

Concentrations of Oligosaccharides in Human Milk and Child Growth

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Abstract

Background: The relationship between human milk oligosaccharides (HMO) and child growth has been investigated only insufficiently with ambiguous results. Therefore, this study examines potential influencing factors of HMO concentrations and how HMOs are associated with child growth parameters.

Methods: Milk samples from the German LIFE Child cohort of healthy children were analyzed for 9 HMOs. Putative associations with maternal and child cofactors and child height, head circumference and BMI between three months and seven years of age were examined. Secretor status, defined as the presence of 2'-fucosyllactose, was investigated for associations with infant outcomes.

Results: Our population consisted of 21 (14.7%) non-secretor and 122 (85.3%) secretor mothers. Maternal age was significantly associated with higher 3'SL concentrations; gestational age was associated with LNT, 6'SL and LNFP-I. Pre-pregnancy BMI was negatively associated with LNnT only in non-secretors. The growth velocity of non-secretors' children was inversely associated with LNnT at 3 months to 1 year ($R=0.95$ [0.90, 0.99], $p=0.014$), 1 to 2 years ($R=0.80$ [0.72, 0.88], $p<0.001$) and 5 to 6 years ($R=0.71$ [0.57, 0.87], $p=0.002$). 2'FL was negatively associated with BMI consistently, reaching statistical significance at 3 months and 4 and 5 years. Children of non-secretors showed higher BMI at 3 months, 6 months, and 3, 6, and 7 years of age.

Conclusion: We found that some associations between HMOs and infant growth may extend beyond the infancy and breastfeeding periods. They highlight the importance of both maternal and infant parameters in the understanding of the underlying associations.

Trial registration: The study is registered with ClinicalTrial.gov: NCT02550236.

Introduction

Human milk is being explored intensively to understand its composition and physiological role for the breastfed infant. Lipids (1,2) have been identified as the most significant source of energy in mature milk. Additional important compounds of human milk are proteins, including enzymes and bioactive proteins like antibodies, nitrogenous compounds, and especially nucleotides, which influence the enzyme activity and the functionality of the immune system, hormones, vitamins, water and carbohydrates, including lactose and oligosaccharides. Together with lipids, lactose is an important source of energy, especially for the developing human brain (3). HMOs form the third largest solid fraction in human milk; they represent about 20% of the total carbohydrates, with an estimated amount of up to 20g/L in colostrum (4,5). HMOs are composed of 5 different monosaccharides (Glc, GlcNAc, Gal, Fuc and Neu5Ac), which are linked together via glycosidic bonds (6) to produce a wide variety of different structures (7).

HMO concentrations are influenced by lactation stage and maternal genetic factors (8–11) and probably to a lesser extent by maternal weight and body mass index (BMI) before pregnancy (8,9,12). HMOs have been investigated for their potential role in the early growth of neonates; however, their effects in early

and later metabolic health are unclear (13–19). To date, only one study has investigated the effects of HMOs on growth parameters beyond infancy (17). The possible underlying biochemical or physiological processes linking HMOs and infant growth are not understood. HMOs are indigestible but can be fermented at least partly by the infant's microbiome (20–22). Thus, they support the maturation of the gastrointestinal tract and the immune system and can protect against the colonization of pathogenic microorganisms by inhibiting their anchoring to human epithelial cells (12,23–25). In preclinical models, various studies have examined the effects of HMOs (sometimes combined with microbiota) on gut epithelial maturation, differentiation and signaling processes (26–28), which can affect nutrient uptake and developmental programming, as exemplified by their effects on bone formation (29). These preliminary observations suggest a potential role of HMOs and microbiota on infant growth.

In this study, we examine the association between maternal factors (maternal age, pre-pregnancy BMI) and the child's birth parameters (anthropometric measurements, gestational age (GA)) and the oligosaccharide composition in breast milk at 3 months postpartum. We also hypothesize associations between HMO concentrations and the child's anthropometric measurements (height, growth velocity, head circumference (HC), BMI) up to 7 years of age.

