Published in final edited form as:
Gut. 2012 October ; 61(10): 1417–1425. doi:10.1136/gutjnl-2011-301404.

The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats

Evelyn Jantscher-Krenn[1,2,3], **Monica Zherebtsov**[2], **Caroline Nissan**[1,2], **Kerstin Goth**[4], **Yigit S Guner**[4], **Natasha Naidu**[5], **Biswa Choudhury**[5], **Anatoly V Grishin**[4], **Henri R Ford**[4], and **Lars Bode**[1,2]

[1]Division of Neonatal Medicine, Department of Pediatrics, University of California–San Diego, San Diego, California, USA

[2]Division of Gastroenterology and Nutrition, Department of Pediatrics, University of California-San Diego, San Diego, California, USA

[3]Institute of Pharmaceutical Sciences, Department of Pharmaceutical Technology, Graz, Austria

[4]Saban Research Institute, Children's Hospital, Los Angeles, California, USA

[5]Glycotechnology Core, University of California–San Diego, San Diego, California, USA

Abstract

Background—Necrotising enterocolitis (NEC) is one of the most common and fatal intestinal disorders in preterm infants. Breast-fed infants are at lower risk for NEC than formula-fed infants, but the protective components in human milk have not been identified. In contrast to formula, human milk contains high amounts of complex glycans.

Objective—To test the hypothesis that human milk oligosaccharides (HMO) contribute to the protection from NEC.

Methods—Since human intervention studies are unfeasible due to limited availability of HMO, a neonatal rat NEC model was used. Pups were orally gavaged with formula without and with HMO and exposed to hypoxia episodes. Ileum sections were scored blindly for signs of NEC. Two-dimensional chromatography was used to determine the most effective HMO, and sequential exoglycosidase digestions and linkage analysis was used to determine its structure.

Results—Compared to formula alone, pooled HMO significantly improved 96-hour survival from 73.1% to 95.0% and reduced pathology scores from 1.98±1.11 to 0.44±0.30 ($p<0.001$). Within the pooled HMO, a specific isomer of disialyllacto-N-tetraose (DSLNT) was identified to be protective. Galacto-oligosaccharides, currently added to formula to mimic some of the effects of HMO, had no effect.

Correspondence to: Dr Lars Bode, University of California–San Diego, Department of Pediatrics, Division of Neonatal Medicine, Division of Gastroenterology and Nutrition, 200 West Arbor Drive, MC 8450, San Diego, CA 92103-8450, USA; lbode@ucsd.edu.
EJK and MZ made equal contributions.

Competing interests None.

Ethics approval IRB of the University of California–San Diego.

Contributors LB, AVG and HRF designed the research; LB, EJK, MZ, KG and YSG conducted the rat intervention studies; LB, YSG and AVG analysed the data; EJK and LB analysed glycans by HPLC-FL; CN and LB conducted the sequential endoglycosidase digestion studies; NN and BC analysed glycans by MALDI-TOF-MS and GC-MS; LB wrote the manuscript. All authors read and approved the final manuscript.

Provenance and peer review Not commissioned; externally peer reviewed.

Conclusion—HMO reduce NEC in neonatal rats and the effects are highly structure specific. If these results translate to NEC in humans, DSLNT could be used to prevent or treat NEC in formula-fed infants, and its concentration in the mother's milk could serve as a biomarker to identify breast-fed infants at risk of developing this disorder.

INTRODUCTION

Necrotising enterocolitis (NEC) is one of the most frequent and fatal intestinal disorders in preterm infants.[1–3] Between 5% and 10% of very-low-birth-weight infants (<1500 g birth weight) develop NEC.[4] More than 25% of them die from the disorder, and the survivors are often faced with long-term neurological complications.[5] NEC aetiology and pathogenesis remain poorly understood, and biomarkers to identify at-risk infants do not exist. Options to treat NEC are limited and include cessation of enteral feeding, antibiotic therapy, and in severe cases surgical removal of the necrotic intestine, all of which are accompanied by devastating long-term complications.[26–8] In 1990 Lucas and Cole reported that formula-fed infants are at a 6- to 10-fold higher risk of developing NEC than breast-fed infants.[9] Despite improvements in formula composition over the past 10–15 years, the gap in NEC risk between formula- and breast-fed infants remains unchanged,[10–13] with a clear advantage for the breast-fed infant. Which components of human milk protect the breast-fed infant from NEC remains unknown. One of the major differences between formula and human milk lies in the quantity and complexity of oligosaccharides. One litre of mature human milk contains between 5 and 15 g of complex oligosaccharides,[14–17] which often exceeds the amount of total protein. In contrast, the oligosaccharide concentration in formula is several orders of magnitude lower and the structures are less complex.

