 0.60 |
| 6'-SL | -0.06 | 0.89 | -0.70 | 0.00 | 0.36 | -0.05 | 0.12 | -0.18 | -0.50 | -0.71 | -0.72 | -0.37 |
| LSTa | -0.39 | 0.49 | -0.69 | 0.40 | 0.45 | 0.41 | 0.12 | -0.07 | -0.19 | -0.57 | -0.58 | -0.66 |
| LSTb | 0.28 | 0.37 | -0.16 | -0.37 | -0.12 | -0.72 | -0.46 | -0.11 | -0.36 | -0.46 | -0.47 | -0.31 |
| LSTc | -0.39 | 0.45 | -0.90 | 0.03 | 0.17 | -0.29 | -0.19 | -0.72 | -0.83 | -0.75 | -0.74 | -0.49 |
| <i>Total</i> | | | | | | | | | | | | |
| Fucosylated | 0.51 | 0.23 | 0.19 | -0.57 | -0.40 | -0.47 | -0.08 | -0.10 | -0.26 | 0.29 | 0.29 | 0.66 |
| Neutral core | 0.00 | 0.64 | -0.46 | 0.02 | 0.05 | -0.18 | -0.40 | 0.02 | -0.31 | -0.38 | -0.52 | -0.41 |
| Acidic | 0.00 | 0.84 | -0.66 | 0.03 | 0.32 | -0.07 | 0.14 | -0.19 | -0.50 | -0.61 | -0.63 | -0.20 |
| Total HMOs | 0.17 | 0.66 | -0.38 | -0.25 | 0.12 | -0.25 | 0.11 | -0.11 | -0.55 | -0.36 | -0.31 | 0.09 |

Significant correlations (p < 0.05) are highlighted in green (positive correlations) and yellow (negative correlations). PG: pre-gestational.

Table A2. Spearman rank correlation coefficient (r) between HMOs concentrations and quantitative clinical variables in Group 3 (Se+Le−; n = 9).

| HMOs Concentrations | Maternal Variables | | | | | | Infant variables | | | | | |
|---------------------|--------------------|-------|-----------------|-------|--------|-----------|------------------|-----------------|-----------------|--------|--------|-------------|
| | Parity | Age | Postpartum Days | BMI | Weight | PG Weight | PG BMI | Gestational Age | Weight at Birth | Weight | Length | Weight Gain |
| <i>Fucosylated</i> | | | | | | | | | | | | |
| 2'-FL | −0.32 | 0.46 | 0.03 | 0.38 | 0.40 | 0.17 | 0.13 | 0.59 | 0.07 | −0.04 | 0.04 | 0.07 |
| 3'-FL | −0.79 | −0.23 | −0.08 | −0.28 | −0.28 | −0.50 | −0.55 | −0.05 | −0.38 | −0.57 | −0.50 | 0.18 |
| LNFP I | 0.79 | 0.01 | −0.12 | 0.28 | 0.38 | 0.48 | 0.68 | −0.17 | 0.33 | 0.29 | 0.32 | −0.61 |
| DFpLNnH | 0.00 | −0.62 | −0.03 | 0.23 | 0.13 | 0.13 | −0.08 | −0.37 | 0.00 | 0.04 | 0.09 | −0.11 |
| <i>Neutral core</i> | | | | | | | | | | | | |
| LNH | 0.79 | −0.15 | 0.00 | −0.20 | −0.18 | 0.10 | 0.23 | −0.48 | 0.21 | 0.21 | −0.02 | −0.36 |
| LNnH | 0.63 | 0.34 | 0.21 | 0.05 | 0.02 | 0.33 | 0.35 | 0.05 | 0.36 | 0.61 | 0.36 | 0.00 |
| LNT+LNnT | 0.79 | −0.26 | −0.11 | −0.22 | −0.13 | 0.03 | 0.18 | −0.49 | 0.19 | 0.00 | −0.05 | −0.50 |
| <i>Acidic</i> | | | | | | | | | | | | |
| 3'-SL | −0.32 | −0.63 | −0.38 | −0.37 | −0.28 | −0.59 | −0.50 | −0.50 | −0.43 | −0.82 | −0.59 | 0.07 |
| 6'-SL | −0.16 | −0.16 | −0.83 | −0.02 | 0.28 | 0.06 | 0.48 | −0.38 | −0.45 | −0.61 | −0.81 | −0.29 |
| LSTa | 0.79 | −0.34 | −0.09 | −0.57 | −0.53 | −0.44 | −0.15 | −0.67 | 0.10 | −0.04 | −0.02 | −0.46 |
| LSTb | 0.79 | −0.55 | 0.09 | 0.32 | 0.25 | 0.30 | 0.23 | −0.44 | 0.50 | 0.14 | 0.23 | −0.82 |
| LSTc | 0.16 | −0.79 | −0.44 | 0.22 | 0.27 | 0.19 | 0.23 | −0.69 | −0.12 | −0.21 | −0.40 | −0.75 |
| <i>Total</i> | | | | | | | | | | | | |
| Fucosylated | 0.32 | 0.56 | −0.15 | 0.37 | 0.53 | 0.40 | 0.55 | 0.39 | 0.40 | 0.04 | −0.09 | −0.32 |
| Neutral core | 0.80 | −0.21 | 0.01 | −0.19 | −0.15 | 0.11 | 0.22 | −0.44 | 0.26 | 0.25 | 0.10 | −0.47 |
| Acidic | −0.16 | −0.48 | −0.74 | −0.18 | 0.09 | −0.13 | 0.20 | −0.55 | −0.47 | −0.67 | −0.72 | −0.41 |
| Total HMOs | 0.32 | 0.43 | −0.22 | 0.43 | 0.63 | 0.57 | 0.75 | 0.28 | 0.33 | −0.04 | −0.13 | −0.36 |