Methods

Study Design and Participants

All data were collected within the LIFE Child study at the Research Centre for Civilization Diseases in Leipzig, Germany (www.ClinicalTrials.gov: NCT02550236). Children and their parents are recruited from the 24th week of gestation to 16 years of age to investigate environmental, metabolic and genetic associations with children's development (30). The study is described in detail elsewhere (30,31).

Between 2011 and 2014, 155 milk samples were collected from 153 nursing mothers at the 3-month baseline visit (Figure 1). The 3-month-visit took place after their 2nd full month and before the end of the 1st week of their 4th month of life. We excluded one sample because of a twin pregnancy and 9 samples because of preterm birth. Two mothers contributing two pregnancies were included. Finally, 145 sample-children combinations were included. Further, 132 follow-up measurements were documented at 6 months, 122 at 1 year, 106 at 2 years, 104 at 3 years, 90 at 4 years, 87 at five years, 59 at 6 years, and 37 at 7 years of age.

Informed written consent was provided by all parents for their children. The study has been conducted per the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Medical Faculty of the University of Leipzig (Reg. No. 264-10-19042010).

Milk Collection, Storage and Analysis

Under private conditions, about 20mL of milk was collected at the 3-month visit using a milk pump (Medela Symphony®). The milk was stored without processing at -80°C at our biobank (30) until its

transport to Nestlé Research, Lausanne, on dry ice. The concentrations of 9 HMOs were determined by liquid chromatography (32): 2'-fucosyllactose (2'FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-fucopentaose-I (LNFP-I), lacto-N-fucopentaose-V (LNFP-V) and lacto-N-neofucopentaose (LNnFP).

Measurements

Maternal pre-pregnancy weight and height and the child's birth parameters were taken from the maternity log ("Mutterpass"). This booklet is updated by medical staff during pregnancy checkups.

Measurements were taken by trained research assistants according to standard procedures.

Height/length was measured using the Dr. Keller II infantometer until one year of age and the Dr. Keller I stadiometer afterward. Weight was determined by using a scale (the seca 757 or the seca 701). HC was measured using non-dilatable measuring tape. BMI was calculated. Height/length, weight, and BMI were transformed to standard deviation scores (SDS) according to the guidelines from the German Working Group on Obesity in Childhood and Adolescence (33). HC measurements were transformed to SDS using German standards from the KiGGS study (34). As a measure of growth, growth velocity was calculated as the standardized difference between the three-month and the one-year measurement and afterward between two consecutive height measures.

Statistical Analysis

All statistical analysis were carried out using R 4.0 (35). Descriptive statistics were given as median [min, max] for HMOs and mean (standard deviation) for the other continuous variables (Table 1). Correlations (r) between the HMOs were investigated using Pearson correlations on the log-transformed values. Secretor status was determined based on the 2'FL concentration, with values of $\leq 53\text{mg/L}$ corresponding to non-secretors and $>53\text{mg/L}$ corresponding to secretors. Differences in means were tested using t-tests. For HMOs, differences in medians were tested using Kruskal-Wallis tests or censored regression models when data below the limit of quantification (LoQ) occurred. Values below the LoQ were set to the LoQ and marked as left censored. Chi-squared tests were applied to test differences in proportions. The associations between the HMOs and anthropometric and maternal parameters were examined using generalized additive models for location, shape, and scale (36,37), with HMO as the outcome and the other variables as predictor variables. Modeling was done separately for each age group, assuming a log-linear relationship between predictor and outcome. Due to the HMO values' considerable skewness, these values were log-transformed. A Box-Cox Cole and Green distribution or its censored equivalent was chosen to describe the outcomes' distributions. Investigating the models' error structure revealed variances according to secretor status. Therefore, variance was modeled dependent on secretor status. Skewness was dependent on secretor status only for LNnT. With evidence of an interaction between the predictor and the secretor status, the respective interaction term was included in the model. The models' appropriateness was checked using different plots (QQ-plot, variance against fitted, variance against covariates, influence vs. cooks distance; plots not shown). Effects are reported as ratios ($R=\exp(\beta)$) or differences (β), including the 95% confidence interval. As non-secretors values of LNFP-I and 2'FL were

below the LoQ ($\leq 15\text{mg/L}$ and 53mg/L , respectively) (32), associations involving LNFP-I and 2'FL were only modeled in the secretor subgroup. P-values ≤ 0.05 were considered to be statistically significant. Essentially, we concentrate on occurring patterns instead of single significant test results.

