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ORIGINAL ARTICLE

Human milk oligosaccharide composition predicts risk of necrotising enterocolitis in preterm infants

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ABSTRACT

Objective Necrotising enterocolitis (NEC) is one of the most common and often fatal intestinal disorders in preterm infants. Markers to identify at-risk infants as well as therapies to prevent and treat NEC are limited and urgently needed. NEC incidence is significantly lower in breast-fed compared with formula-fed infants. Infant formula lacks human milk oligosaccharides (HMO), such as disialyllacto-N-tetraose (DSLNT), which prevents NEC in neonatal rats. However, it is unknown if DSLNT also protects human preterm infants.

Design We conducted a multicentre clinical cohort study and recruited 200 mothers and their very low birthweight infants that were predominantly human milk-fed. We analysed HMO composition in breast milk fed to infants over the first 28 days post partum, matched each NEC case with five controls and used logistic regression and generalised estimating equation to test the hypothesis that infants who develop NEC receive milk with less DSLNT than infants who do not develop NEC.

Results Eight infants in the cohort developed NEC (Bell stage 2 or 3). DSLNT concentrations were significantly lower in almost all milk samples in NEC cases compared with controls, and its abundance could identify NEC cases prior to onset. Aggregate assessment of DSLNT over multiple days enhanced the separation of NEC cases and control subjects.

Conclusions DSLNT content in breast milk is a potential non-invasive marker to identify infants at risk of developing NEC, and screen high-risk donor milk. In addition, DSLNT could serve as a natural template to develop novel therapeutics against this devastating disorder.

Significance of this study

What is already known on this subject?

- Necrotising enterocolitis (NEC) is one of the most frequent and often fatal intestinal disorders in premature infants.
- Breast-fed infants are at a 6-fold to 10-fold lower risk of developing NEC than formula-fed infants.
- Human milk oligosaccharides (HMO), complex glycans that are highly abundant in breast milk but not in infant formula, prevent NEC in a neonatal rat model.
- Of the more than 150 HMO described to date, a single oligosaccharide, disialyllacto-N-tetraose (DSLNT), is responsible for the beneficial effects in neonatal rats.

What are the new findings?

- A prospective cohort study on 200 mothers and their very low birthweight infants that were predominantly human milk-fed supports the preclinical results in the neonatal rat model.
- Infants who developed NEC received less DSLNT with the milk than infants who did not develop NEC.
- Even though DSLNT concentration is occasionally low in individual milk samples consumed by control infants, the ability to discriminate between NEC cases and control infants is enhanced when samples from multiple consecutive days are averaged.

How might it impact on clinical practice in the foreseeable future?

- Low DSLNT concentrations in the mother's milk might become a non-invasive biomarker to identify breast-fed infants at risk of developing NEC.
- Donor milk and human milk fortifiers and other products might be screened for low DSLNT concentrations to avoid feeding them to infants at risk to develop NEC.
- DSLNT might be a natural template to develop novel therapeutics to help prevent NEC.

BACKGROUND

Necrotising enterocolitis (NEC) is one of the most common and devastating intestinal disorders in preterm infants.[1] It affects 5%–10% of all very low birthweight infants (VLBW; birth weight under 1500 g) and leads to a severe and often fatal destruction of the infant's intestine. More than a quarter of the affected infants die from NEC, and the survivors are often faced with long-term neurological complications.[1 2] Markers to identify at-risk infants as well as therapies to meet the clinical needs for this special and highly vulnerable population are extremely limited, and urgently needed.

Human milk-fed infants are at a 6-fold to 10-fold lower risk of developing NEC than formula-fed infants.[3–5] Several different human milk components attenuate NEC in preclinical models in rodents or piglets,[6–8] but it is not known whether

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these results translate to benefit the human neonate. Data from in vitro models and our own preclinical studies in neonatal rats suggest that human milk oligosaccharides (HMO) contribute to the reduced NEC incidence in human milk-fed infants.[9–11] HMO are complex glycans that represent the third most abundant component of human milk, but are currently not present in infant formula.[12] Feeding neonatal rats with HMO significantly improves survival and attenuates NEC pathology scores.[11] While more than 150 different HMO have been described so far, we identified one specific HMO called disialyllacto-N-tetraose (DSLNT) that was most effective in preventing NEC in neonatal rats. Closely related HMO like sialyllacto-N-tetraose (LSTb) or lacto-N-tetraose (LNT) that lack one or both sialic acid residues had no effect,[11] suggesting a highly structure-specific mechanism.