Human milk oligosaccharides (HMO) are built from five monosaccharides: glucose (Glc), galactose (Gal), N-acetyl-glucosamine (GlcNAc), fucose (Fuc) and sialic acid (in humans, exclusively N-acetyl-neuraminic acid, NeuAc). All HMO contain lactose at the reducing end that can be elongated with the disaccharides lacto-N-biose (Galβ1–3GlcNAc, type I) or N-acetyl-lactosamine (Galβ1–4GlcNAc, type II) in β1–3 or β1–6 linkages. Lactose or the elongated oligomers can be fucosylated in α1–2, α1–3 and/or α1–4 linkages and/or sialylated in α2–3 and/or α2–6 linkages (see figure 1B for examples). To date, more than 150 different HMO have been identified,[18–21] but not all women produce the entire set of oligosac-charides,[2223] which depends on the expression of the respective glycosyltransferases. The interpersonal variation in HMO composition might explain why some infants are at higher risk of developing certain disorders despite being breast-fed.

HMO are prebiotics and antimicrobials that alter the infant's intestinal microbiota composition.[14–17] In addition, our results from in vitro studies suggest that HMO may modulate the infant's immune system and reduce mucosal neutrophil infiltration[24] and activation.[25] Since bacterial colonisation and excessive mucosal neutrophil activity are key features in NEC pathogenesis, we hypothesise that HMO contribute to the lower NEC risk in breast-fed infants compared to infants that receive formula that does not contain the potentially beneficial HMO. Studies to test this hypothesis in human infants are currently unfeasible due to the limited availability of isolated and purified HMO. We calculated that a controlled and statistically powered clinical intervention study would need to recruit at least 600 formula-fed very-low-birth-weight infants and require at least 10 kg purified HMO, which is simply not available. Instead, we took an alternative approach, isolated several grams of HMO from pooled human milk and determined whether they reduce NEC in an established model of the disorder in neonatal rats.

Our studies confirmed that formula that lacks HMO reduces survival rates and causes NEC in neonatal rats. Adding pooled HMO to the same formula significantly improved survival

and reduced NEC risk. Galacto-oligosaccharides (GOS), which are currently added to commercial infant formula to mimic the prebiotic effects of HMO but are structurally very different from the naturally occurring HMO, had no effect, suggesting that the beneficial effects of HMO are structure-specific and independent of their prebiotic properties. Subsequently, we identified a single oligosaccharide out of the pooled HMO that reduced NEC risk in the animal model and fully elucidated its molecular structure. If our results from the rat model translate to human infants, it would provide one explanation for why infants that receive human milk remain at a lower risk of developing NEC than those infants that receive formula that lacks HMO.

MATERIALS AND METHODS

Isolation of pooled HMO

Human milk was obtained from 12 healthy volunteers of preterm infants recruited at the University of California–San Diego Medical Center, San Diego, California, USA, after approval by the university's institutional review board. After centrifugation the lipid layer was removed and proteins were precipitated from the aqueous phase by addition of ice-cold ethanol and subsequent centrifugation. Ethanol was removed from the HMO-containing supernatant by roto-evaporation. Lactose and salts were removed by gel filtration chromatography over a BioRad P2 column (100 cm×16 mm, Bio-Rad, Hercules, California, USA) using a semi-automated fast protein liquid chromatography (FPLC) system. GOS syrup (Vivinal, dry matter 75%) was provided by Friesland Campina Domo (Amersfoort, The Netherlands). Disialyllacto-N-tetraose (DSLNT) was purchased from Dextra (Reading, UK).

HMO fractionation by two-dimensional chromatography

Pooled HMO were separated by charge using anion exchange chromatography over QAE gravity columns (Sigma Aldrich, St Louis, Missouri, USA). Lyophilised pooled HMO were dissolved in 2 mM Tris and applied to equilibrated columns. Neutral, −1, −2, −3 and −4 charged HMO were eluted with 2 mM Tris containing 0, 20, 70, 100 and 400 mM NaCl, respectively. Tris and NaCl were removed by gel filtration chromatography over a P2 column. Separation was monitored by fluorescence high-performance liquid chromatography (HPLC-FL) as described below. Differently charged HMO fractions were further separated by size using P2 gel filtration chromatography (100 cm×16 mm) and monitored by HPLC-FL. Fractions that contained the same, but no other HMO were pooled and lyophilised.