Table 1. Descriptive statistics of the cohort stratified by secretor group and overall given as median [min, max] for HMOs and mean (standard deviation) for the other continuous variables. Group differences were tested using Kruskal-Wallis-tests (HMOs) and t-tests (other variables).

	Secretor (n=124)	Non-secretor (n=21)	p- value	n=145	n missing) (non-
Maternal age	30.3 (4.12)	30.2 (4.95)	0.898	30.3 (4.24)	143
Pre-pregnancy BMI	23.2 (3.78)	22.7 (3.83)	0.642	23.2 (3.78)	124
Gestational age	40.0 (1.15)	40.2 (1.26)	0.494	40.0 (1.16)	145
Birth weight	3467 (473)	3646 (486)	0.13	3493 (477)	145
Birth length	50.3 (2.37)	50.7 (2.28)	0.475	50.4 (2.36)	144
Birth head circumference	34.9 (1.51)	35.3 (1.33)	0.301	35.0 (1.48)	123
HMOs					
2'FL	2038 [852;4719]	7.37 [3.90;32.2]	<0.001	1931 [3.90;4719]	145
3-FL	785 [7.59;2140]	2544 [299;3602]	<0.001	916 [7.59;3602]	145
3'SL	136 [75.2;388]	160 [75.8;274]	0.026	138 [75.2;388]	145
6'SL	151 [44.5;407]	139 [37.4;507]	0.39	150 [37.4;507]	145
LNT	547 [173;1830]	699 [338;1988]	0.01	567 [173;1988]	145
LNnT	149 [36.1;402]	66.9 [16.1;132]	<0.001	137 [16.1;402]	145
LNFP-I	473 [91.6;2372]	2.00 [2.00;6.43]	<0.001	416 [2.00;2372]	145
LNFP-V	44.6 [7.10;145]	197 [69.7;401]	<0.001	50.1 [7.10;401]	145
LNnFP	16.6 [5.60;69.1]	17.6 [5.60;50.9]	0.848	16.7 [5.60;69.1]	145

BMI, Body Mass Index; HMO, Human Milk Oligosaccharide; 2'FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP-I, lacto-N-fucopentaose-I; LNFP-V, lacto-N-fucopentaose-V; LNnFP, lacto-N-neofucopentaose

Results

Descriptive Statistics and Correlations Between HMOs

The cohort consists of 21 (14.7%) non-secretor and 122 (85.3%) secretor mothers. Two of the secretor mothers took part with two singleton pregnancies (Figure 1). 2'FL, LNFP-I and LNnT concentrations were significantly lower in non-secretors, while 3-FL, 3'SL, LNT and LNFP-V concentrations were higher. Further descriptive statistics are given in Table 1.

Correlation was highest between 2'FL and LNFP-I ($r=0.95$, $p<0.001$). Both were also positively correlated to LNnT (2'FL: $r=0.56$, $p<0.001$; LNFP-I: $r=0.61$, $p<0.001$) and negatively correlated to LNFP-V (2'FL: $r=-0.66$, $p<0.001$; LNFP-I: $r=-0.62$, $p<0.001$) and 3-FL (2'FL: $r=-0.54$, $p<0.001$; LNFP-I: $r=-0.64$, $p<0.001$) (Figure 2).