While the data are encouraging, the validity of available preclinical NEC models in rodents or piglets is limited.[13] Animals are exposed to external hypoxic and/or hypothermic insults that are rather artificial, and the use of animals itself is a limitation due to interspecies differences in GI development, anatomy and physiology. Thus, advancing a potential therapeutic like DSLNT from controversial preclinical models to clinical treatment trials carries a tremendous risk of failure. To help close the gap between animal models and clinical intervention studies, we used an intermediate approach and conducted a multicentre clinical prospective cohort study with mothers and their VLBW infants fed predominantly human milk. The study is based on the observation that some infants still develop NEC despite receiving predominantly human milk. HMO composition in human milk varies between women and over the course of lactation. This led us to hypothesise that human milk fed to infants who develop NEC contains less DSLNT than human milk fed to infants who do not develop NEC.

METHODS

Cohort and samples

We recruited 200 mothers and their VLBW infants (birth weight under 1500 g) at five different sites in North America (University of California, San Diego (UCSD), California, USA; Sunnybrook Health Sciences Centre, Toronto; Loma Linda University Children's Hospital, Loma Linda, California, USA; Cook Children's Health Care System, Forth Worth, Texas, USA; Rush University Medical Center, Chicago, Illinois, USA). Only infants who predominantly received human milk for at least the first 28 days of life were included. Infants were excluded if they received infant formula or had known congenital bowel anomalies such as gastroschisis. An aliquot of the milk that the infant received on a given day (not necessarily the milk that the mother produced that day) was collected every 2–3 days for the first 28 days of life, the time period when most NEC cases occur. No samples were collected if the infant was not fed that day or if there was insufficient milk to collect a 30 µL aliquot. Milk samples were stored at 4°C for <1 hour and at −20°C until shipment to the Bode lab at UCSD for HMO analysis. Basic demographic data were recorded for the mother (age, gravidity, parity, medications, medical diagnoses) and for the infant (gestational age, birth weight, sex, medications, medical diagnoses). NEC was diagnosed based on the modified Bell staging system.[14] Further clinical details for each NEC case are found in the Case Descriptions in the online supplementary appendix.

Once all 200 mother-infant pairs were recruited and all samples were collected, each NEC case was matched with five controls (mothers and their infants who did not develop NEC). Controls were selected from the same study site to minimise location effects. In addition to location, matching criteria included gestational age, birth weight, mode of delivery, race and ethnicity as well as availability of milk samples throughout the 28-day collection period. An example of case-control matching is shown in online supplementary figure S1 in the appendix. The institutional review boards at all five participating study sites approved the research protocol, and parents of all participants gave their written informed consent.

Human milk oligosaccharide analysis

HMO composition was analysed in all available milk samples from all NEC cases and the respective matched controls. Analytical staff was blinded to the clinical metadata associated with each sample. HMO analysis was performed by high-performance liquid chromatography (HLPC) after fluorescent derivatisation with 2-aminobenzamide (2AB) as previously described.[15 16] The non-HMO oligosaccharide raffinose was added to each milk sample as internal standard at the very beginning of sample preparation to allow for absolute quantification. The following individual HMO were detected based on retention time comparison with commercial standard oligosaccharides and mass spectrometry analysis: 2′-fucosyllactose (2′FL), 3-fucosyllactose, 3′-sialyllactose, LNT, lacto-N-neotetraose (LNnT), lacto-N-fucopentaose (LNFP)1, LNFP2 and LNFP3, sialyl-LNT b (LSTb) and LSTc, difucosyl-LNT (DFLNT), DSLNT, fucosyl-lacto-N-hexaose, difucosyl-lacto-N-hexaose, fucosyl-disialyl-lacto-N-hexaose and disialyl-lacto-N-hexaose. In addition to absolute concentrations, the proportion of each HMO per total HMO concentration (sum of all integrated HMO) was calculated and expressed as relative abundance (% of total). Secretor status was defined by the presence of 2′FL. Simpson's diversity index D was calculated as the reciprocal sum of the square of the relative abundance of each of the measured HMO. HMO equitability (evenness, E) was calculated by dividing the actual D index for each sample by Dmax (maximum D index in the theoretical case that all measured HMO have the same relative abundance).