Significant correlations ($p < 0.05$) are highlighted in green (positive) and yellow (negative). PG: pre-gestational.

Table A3. Spearman rank correlation coefficient (r) between HMOs concentrations and quantitative clinical variables in Se+ women (n = 68).

| HMOs Concentrations | Maternal Variables | | | | | | Infant Variables | | | | | |
|---------------------|--------------------|-------|-----------------|-------|--------|-----------|------------------|-----------------|-----------------|--------|--------|-------------|
| | Parity | Age | Postpartum Days | BMI | Weight | PG Weight | PG BMI | Gestational Age | Weight at Birth | Weight | Length | Weight Gain |
| <i>Fucosylated</i> | | | | | | | | | | | | |
| 2'-FL | -0.04 | 0.00 | -0.10 | 0.30 | 0.29 | 0.23 | 0.25 | -0.04 | 0.11 | -0.04 | 0.08 | -0.15 |
| 3'-FL | 0.09 | 0.10 | 0.31 | -0.23 | -0.14 | -0.13 | -0.20 | -0.08 | -0.09 | 0.16 | 0.07 | 0.17 |
| LNFP I | -0.04 | -0.18 | -0.43 | 0.24 | 0.21 | 0.16 | 0.18 | 0.06 | 0.09 | -0.30 | -0.09 | -0.28 |
| LNDFH I | 0.04 | -0.04 | -0.07 | -0.08 | -0.02 | 0.06 | 0.02 | 0.18 | 0.09 | -0.19 | 0.11 | -0.28 |
| LNDFH II | 0.10 | 0.09 | 0.29 | -0.16 | -0.09 | -0.05 | -0.10 | -0.10 | -0.10 | 0.09 | 0.05 | 0.00 |
| DFLNHc | 0.01 | 0.15 | -0.08 | 0.03 | -0.02 | 0.02 | 0.05 | 0.13 | 0.07 | -0.03 | 0.13 | 0.04 |
| DFpLNnH | 0.07 | -0.14 | -0.01 | -0.04 | -0.02 | 0.07 | 0.05 | -0.11 | -0.16 | -0.15 | -0.17 | -0.14 |
| <i>Neutral core</i> | | | | | | | | | | | | |
| LNH | 0.05 | 0.04 | -0.34 | 0.09 | -0.03 | -0.03 | 0.04 | 0.08 | 0.11 | -0.15 | -0.16 | 0.03 |
| LNnH | -0.01 | 0.13 | 0.12 | 0.19 | 0.08 | 0.13 | 0.24 | -0.02 | 0.13 | 0.13 | 0.09 | 0.06 |
| LNT+LNnT | 0.12 | -0.14 | -0.45 | -0.10 | -0.15 | -0.12 | -0.11 | 0.07 | 0.10 | -0.35 | -0.26 | -0.14 |
| <i>Acidic</i> | | | | | | | | | | | | |
| 3'-SL | -0.06 | -0.03 | -0.28 | -0.14 | -0.16 | -0.22 | -0.22 | -0.12 | -0.18 | -0.37 | -0.33 | -0.24 |
| 6'-SL | 0.07 | 0.04 | -0.73 | -0.02 | -0.01 | 0.01 | -0.03 | 0.14 | 0.00 | -0.51 | -0.31 | -0.30 |
| LSTa | 0.01 | 0.03 | -0.53 | -0.18 | -0.19 | -0.18 | -0.20 | 0.14 | 0.02 | -0.39 | -0.24 | -0.21 |
| LSTb | 0.14 | -0.22 | -0.23 | -0.04 | -0.04 | -0.07 | -0.08 | -0.06 | 0.11 | -0.28 | -0.16 | -0.25 |
| LSTc | 0.06 | -0.06 | -0.72 | -0.07 | -0.06 | 0.00 | -0.05 | 0.15 | 0.02 | -0.53 | -0.27 | -0.39 |
| <i>Total</i> | | | | | | | | | | | | |
| Fucosylated | -0.06 | -0.12 | -0.28 | 0.24 | 0.25 | 0.22 | 0.21 | 0.08 | 0.12 | -0.25 | -0.01 | -0.33 |
| Neutral core | 0.09 | -0.07 | -0.42 | -0.01 | -0.10 | -0.10 | -0.05 | 0.11 | 0.15 | -0.29 | -0.24 | -0.07 |
| Acidic | 0.03 | -0.03 | -0.80 | -0.08 | -0.08 | -0.07 | -0.11 | 0.16 | -0.03 | -0.63 | -0.39 | -0.43 |
| Total HMOs | -0.02 | -0.11 | -0.44 | 0.24 | 0.25 | 0.23 | 0.20 | 0.10 | 0.14 | -0.37 | -0.10 | -0.39 |