Maternal Parameters, Gestational Age and Birth Parameters

The maternal age at birth was positively associated only with 3'SL ($R=1.06$ [1.00,1.11] for every five years older, $p=0.03$). Pre-pregnancy BMI was negatively associated with LNnT in non-secretors ($R=0.93$ [0.90,0.97], $p<0.001$); no association was found in secretors. GA was positively associated with LNT ($R=1.08$ [1.01,1.15], $p=0.03$), 6'SL ($R=1.09$ [1.03,1.16], $p=0.005$) and LNFP-I ($R=1.15$ [1.03,1.28], $p=0.012$, only secretors). LNFP-I ($R=1.2$, [1.05,1.37], $p=0.008$, only secretors) and 3-FL ($R=0.92$ [0.86,0.98], $p=0.011$) were significantly associated with birth length (Supplementary Table S1).

Height-SDS and Growth Velocity

The interaction between height-SDS and secretor status was significant for LNT. The association of height-SDS with LNT had a negative direction in non-secretors ($0.78 \leq R \leq 1.01$) and a positive direction in secretors ($1.00 \leq R \leq 1.09$; Supplementary Table S2). However, the effects reached significance only in non-secretors at 2Y. LNFP-I was positively associated with height-SDS effects at 3M, 6M and 1Y ($R \approx 1.2$, $p \leq 0.02$); afterward, no further associations were found. Besides, there were consistently positive associations between height SDS and LNnT. However, statistical significance was only reached at 6Y. There was no evidence of associations between height-SDS and 2'FL, 3-FL, 3'SL, 6'SL, LNnFP, or LNFP-V.

The interaction between growth velocity and secretor status was significant for LNnT. In non-secretors, we found negative effects at 3M–1Y ($R=0.95$ [0.90,0.99], $p=0.01$), 1Y–2Y ($R=0.80$ [0.72,0.88], $p<0.001$) and in 5Y–6Y ($R=0.71$ [0.57,0.87], $p=0.002$).

LNT and LNFP-V were negatively associated with growth velocity from 3M–1Y (LNT: $R=0.97$ [0.95,1.00], $p=0.02$; LNFP-V: $R=0.97$ [0.95,1.00], $p=0.04$). LNFP-I and 3'SL were negatively associated with growth

velocity from LNFP-I: 1Y–2Y ($R=0.90$ [0.83,0.97] $p=0.008$) and 3'SL: 4Y–5Y ($R=0.95$ [0.90,1.00] $p=0.045$). No significant associations were found for growth velocity and 2'FL, 3-FL, 6'SL and LNnFP (Supplementary Table S3).

BMI-SDS

The interaction between BMI-SDS and secretor status was significant for 3'SL, 6'SL, LNT, LNFP-V and LNnFP. For non-secretors, we found consistently negative associations between BMI-SDS and 3'SL, 6'SL, LNT and LNFP-V at all time points (Supplementary Table S4). However, statistical significance was reached only for LNT and LNFP-V at 2Y. Besides, we found consistently positive associations between BMI-SDS and LNnFP from 6M onward. Statistical significance was reached at 02Y, 05Y and 06Y. We did not find evidence of associations between BMI-SDS and the HMOs in secretors when the models included the interaction term.

2'FL showed consistently negative associations with BMI-SDS ($0.92 \leq R \leq 1$; Supplementary Table S4); statistical significance was reached at 3M, 4Y and 5Y. The 3-FL was consistently positively associated with BMI-SDS between 3M and 6Y; however, the results did not reach statistical significance. For LNnT and LNFP-I, no consistent patterns were found.

Head Circumference

The interaction between HC and secretor status was significant for LNFP-V and LNnFP. LNFP-V showed consistent, significantly negative associations with HC between 3M and 7Y, with effect sizes varying between 0.63 and 0.92 in non-secretors (Supplementary Table S5). Secretors had no notable pattern. LNnFP showed consistently positive effects on HC from 3M–7Y, with effect sizes between 1.12 and 2.09 in non-secretors. In general, the effect sizes increased with age. Statistical significance was reached from 2Y–6Y. Again, we found no notable patterns in secretors.