Statistical analysis

Initial comparison of HMO levels was done with the Mann-Whitney U test, Wilcoxon test and Kruskal-Wallis test, since the distributions of HMO concentrations were non-normal according to Shapiro-Wilk tests. Univariate logistic regression models were used to prescreen clinical covariates, including gestational age, birth weight, mode of delivery, race, etc. To test if HMO significantly influenced the onset of NEC, we estimated its effect using generalised estimating equation (GEE) models to account for longitudinal measurement.[17] GEE allows non-linear relations between the outcome and covariates, and accounts for unknown correlation among repeated measurements from the same subject. Here we used GEE with logit link and exchangeable correlation structure, by assuming the within-subject correlation between any two time-points is ρ. To stabilise the variance and equalise the range, we standardised each HMO measurement, for example, DSLNT, LNFP1. We also used the square root of days post partum (DPP) to linearise the relationship over time. NEC status (ie, Bell stage) was used as outcome, and the Wald test was used to assess statistical significance of model components. To reduce variation and allow comparison between HMO, oligosaccharide concentrations were standardised by subtracting the mean and dividing by the SD.

We first prescreened each HMO using a univariate GEE model. Any HMO with $p<0.2$ were further analysed to assess their joint contribution to NEC onset in combination with

significant clinical covariates to construct final GEE model. Quasi-likelihood under the independence model criterion (QIC) was used to compare multivariate models. QIC and Wald statistics informed a backward elimination process of model selection. Detailed information on statistical analysis is provided in the online supplementary appendix.

RESULTS

Eight of the 200 recruited infants developed NEC Bell stage 2 or 3, and two infants were diagnosed with NEC Bell stage 1. Table 1 shows the case-control characteristics stratified by study site A to E, and Case Descriptions in the online supplementary appendix provide detailed clinical information for each NEC case. We analysed the HMO composition in a total of 636 milk samples (15 samples from Bell stage 1, 38 from stage 2, 25 from stage 3 and 558 from controls).

DSLNT concentrations are significantly lower in milk fed to NEC cases compared with controls

We first compared the concentrations of each HMO from all 636 milk samples using the Mann-Whitney U test, Wilcoxon test and Kruskal-Wallis test. Figure 1A shows that DSLNT concentrations in NEC cases (Bell stage 2–3) were significantly lower than DSLNT concentrations in controls. However, there was no significant difference between NEC cases and controls when looking at the sum of all HMO (figure 1B) or any of the individual HMO other than DSLNT (see online supplementary figure S2 in the appendix).

While the first analysis combined HMO results from all NEC cases and compared it with results from all controls, we next examined HMO concentrations for each separate NEC case and its associated controls. For each milk sample, the fold change for each HMO was calculated relative to its average concentration in the associated control group (figure 2). Controlling for known clinical factors through our case-control matching further suggested that DSLNT exhibits a consistently lower concentration in all NEC cases, throughout the length of the study (figure 2A). Furthermore, the magnitude of deficiency worsened with Bell stage. Indeed, NEC Bell stage 3 cases showed the lowest DSLNT concentration compared with controls, and Bell stage 1 cases exhibited the weakest effect. The oligosaccharides LSTb and LNT (structurally similar to DSLNT but with reduced sialylation) did not show consistent differences in the case-control matching (figure 2B, C). Similarly, total HMO concentration did not differ considerably between cases and their clinically matched controls (figure 2D).

A robust statistical assessment of all measured HMO shows DSLNT as the primary contributor

To test for associations between clinical covariates and NEC onset, we used univariate logistic regression model to prescreen each clinical covariate measured at birth. None of the clinical covariates (ie, study site, delivery mode, race/ethnicity or gender) or characteristics of the infant at birth (ie, gestational age or birth weight) was significantly associated with NEC onset in our cohort (figure 3A). The insignificance of birth characteristics indicated that our cohort was successfully matched for variance due to birth weight, gestational age and location, and potential confounding based on these covariates was minimised. Our analysis concentrated on Bell stage 2 and 3 as Bell stage 1 is generally considered less specific for NEC diagnosis.

We then assessed the contribution of each HMO to the onset of NEC using a univariate GEE to account for repeated measurements. In this analysis, DSLNT clearly contributed ($p<0.001$; figure 3B), with an OR of 0.86, suggesting that lower concentrations increase the risk of developing NEC. Additionally, HMOs such as LNFP1, LNFP3 and DFLNT were selected ($p<0.2$) from the univariate screening for further examination. A final multivariate model demonstrated that DSLNT has a significantly protective contribution (OR 0.84; 95% CI 0.79 to 0.88; $p=0.001$), while LNFP1 and DFLNT are associated with a decreased (OR 0.91; 95% CI 0.84 to 0.97; $p=0.006$) and increased (OR 1.15; 95% CI 1.01 to 1.28; $p=0.022$) risk of NEC, respectively, albeit to a much lesser