Oligosaccharide profiling by HPLC

HMO and GOS were fluorescently labelled with 2-amino-benzamide (2AB) and separated by HPLC on an amide-80 column (4.6 mm ID×25 cm, 5 μm, Tosoh Bioscience, Tokyo, Japan) with a 50 mM ammonium formate/acetonitrile buffer system (Bode *et al*, personal communication). Separation was monitored by a fluorescence detector at 360 nm excitation and 425 nm emission. Peak annotation was based on standard retention times and mass spectrometric (MS) analysis on a Thermo LCQ Duo Ion trap mass spectrometer equipped with a Nano-ESI-source.

HMO analysis by MALDI-TOF mass spectrometry

2AB-labelled HMO peaks were collected, dried and mixed with super-DHB matrix in a 1:1 ratio and spotted on MALDI plates for analysis. Spectra were acquired in positive ion mode.

HMO analysis by sequential exoglycosidase digest

Linkage promiscuous neuraminidase (α2–3>6,8,9; *Arthrobacter ureafaciens*) was purchased from Sigma Aldrich (St Louis, Missouri, USA); α2–3-specific neuraminidase (*Salmonella typhimurium*), β1–3 galactosidase (*Xanthomonas manihotis*), β1–4 galactosidase (*Bacteroides fragilis*) and β-N-acetyl-glucosaminidase (GlcNAcase, *X manihotis*) were obtained from New England Biolabs (Ipswich, Massachusetts, USA). All enzymes were used at concentrations and incubation times according to the manufacturers' protocols.

HMO linkage analysis by gas chromatography mass spectrometry (GC–MS)

The unknown HMO 2 was dissolved in dimethylsulphoxide and par-O-methylated by sequential addition of sodium hydroxide and methyl iodine. Chloroform was added and the reaction stopped by the addition of water. The methylated glycan was extracted in the chloroform layer, dried and hydrolysed with 4N trifluoroacetic acid at 100°C for 6 h. Acids were removed with 50% isopropanol:water under dry nitrogen flush. Hydrolysed samples were reduced overnight by sodium borohydride in 1M ammonium hydroxide. Excess borohydride was neutralised by 30% acetic acid and boric acid was removed as methyl borate. Samples were treated with 1:1 acetic anhydride:pyridine at 100°C for 1 h. Pyridine and acetic anhydride were removed by nitrogen flush. Partially methylated alditol acetates were extracted with dichloromethane, analysed by GC–MS with a DB-5 capillary column, and identified by a combination of established retention times and mass fragmentation patterns.

Induction and evaluation of NEC in neonatal rats

The NEC model in neonatal rats was originally described by Barlow *et al*[26] and later modified.[27] Briefly, pregnant time-dated Sprague-Dawley rats were induced at term using Pitocin (1–2 U per animal). Immediately after birth, neonates were randomised into one of the different study groups. Animals in the dam-fed (DF) group remained with the dam. All other animals were separated from the dam, housed in a temperature- and humidity-controlled incubator and orally gavaged with a special rodent formula (0.2 ml) twice daily. The formula approximates the protein and caloric content of rat breast milk and consists of 15 g Similac 60/40 (Ross Pediatrics, Columbus, Ohio, USA) in 75 ml of Esbilac canine milk replacer (Pet-Ag, Hampshire, Illinois, USA). All animals, dam-fed and gavaged, were exposed to 10 min of hypoxia (5% O_2, 95% N_2) thrice daily in a modular chamber. All animals were sacrificed 96 h post-partum; their intestines were collected and inspected for the presence of gross necrotic changes or *Pneumatosis intestinalis*. A 0.5 cm section of the terminal ileum was prepared for H&E staining per standard protocols and scored blindly by three investigators based on morphological changes that included epithelial sloughing, villus oedema, infiltration of neutrophils, apoptosis of villus enterocytes, crypt hyperplasia and misaligned nuclei in the epithelium. If at least one pathology sign was observed, a score of 0.5–1.5 was assigned depending on severity. Two or three signs together resulted in a score of 2–3. The maximum score of 4 was given in case of complete obliteration of the epithelium with or without intestinal perforation. Pathology scores were plotted for each animal and the mean calculated per group. Each intervention was tested in at least two independent sets of experiments with a total of 8–26 animals per intervention group. Differences between the groups were calculated by one-way ANOVA with the Kruskal–Wallis test and Dunn's multiple comparison test. Significance was defined as $p<0.05$.