LNFP-I was consistently positively related to HC-SDS from 6M–7Y with effect sizes between 1.02 and 1.20. However, most of the effects did not reach significance. There were no notable pattern effects for 2'FL, 3-FL, 3'SL, 6'SL or LNT (Supplementary Table S5).

Comparisons in Children of Non-secretors vs. Secretors

Children of non-secretor mothers had a significantly higher BMI-SDS at 3M ($\beta=0.8$ [0.4,1.2], $p<0.001$) and 6M ($\beta=0.8$ [0.4,1.2], $p<0.001$). At 1Y, the BMI-SDS was still 0.4 [-0.1,1.0] higher in children of non-secretors, but the effect did not reach significance ($p=0.1$). At 2Y, there was no difference in BMI-SDS between both groups. Afterward, the non-secretor group had consistently higher mean BMI-SDS, reaching significance at 3Y and 7Y (Figure 3).

At 3M and 6M, non-secretor children showed a higher height-SDS (3M: $\beta=0.5$ [-0.14,1.05; 6M: $\beta=0.4$ [-0.09,0.94]), $\beta=0.4$). However, the effect was not significant ($p=0.1$). HC-SDS was astoundingly higher in non-secretors at 3M ($\beta=1.3$ [0.6,2.0], $p<0.001$), 6M ($\beta=1.0$ [0.4,1.6], $p=0.001$), and 1Y ($\beta=0.7$ [0.3,1.2],

$p=0.002$). Even afterwards, HC-SDS stayed higher in non-secretors with effect sizes between $\beta=0.4$ and $\beta=1.0$, reaching significance at 3Y, 6Y, and 7Y (Figure 3).

Discussion

HMOs are indigestible but can be fermented, at least partially, by the infant's microbiome (20–23,38–40). This promotes the growth and activity of commensal bacteria such as *Bifidobacterium* and *Bacteroides spp.* and supports the gastrointestinal tract's maturation and the immune system (41). HMOs may also reduce the risk of infections by protecting against colonization with pathogenic microorganisms. It is proposed that they can act as decoys, inhibiting the pathogen anchoring to the human epithelial cells (24,25,42,43).

Recent studies proposed that HMOs, besides their antimicrobial effects, may be involved in an infant's growth and development. Sialylated oligosaccharides may exert a microbiota-dependent promotion of anabolic function in animal models by increasing the nutrient's efficiency, promoting better growth and physical development (44,45). Given the HMO-microbiome interaction and the microbiome's proposed effect on nutrient efficiency, the HMO composition of breast milk may also affect the infant's growth. Previous studies (14,15,17–19) investigating associations between HMOs and infant growth obtained conflicting results. Alderete et al. identified associations between LNFP-I and lower infantile weight, but not with pre-pregnancy BMI (15). In contrast, more recent studies found 2'FL positively associated with both child growth and pre-pregnancy BMI (17,18). Other studies reported no associations between HMO composition or secretor status with child growth (14,19).

Despite the variability in results, two recent studies indicated a role of sialylated HMOs in infant growth considering maternal BMI (19,46). Binia et al. found moderate associations between HMOs and infant growth and body composition during the first 4 months of life in a cohort of predominantly healthy babies and mothers with normal BMI. They reported significant associations of higher growth rate during the 4 months of lactation with higher 3'SL, expressed as Area Under the Curve of HMO concentrations at all visits, a potentially better measure of HMO exposure. Saben et al. confirmed the positive association of several sialylated HMOs, including 3'SL but also total acidic HMOs with infant growth during the first 6 months of life, including also obese mothers. The growth and body composition of the healthy infants were independent of maternal pre-pregnancy BMI. Interestingly, Saben et al. used calculated milk and HMO intake and not only concentrations, an attempt again to better quantify exposure to HMOs. The study was limited to one single time-point of HMO quantification at 2 months. Both studies lacked the longer follow-up of infant growth, which could be a better indicator of future risk to obesity. Finally, neither of these two studies confirmed the previous observations by Lagström et al. (17) and Larsson et al. (18) on the positive association of 2'FL and the negative association of LNnT with infant growth.