Table 1 Case-control characteristics

Case ID	Bell stage	Gestational age (weeks+days)	Birth weight (g)	Gender	Delivery mode	Race/ethnicity
A003	2	29+2	1350	M	C	A
Controls		29+0.6±4.4	1120±234	(M, M, M, M, F)	(C, C, C, C, C)	(H, H, H, Ca, A)
A029	2	27+3	1069	M	C	Ca
Controls		27+2.1±3.5	1022±103	(M, F, M, M, F)	(V, V, C, C, V)	(H, NS, NS, AA, AA)
A066	1	26+6	961	M	C	H
Controls		26+4.9±0.7	950±173	(F, M, M, M, F)	(C, C, C, C, C)	(Ca, Ca, Ca, H, Ca)
B032	3	31+1	1160	F	V	AA
Controls		31+6.4±7.6	1382±96	(F, F, M, F, M)	(V, V, C, C, V)	(AA, A, H, Ca, AA)
C005	3	26+3	1040	M	C	AA
Controls		26+0.4±8.2	766±228	(F, F, F, F, M)	(V, C, V, C, V)	(H, AA, AA, Ca, AA)
C024	2	27+1	830	F	C	AA
Controls		26+4.6±3.2	928±120	(M, F, F, M, F)	(C, V, C, C, C)	(AA, Ca, H, Ca, AA)
C027	2	29+5	1370	F	C	AA
Controls		29+2.8±2.7	1276±142	(M, F, M, M, F)	(V, C, C, C, C)	(Ca, AA, Ca, AA, H)
D015	1	26+1	813	M	V	NS
Controls		26+3.2±2.4	771±224	(M, M, M, M, M)	(C, C, C, C, C)	(NS, NS, NS, NS, NS)
D024	2	26+2	770	F	C	NS
Controls		26+0.8±5.0	790±116	(F, F, F, F, F)	(V, C, V, C, C)	NS
E002	3	25+0	1040	M	V	H
Controls		26+5.1±17.5	823±197	(F, F, M, M, F)	(C, C, C, V, V)	(Ca, AA, H, H, Ca)

A, Asian; AA, African-American; C, caesarean; Ca, Caucasian; H, Hispanic; NS, not specified; V, vaginal.