RESULTS

Pooled HMO, but not GOS improve survival and reduce NEC in neonatal rats

The primary objective of this study was to assess whether HMO affect NEC in neonatal rats. Therefore, we randomised rat pups at birth into different study groups. The first group stayed with the dam for the entire duration of the study (dam-fed, DF), but was exposed to hypoxia thrice a day together with all the other groups. The second group was fed formula that did not contain HMO (formula-fed, FF). The third group was fed formula supplemented with HMO at 10 mg/ml (FF+HMO), the average HMO concentration in mature human milk. The fourth group was fed formula supplemented with GOS at 8 mg/ml (FF +GOS), comparable to the GOS concentration in infant formula with prebiotics. HPLC-FL analysis of the isolated, pooled HMO showed that 2′-fucosyllactose (2′FL), lacto-N-fucopentaose 1 (LNFP 1) and lacto-N-tetraose (LNT) were the major oligosaccharides (figure 1A,B). In addition, the pooled HMO contained several complex, fucosylated and/or sialylated oligosaccharides. In comparison and as expected, the HPLC-FL profile of GOS looked strikingly different (figure 1C) and contained mostly tri-and tetra-saccharides and hardly any complex glycans.

Comparable to published data derived from the same neonatal rat model,[27–29] all DF pups, but only 19 of 26 FF pups (73.1%) survived the first 96 h post-partum (figure 2A). Most intriguingly, the addition of HMO greatly improved survival (19 of 20 pups, 95.0%). GOS, however, had no effect (13 of 17 pups, 76.5%).

DF pups gained weight faster than FF pups, but the addition of HMO or GOS did not improve weight gain (data not shown), suggesting that improved survival was independent of weight gain. Macroscopic evaluation 96 h post-partum showed that the intestines of most FF and FF+GOS pups were darker, with patchy necrosis and evidence of haemorrhagic intestine as well as intramural gas cysts (*Pneumatosis intestinalis*), which are characteristic signs of NEC (figure 2B) and were absent from the intestines of all DF and most FF+HMO pups. Microscopic evaluation of H&E-stained ileum sections confirmed the macroscopic observations (figure 2C). While the ileum of most DF and FF+HMO pups showed a normal, healthy microscopic architecture, some of the sections from FF and FF+GOS pups showed complete destruction. While the mean pathology score (±SD) was 0.15±0.34 in the DF group, it increased significantly to 1.98±1.11 in the FF group ($p<0.001$) (figure 2D). Pups that received HMO with their formula (10 mg/ml) had a mean pathology score of 0.44±0.30, which was significantly lower than in the FF group ($p<0.001$), but statistically not different from that of DF pups. Pups that received HMO at a 10-fold lower concentration (1 mg/ml) had a mean pathology score of 0.64±0.54, which was still significantly lower than that in the FF group ($p<0.001$), but slightly higher than in the DF controls ($p<0.05$). GOS had no effect on pathology scores (1.69±0.90). These results demonstrate for the first time that oligosaccharides isolated from human milk improve survival and reduce NEC in a neonatal rat model of the disease.

Exposure to HMO in the first 24 h post-partum is required, but not sufficient to reduce NEC

To assess whether or not HMO have to be present in all feedings to be protective, we fed a group of pups with formula that did not contain HMO for the first 24 h and then switched to formula that was supplemented with HMO for the remaining 72 h (figure 3). To our surprise, pathology scores (1.72±1.06) were not different from pups that received unsupplemented formula for the entire duration of the study (1.97±1.15). Another group of pups received formula with HMO for the first 24 h and formula without HMO for the remaining 72 h. Again, pathology scores (2.04±0.80) were not different from the group that received unsupplemented formula for the entire time. Together, these results indicate that

exposure to HMO in the first 24 h post-partum is required, but not sufficient to protect from NEC.

A single, disialylated HMO reduces NEC

Since more than 150 structurally different HMO have been identified so far, we wondered whether all HMO are protective or whether the effect depends on a specific structural epitope. First, we used anion exchange chromatography to separate the pooled HMO by charge based on the number of sialic acid moieties on the individual HMO. As confirmed by HPLC-FL (data not shown), we generated five distinct HMO fractions with oligosaccharides that contained either zero, one, two, three or four sialic acids and had a net charge of 0, −1, −2, −3 or −4, respectively. We then tested these fractions in the rat model at their respective concentrations in pooled HMO at 10 mg/ml (figure 4A). Adding the neutral (0) HMO fraction to the formula lowered pathology scores to 1.18±0.50 ($p<0.05$). While the −1, −3 and −4 charged HMO fractions had no effect, the −2 charged fraction lowered pathology scores to 0.44±0.42, which was significantly different from the FF group ($p<0.001$), but not different from DF controls. These results showed that not all HMO are protective and that the effects depend on the presence of two sialic acids.