Despite measuring HMOs only at 3 months postpartum, limiting insights on associations between growth and the changing HMO exposure over time, we could include growth data from birth until 7 years of age. Indeed, we found higher BMI and HC SDS in non-secretors' than secretors' infants. Although significance

was not achieved at all time points, the probability of only positive results is $p < 0.002$. Growth velocity but not BMI was inversely correlated with LNnT at 3M–1Y and 1Y–2Y in non-secretors, supporting the findings from Lagström et al. (17). Regarding the association between infant growth and sialylated HMOs 3'SL and 6'SL, we found a negative association with BMI-SDS in non-secretors but not with growth velocity, as previously reported (19,46).

Included mothers had a mean BMI of 23.2 (3.78) kg/m² and a mean age of 30 years, similar to other studies' populations (19,46). In line with previous results, we found a negative association between pre-pregnancy BMI and LNnT in non-secretors (17). However, other studies reported a positive or no association (8,9,46). This highlights the variability of reported HMO associations, reflecting possible differences in methods or non-measured confounders. Therefore, future studies examining HMOs' role in growth and metabolic health should consider maternal physiology and other human milk components, such as proteins and lipids.

One *in vivo* intervention study with sialylated oligosaccharides (45) did report growth recovery following treatment with sialylated oligosaccharides in animal models of undernutrition. Recent clinical studies testing infant formula containing HMO effect on growth and body composition showed no differences in infant growth and development at least during the first months of life (47,48). The conflicting reported results from recent observational data call for hypothesis-driven studies to test the specific role of groups rather than single HMOs in influencing early growth and composition.

Conclusion

Our results suggest that associations between HMOs and infant growth may extend beyond the breastfeeding period and interventional studies are needed to elucidate their influence on infant weight, height and body composition. Our study also confirms the value of long-term follow-up of breastfed infants and the inclusion of both maternal and infant factors to understand the role of HMOs in growth and development.(8, 10)

Abbreviations

HMO, Human Milk Oligosaccharide; 2'FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP-I, lacto-N-fucopentaose-I; LNFP-V, lacto-N-fucopentaose-V; LNnFP, lacto-N-neofucopentaose; BMI, Body Mass Index; HC; Head Circumference; GA, Gestational Age; SDS, Standard Deviation Score; Glc, Glucose; GlcNAc, N-Acetylglucosamine; Gal, Galactose; Fuc, Fucose; Neu5Ac, N-Acetylneuraminic acid; LoQ, Limit of Quantification

Declarations

Ethics approval and consent to participate: The study has been conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Medical Faculty of the University of Leipzig (Reg. No. 264-10-19042010). Informed written consent was provided by all parents for their children.

Consent for publication: Not applicable.

Availability of data and materials: The legal requirements and the given informed consent do not allow public sharing of the dataset. Interested researchers can contact the research data management of the Medical Faculty, University Leipzig: rdm@medizin.uni-leipzig.de for further information. The dataset ID is PV450.

Competing interests: Aristeia Binia, Sean Austin and Norbert Sprenger are employees of Société des Produits Nestlé. All other authors declare no conflict of interest.

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Authors' contributions: WK and AB conceived of the presented idea. NG, AJ, and CH were in charge of study organization and data collection. AB, SA, and NS were in charge of HMO analyses. PM and MV performed the statistical analyses, interpreted the results, wrote the first draft of the manuscript, and designed the visualizations. All authors discussed and contributed to the manuscript. All authors reviewed the results and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Figures