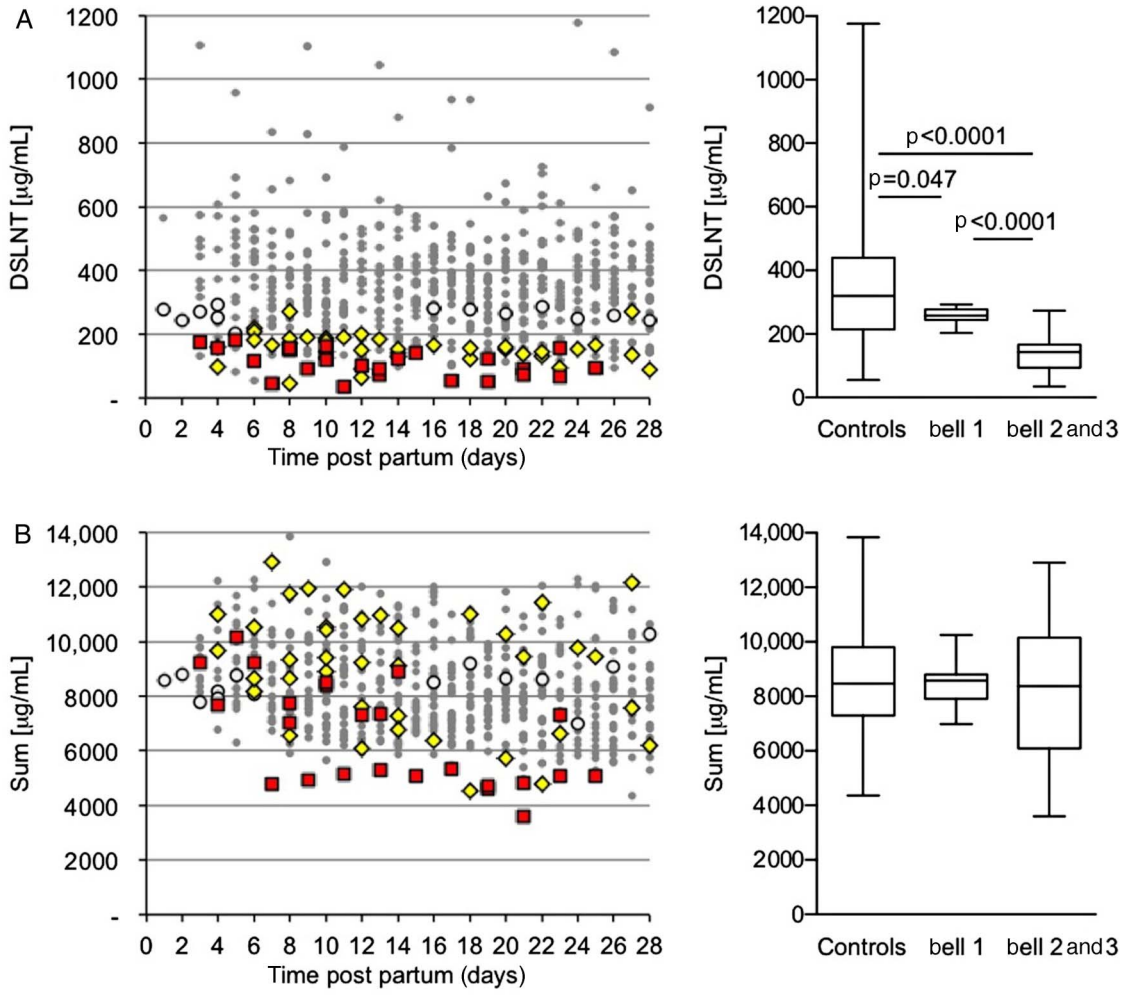


Figure 1 Disialyllacto-N-tetraose (DSLNT) concentrations are consistently lower in necrotising enterocolitis (NEC) cases. Concentrations of DSLNT alone (A) and all human milk oligosaccharides (HMO) combined (B) in human milk were plotted as a function of days post partum when the milk was given to infants who either developed NEC (red squares: Bell stage 3; yellow diamonds: Bell stage 2; grey circles: Bell stage 1 or did not develop NEC and served as controls (small grey circles). Box plots on the right show median with 25/75 quartiles and whiskers for min/max values. The Bell stage groups were compared by Mann-Whitney U test and Kruskal-Wallis test. Distribution normality was rejected by the Shapiro-Wilk test ($p<0.001$). DSLNT concentrations in human milk were significantly lower in infants who developed NEC (stage 2 and 3 combined) when compared with controls. However, there was no significant difference in total HMO concentration (sum of all integrated individual HMO), and there were also no differences in any HMO other than DSLNT (see online supplementary figure S2).

extent than DSLNT, as detailed in figure 3C and online supplementary table S1. LNFP3 did not significantly contribute to univariate or multivariate models (see online supplementary table S1) and its removal did not considerably increase QIC (see online supplementary table S2). Therefore, LNFP3 was excluded from the final multivariate model. A minimal model accounting only for DSLNT and DPP, provided only a small increase in QIC relative to the final model with three HMO, further supporting the dominant contribution of DSLNT to NEC (see online supplementary table S2).

NEC cases exhibit consistently perturbed HMO concentration over time

In addition to highlighting HMO that are associated with NEC, the univariate DSLNT model and multivariate models were capable of identifying individual milk samples associated with NEC cases (see online supplementary figure S3). Indeed, the models could identify potentially problematic milk samples or identify infants who may benefit from intervention prior to the possible onset of NEC. Furthermore, we note that DSLNT concentration tends to be significantly perturbed in NEC cases for multiple consecutive days (see online supplementary figure S4 in the appendix). Thus, even though DSLNT concentration is occasionally diminished in individual milk samples consumed by control infants (see online supplementary figure S4A), the ability to discriminate between NEC cases and control infants is enhanced when samples from multiple consecutive days are averaged (figure 4, see online supplementary figure S4B and S5 in the appendix).

DISCUSSION

For decades, efforts have been made to understand why human milk-fed infants are at significantly lower risk to develop NEC. While human milk components have previously been shown in preclinical models to provide a protective effect,[6–8 11] none of these findings, until now, has been validated in human infants. In this study, we demonstrated in a clinical cohort that DSLNT may provide a significant protective effect against the onset of NEC. These results validate our earlier observation of the protective effect of DSLNT on neonatal rats.[11] DSLNT deficiency was identified as a major contributor to NEC from a panel of the 16 most