We analysed the −2 charged HMO fraction by HPLC-FL and detected four distinct peaks (figure 4B) which we collected and analysed by MALDI-TOF-MS (figure 4C). The m/z value of peak 1 corresponded to the 2AB-labelled sodium adduct of an oligo-saccharide containing three hexoses, one N-acetyl-hexosamine and one N-acetyl-neuraminic acid, likely a monosialylated lacto-N-tetraose (Galβ1–3GlcNAcβ1–3Galβ1–4Glc) or lacto-N-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc). Since peak 1 contained only one sialic acid and we had shown that the monosialylated (−1) HMO fraction had no significant effect on reducing NEC pathology scores, we assumed that peak 1 was a spillover from the −1 charged HMO fraction and disregarded this oligosaccharide in future analyses. Peak 2 was different from peak 1 only by the addition of one N-acetyl-neuraminic acid and was likely disialylated lacto-N-tetraose or lacto-N-neotetraose. Peaks 3 and 4 contained one additional hexose and one additional N-acetyl-hexosamine, which likely represent an extension of the HMO backbone by the disaccharides N-acetyl-lactosamine (Galβ1–4GlcNAc) or lacto-N-biose (Galβ1–3GlcNAc). Peak 3 was different from peak 4 only by the addition of a fucose moiety. In the following, the oligosaccharides represented by peaks 2, 3 and 4 are called HMO 2, HMO 3 and HMO 4, respectively.

Next, we used gel exclusion chromatography to further separate the oligosaccharides in the −2 charged HMO fraction by size. While we were unable to separate HMO 3 and 4 from each other, we separated HMO 3+4 from HMO 2 (figure 4D). We then pooled the subfractions containing either HMO 2 or HMO 3+4 and tested them in the rat model at their original concentrations in pooled HMO at 10 mg/ml (figure 4E). While HMO 3+4 had no effect, HMO 2 reduced pathology scores to 0.64±0.41, which was significantly lower than that of the FF group ($p<0.001$), but not different from DF controls.

The NEC-protective HMO is DSLNT

The results of our two-dimensional chromatography approach showed that a distinct disialylated HMO protects neonatal rats from NEC. While MALDI-TOF-MS provided the first insights into the overall composition of the protective HMO, we used HPLC-FL after sequential exoglycosidase digestion to determine the exact positions and linkages of the different monosaccharide residue. First, we determined whether the two sialic acids are bound in an α2–3 or α2–6 linkage. Incubating HMO 2 with an α2–3-specific neuraminidase caused a complete shift of the HMO 2 peak in the HPLC-FL chromatogram (figure 5A), indicating that at least one sialic acid is bound in an α2–3 position. Incubating HMO 2 with

a linkage promiscuous neuraminidase that cleaves both α2–3- and α2–6-bound sialic acid resulted in an even bigger shift of the HMO 2 peak (figure 5A). Together, these results indicate that one sialic acid is bound in an α2–3 linkage and one in an α2–6 linkage. After removal of both sialic acids, we used linkage specific galactosidases to determine whether the terminal monosaccharide is indeed galactose and whether the underlying HMO backbone is a type I (Galβ1–3GlcNAc-R) or type II chain (Galβ1–4GlcNAc-R). β1–3-specific galactosidase digestion resulted in a complete peak shift; β1–4-specific galactosidase digestion had no effect, confirming the presence of terminal galactose in a type I chain (figure 5B). Next, we used a β-N-acetyl-glucosaminidase and confirmed that the subterminal monosaccharide is indeed GlcNAc (figure 5C). The remaining disaccharide was cleaved by a β1–4-specific galactosidase (data not shown), verifying that lactose forms the reducing end of HMO 2.

After elucidating the position and some of the linkages in the HMO 2 backbone, we determined the positions of the two sialic acids. The β1–3-specific galactosidase removed the terminal galactose only after pretreatment with the linkage promiscuous neuraminidase or the α2–3-specific neuraminidase (data not shown), suggesting that the terminal galactose is capped by α2–3-linked sialic acid. Removal of the subterminal GlcNAc was only possible after pretreatment with the linkage promiscuous but not the α2–3-specific neuraminidase (data not shown), suggesting that the second sialic acid is bound to the subterminal GlcNAc in α2–6 linkage.