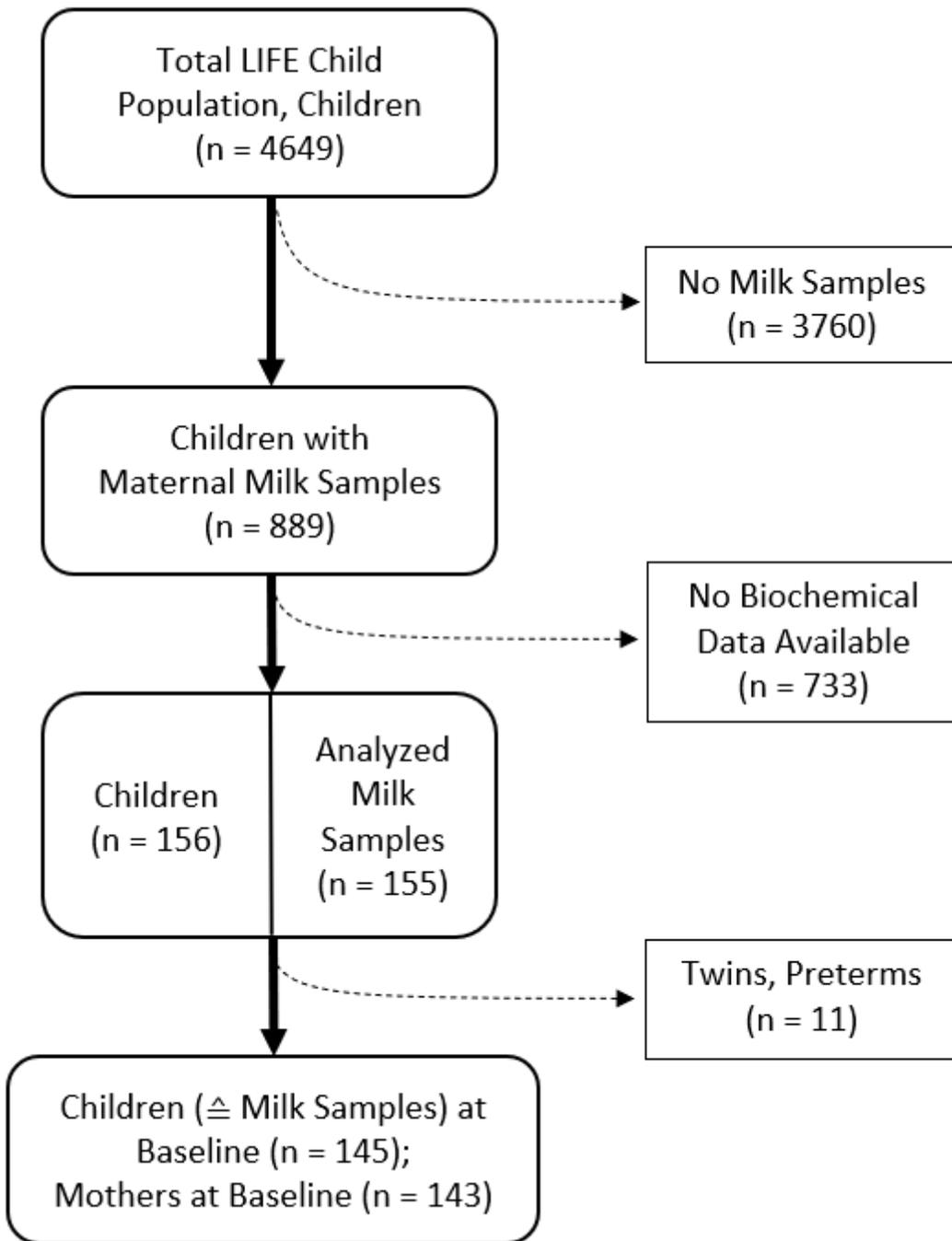


Figure 1

Flow chart of the study design.