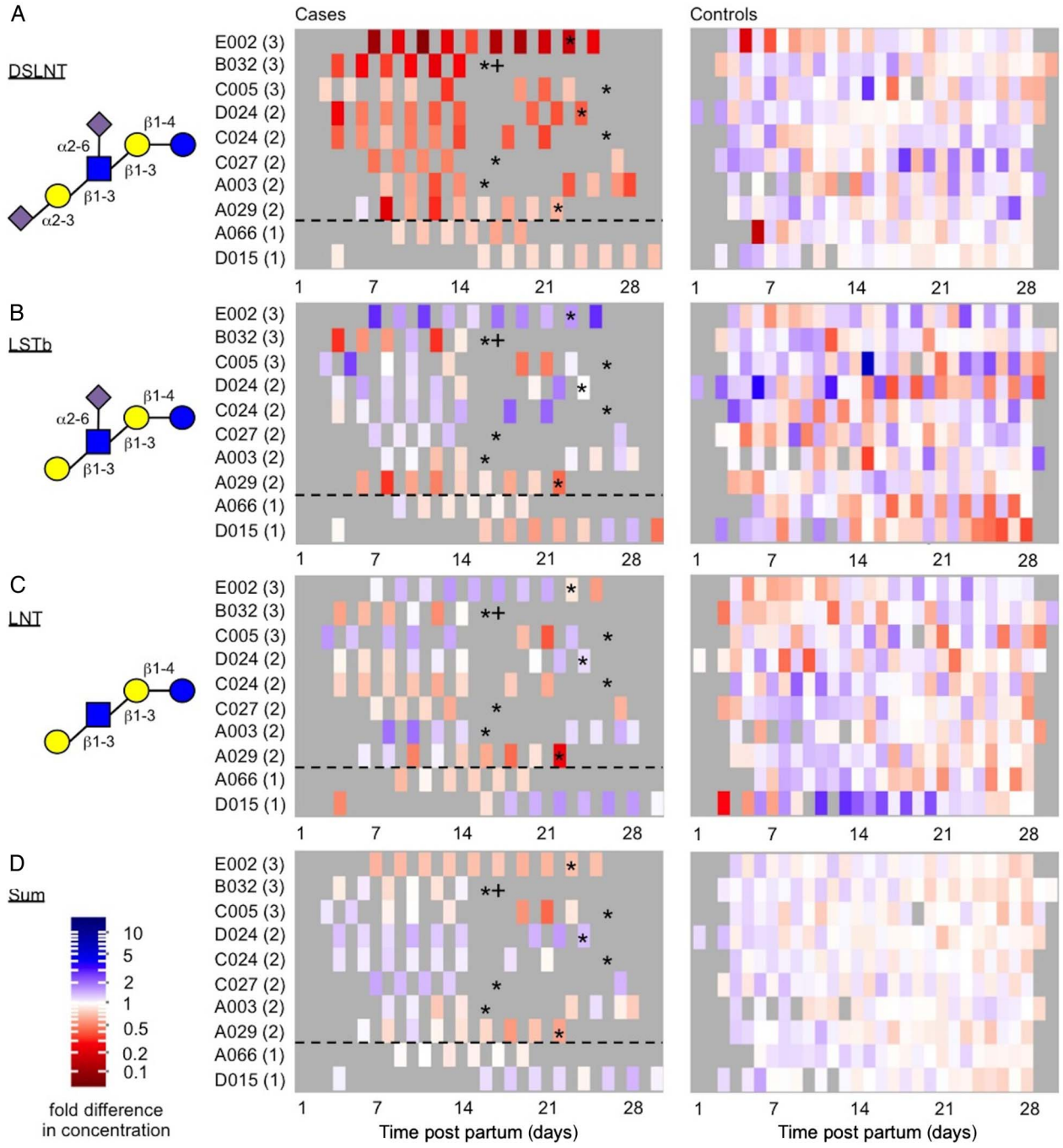


Figure 2 Disialyllacto-N-tetraose (DSLNT) concentrations are uniquely and consistently low in necrotising enterocolitis (NEC) cases (left) when compared with controls (right). Samples in each row are case-control matched by study site, gestational age, birth weight and other NEC-relevant factors. For each milk sample collected over the first 28 days post partum, the fold difference of human milk oligosaccharides (HMO) concentration relative to the associated matched control sample average is illustrated for DSLNT (A), sialyllacto-N-tetraose (LSTb) (B), lacto-N-tetraose (LNT) (C) and the sum of all integrated HMO (D). DSLNT was lowest in cases with a Bell stage of 3 and 2 at concentrations an order of magnitude lower than matched control averages. Bell stage 1 cases showed slightly lower concentrations than their matched controls. Structurally similar HMOs with reduced sialylation, such as LSTb and LNT, failed to exhibit consistent variations in concentration in NEC cases compared with matched controls. Number in parentheses after case codes denotes NEC Bell stage. (*) denotes the day of NEC onset, (+) denotes the day of death due to NEC. Oligosaccharide structure nomenclature: blue circles: glucose; yellow circles: galactose; blue squares: N-acetylglucosamine; purple diamonds: sialic acid.