Significant correlations ($p < 0.05$) are highlighted in green (positive) and yellow (negative). PG: pre-gestational.

Table A4. Spearman rank correlation coefficient (r) between HMOs concentrations and quantitative clinical variables in Se- women (n = 10).

| HMOs Concentrations | Maternal Variables | | | | | | Infant Variables | | | | | |
|---------------------|--------------------|-------|-----------------|-------|--------|-----------|------------------|-----------------|-----------------|--------|--------|-------------|
| | Parity | Age | Postpartum Days | BMI | Weight | PG Weight | PG BMI | Gestational Age | Weight at Birth | Weight | Length | Weight Gain |
| <i>Fucosylated</i> | | | | | | | | | | | | |
| 3'-FL | 0.43 | -0.24 | 0.18 | -0.18 | -0.36 | -0.33 | 0.20 | -0.37 | 0.07 | 0.31 | 0.32 | 0.29 |
| LNDFH II | 0.52 | 0.14 | -0.22 | 0.22 | -0.10 | -0.29 | 0.38 | -0.14 | -0.02 | -0.14 | -0.08 | -0.25 |
| DFpLNnH | -0.09 | -0.19 | 0.26 | -0.42 | -0.19 | -0.37 | -0.28 | -0.10 | -0.13 | 0.29 | 0.31 | 0.14 |
| <i>Neutral core</i> | | | | | | | | | | | | |
| LNH | -0.26 | 0.07 | -0.35 | -0.25 | 0.05 | -0.03 | -0.49 | -0.43 | -0.33 | -0.43 | -0.59 | 0.11 |
| LNnH | -0.26 | -0.28 | 0.18 | -0.57 | -0.35 | -0.25 | -0.62 | -0.39 | -0.30 | 0.12 | 0.01 | 0.36 |
| LNT+LNnT | -0.09 | 0.78 | -0.24 | -0.10 | -0.12 | -0.18 | -0.41 | 0.33 | -0.28 | -0.33 | -0.35 | -0.14 |
| <i>Acidic</i> | | | | | | | | | | | | |
| 3'-SL | 0.35 | 0.44 | -0.28 | -0.12 | -0.22 | -0.23 | -0.12 | -0.10 | -0.35 | -0.43 | -0.46 | 0.57 |
| 6'-SL | 0.09 | 0.70 | -0.78 | 0.37 | 0.08 | -0.11 | 0.14 | -0.23 | -0.43 | -0.81 | -0.81 | -0.46 |
| LSTa | -0.43 | 0.53 | -0.51 | 0.15 | 0.24 | 0.37 | -0.15 | 0.04 | -0.28 | -0.48 | -0.48 | -0.32 |
| LSTb | 0.09 | 0.54 | -0.11 | -0.23 | -0.42 | -0.65 | -0.52 | 0.14 | -0.35 | -0.38 | -0.38 | -0.04 |
| LSTc | -0.26 | 0.32 | -0.91 | 0.15 | 0.07 | -0.28 | -0.18 | -0.69 | -0.75 | -0.83 | -0.83 | -0.54 |
| <i>Total</i> | | | | | | | | | | | | |
| Fucosylated | 0.61 | -0.01 | 0.01 | -0.08 | -0.33 | -0.39 | 0.24 | -0.22 | 0.00 | 0.12 | 0.12 | 0.14 |
| Neutral core | -0.26 | 0.68 | -0.32 | -0.20 | -0.12 | -0.16 | -0.58 | 0.15 | -0.45 | -0.31 | -0.40 | -0.07 |
| Acidic | -0.09 | 0.83 | -0.63 | 0.23 | 0.07 | -0.02 | 0.00 | -0.01 | -0.50 | -0.55 | -0.57 | -0.11 |
| Total HMOs | 0.26 | 0.49 | -0.50 | 0.17 | -0.18 | -0.33 | 0.16 | -0.21 | -0.43 | -0.48 | -0.44 | -0.18 |

Significant correlations (p < 0.05) are highlighted in green (positive) and yellow (negative). PG: pre-gestational.

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