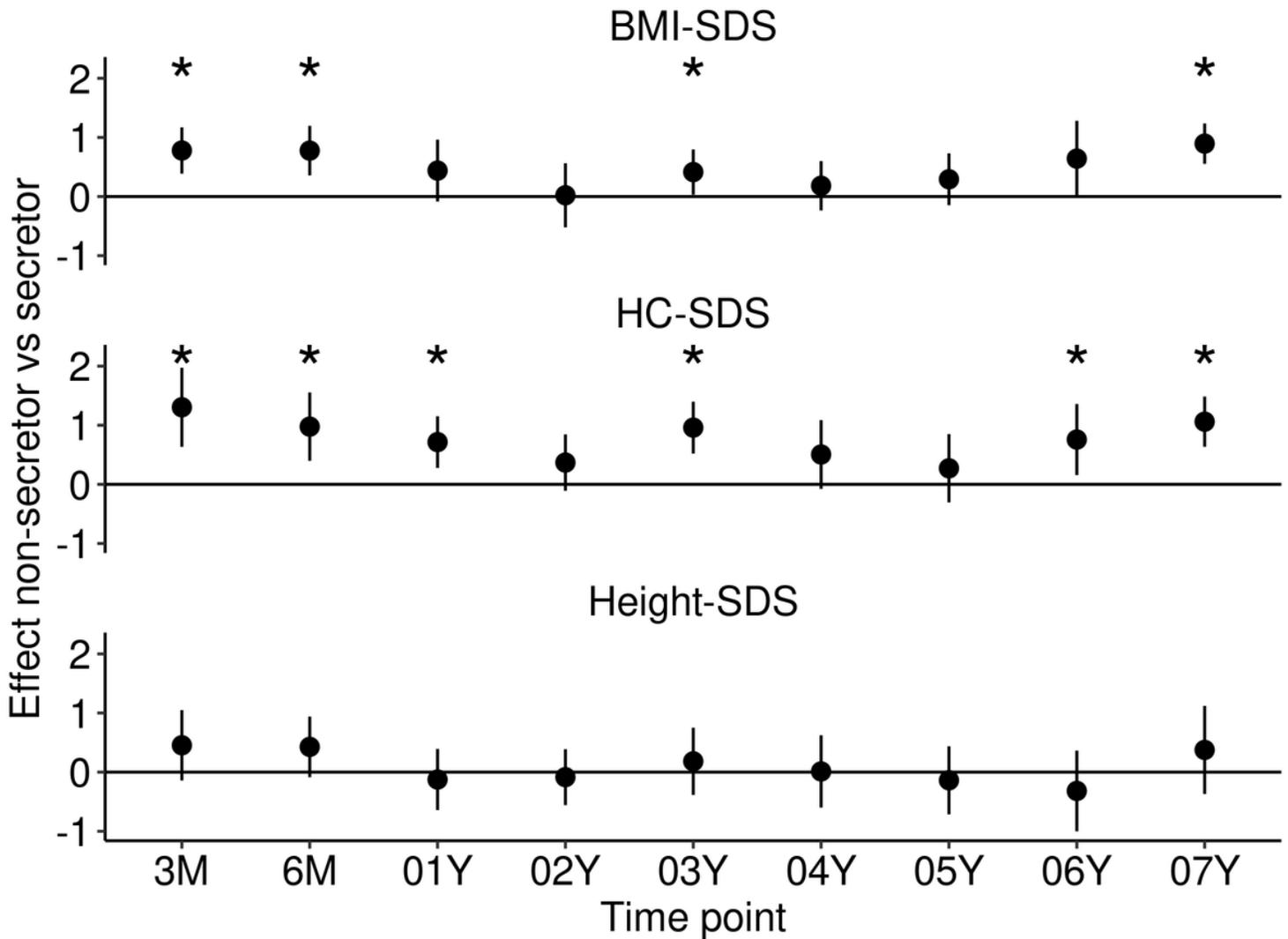


Figure 3

Mean differences in BMI-SDS, HC-SDS, and Height-SDS between the secretor and non-secretor group and 95%-confidence intervals. Mean SDS-differences >0 represent higher values in non-secretors compared to secretors. From 3 months to 7 years of age BMI-SDS and HC-SDS were higher in non-secretors, especially at 3M, 6M, 01Y, and 03Y. Body Mass Index; HC, Head Circumference; SDS, Standard Deviation Score.

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