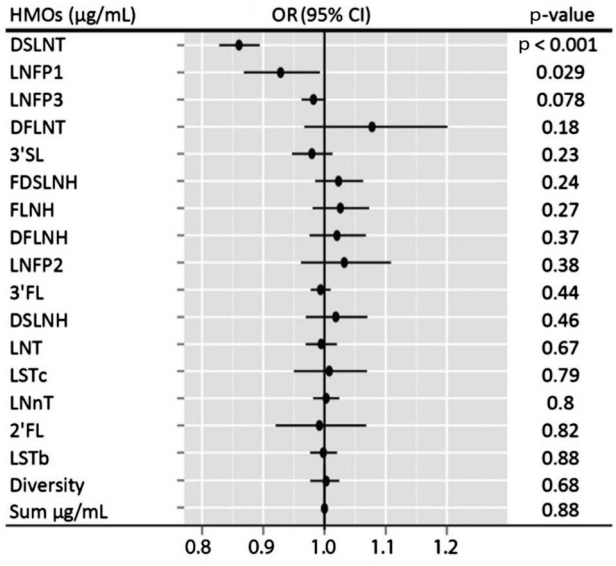
abundant HMO, which represent >95% of the HMO in human milk. In this panel, additional HMO (ie, LNFP1 and DFLNT) were also found to provide a lesser contribution. It is important to note that we did not measure total milk volume fed to each infant per feeding or per day. The analysis purely focuses on HMO concentrations and not on absolute HMO amounts received.

The role of DSLNT and other HMO were robustly quantified using GEE. DSLNT deficiency is significantly associated ($p=0.001$) with NEC onset with an OR of 0.84. LNFP1 and DFLNT were also found to have a significant protective (OR 0.91) and harmful (OR 1.14) contribution, respectively (figure 3C). These contributions were stable across all

A Univariate regression

Clinical covariates	p-value
Delivery	0.69
Gender	0.96
Secretor status	0.5
Location	0.96
Race/ethnic	0.34
Birth weight	0.28
Gestational age	0.77

B Univariate GEE model



C Final model

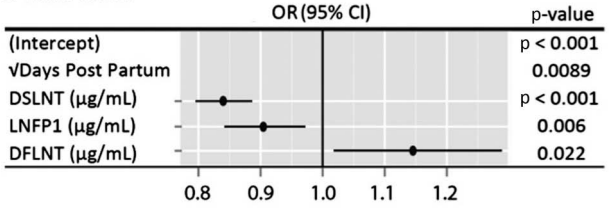


Figure 3 Univariate logistic regression screening of (A) birth characteristics (ie, birth weight and gestational age) were effectively controlled through case-control matching, and therefore, no clinical covariate showed a significant association with necrotising enterocolitis (NEC). (B) Univariate temporal logistic generalised estimating equation (GEE) screening of human milk oligosaccharides (HMOs) revealed several candidate associations, with disialyllacto-N-tetraose (DSLNT) being the predominant HMO. (C) The final multivariate temporal GEE model demonstrated that DSLNT, lacto-N-fucopentaose (LNFP1) and difucosyl-LNT (DFLNT) each contribute significantly to a final multivariate model. The OR is the exponentiated coefficient and the 95% CI describes the range of possible OR. For panel A, the p value represents the significance based on the χ^2 distribution, while p values in panels B and C were calculated from the Wald statistic of each coefficient, evaluated along a normal distribution.

multivariate models (see online supplementary table S1). Furthermore, these HMO were consistently dysregulated in NEC cases. When considering dysregulation over multiple consecutive days, the separation between cases and controls increased (see online supplementary figures S2–S4), suggesting that prolonged dysregulation of HMO is more indicative of NEC onset. Interestingly, NEC Bell stage 1 did not correlate with DSLNT deficiency, supporting the lack of specificity of Bell

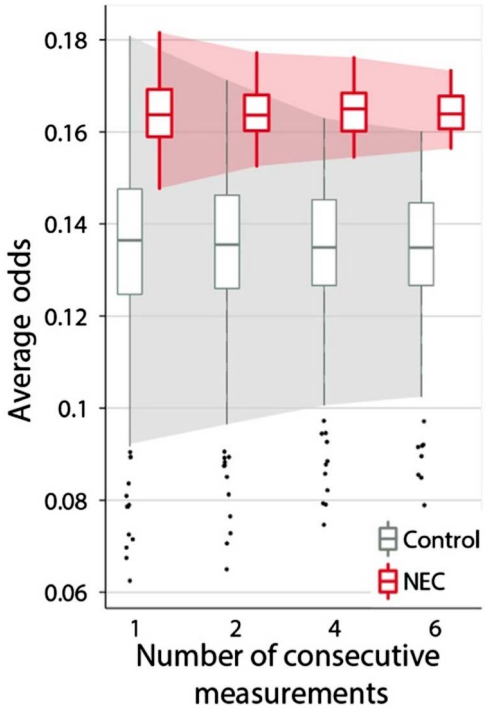


Figure 4 Aggregation of disialyllacto-N-tetraose (DSLNT) concentration for multiple days enhances the identification of high-risk infants. Infants who will develop necrotising enterocolitis (NEC) are more readily identifiable when DSLNT concentration from multiple consecutive milk samples for each subject is aggregated using the geometric mean. When we combine the computed odds for 2, 4 and 6 consecutive milk samples from the same individual, separation of cases and controls increased and variance in the average odds decreased.

stage 1 in diagnosing NEC or suggesting that DSLNT deficiency only impacts infants' risk for more advanced NEC.

The underlying mechanisms of how HMO such as DSLNT attenuate NEC risk remain to be elucidated. Although HMO have profound effects on infant microbiota composition,[18–20] the importance of microbiota composition on NEC onset and development is poorly understood.[21–26] Whether microbial dysbiosis is a causative event or merely a marker of intestinal disease remains unknown.[27] Instead, HMO may have direct effects on infant intestinal epithelial or immune cells, which might directly attenuate NEC risk, and also indirectly alter microbiota composition. The observation that the effects of DSLNT are highly structure-specific (removal of just one sialic acid renders the oligosaccharide ineffective in neonatal rats[11] and these truncated oligosaccharides are no longer associated with NEC risk in the cohort study) indicates a potentially receptor-mediated mechanism.

The study recruited 200 mothers and their VLBW infants, of which 8 (4%) developed NEC Bell stage 2 or 3. NEC incidence in VLBW infants in North America typically varies between <5 and up to 10%, but that includes both human milk-fed as well as formula-fed infants. Since NEC incidence is 6-fold to 10-fold lower in predominately human milk-fed infants compared with formula-fed infants,[3–5] the 4% NEC incidence reported in this study is well within the anticipated range.

While the results from this study indicate that higher DSLNT concentrations in mother's milk lower the infant's risk to develop NEC, larger cohort studies with more detailed maternal data will be needed to identify maternal factors (genetics, nutrition, stress, etc) that influence DSLNT synthesis.

Although selection bias is a common limitation of case-control studies, this has been minimised in two ways: first by

prospective enrolment at five different locations before identification of cases or controls, and, second, by matching cases with controls from their own location, and therefore with the most similar unmeasured exposures.

Current practice aims to reduce the risk of NEC through the administration of human milk from the mother or a donor. While human milk in general reduces the risk of NEC, it remains vastly unknown how the natural variation in the concentration of specific human milk components contributes to NEC risk. Our data suggest that feeding preterm infants with human milk rich in DSLNT lowers NEC risk, while feeding human milk deficient in DSLNT increases NEC risk. While cohort association studies cannot exclude that DSLNT is simply a proxy for other maternal or infant markers, the data are consistent with results from preclinical studies showing that supplementation with DSLNT (alone and without any other confounders) improves outcome measures in neonatal rodents.[11] The combined datasets from preclinical study and mother-infant cohort increase our confidence that feeding human milk rich in DSLNT lowers NEC risk. However, larger cohort studies as well as clinical intervention studies are needed to validate the association of DSLNT with reduced NEC risk. If confirmed, low DSLNT concentrations in the mother's milk might become a non-invasive marker to identify breast-fed infants at risk of developing NEC. Donor milk, human milk fortifiers and other products might be screened for low DSLNT concentrations to avoid feeding them to infants at risk to develop NEC. In addition, DSLNT might serve as a natural template to develop novel therapeutics to help prevent NEC.[28]

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Contributors LB and JHK designed research; CAA, LB, EA, ABB, ECHS, ALP and JHK recruited patients, collected samples and conducted research; CAA, BPK, NEL, JH and LB analysed data and performed statistical analysis; BPK, JH, JHK, NEL and LB wrote the manuscript; LB had primary responsibility for final content. All authors read and approved the final version of the manuscript.

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