In addition, we used GC–MS analysis of partially methylated alditol acetate (PMAA) derivatives and confirmed the presence of 3-linked galactose, 4-linked glucose and 3,6-linked GlcNAc (figure 5D). The combined data of sequential exoglycosidase digestions and PMAA linkage analysis unambiguously identified HMO 2 as DSLNT with the isomeric configuration NeuAcα2–3Galβ1–3 (NeuAcα2–6) GlcNAcβ1–3Galβ1–4Glc (figure 5E).

Sialic acid is required for the NEC-protective effects of DSLNT

Based on HPLC-FL analysis of the pooled HMO (figure 1A), the DSLNT concentration was about 300 μM in formula that we had supplemented with pooled HMO at 10 mg/ml. We purchased commercially available DSLNT, added it to formula at 300 μM, and confirmed that it significantly reduced NEC pathology scores to 0.60±0.52 compared to 1.90±1.13 in the FF group ($p<0.001$) (figure 6). We used linkage promiscuous neuraminidase to remove both sialic acids from DSLNT and found that it no longer reduced NEC. When pretreated with α2–3-specific neuraminidase, DSLNT also lost its protective effects. These results indicate that both sialic acids are required for DSLNT to protect neonatal rats from NEC.

DISCUSSION

We report that a single HMO, DSLNT, improves survival and reduces NEC in neonatal rats. We recently reviewed possible underlying mechanisms for the role of HMO in NEC prevention,[16] but how DSLNT reduces NEC remains to be elucidated, which is beyond the scope of this study. Based on our results, it appears unlikely that the protection is due to a prebiotic effect. GOS, which are supposed to mimic the prebiotic effects of HMO, had no effect on NEC, and even the effects of HMO were highly structure-dependent. These results suggest that the underlying mechanisms are mediated by specific receptors—that is, lectins, which could be expressed by either the host or certain intestinal microorganisms. In previous in vitro and ex vivo studies we have shown that sialylated HMO reduced selectin-mediated neutrophil infiltration[24] and activation,[25] processes in the host's immune system that are involved in NEC pathogenesis.[30] Thus, we hypothesised that an HMO-mediated reduction in neutrophil infiltration and activation contributes to the protection from NEC. However, selectin ligands need to be not only sialylated but also fucosylated as demonstrated in

patients with congenital disorder of glycosylation type IIc.[31] The underlying genetic defects in these patients' intracellular fucose metabolism decreases the fucosylation of cell surface proteins,[32] which include selectin ligands. As a consequence, neutrophil motility and extravasation are impaired and lead to recurrent infections.[31] Since DSLNT is not fucosylated, it appears unlikely that it interferes with selectin-mediated neutrophil infiltration and activation. In fact, we did not observe any difference in mucosal neutrophil infiltration in our rat model (data not shown). These results suggest that selectin-independent mechanisms are involved. Siglecs, sialic acid binding immunoglobulin-like lectins that are expressed on different cell types in the haematopoietic and immune system, specifically bind to sialic acid containing ligands[33] and thus represent potential targets for DSLNT. Koliwer-Brandl *et al* have shown that DSLNT inhibits siglec-4 but not siglec-2 binding to glycosylated surfaces.[34] However, the role of siglecs in intestinal physiology in general and NEC pathogenesis in particular has not yet been investigated. Identifying which molecules interact with DSLNT to reduce NEC could provide additional insights into NEC aetiology and pathogenesis. In addition, these molecules could become targets for the development of new and desperately needed drugs to prevent and treat this devastating disorder.

Due to the limited amount of available HMO, the neonatal rat NEC model was the most feasible approach to test our hypothesis that HMO protect against NEC and to identify individual protective HMO. The rat model itself, however, has its limitations (reviewed in Sodhi *et al*[35]), and whether our results translate to human infants needs to be confirmed. Now, that we have identified a single HMO and determined its structure as a specific DSLNT isomer, it will be possible to generate this oligosaccharide by chemical and/or enzymatic synthesis in quantities needed for human intervention studies. If these studies confirm the benefits of DSLNT for human infants, it will be a valuable supplement to prevent NEC in infants that do not receive human milk.

Our results are in line with a recent study on the effectiveness of human milk-based fortifiers (HMF) in feeding extremely premature infants.[36] Mother's milk fortified with HMF reduced the risk of overall NEC compared to mother's milk fortified with bovine milk-based supplements. While HMF is enriched in HMO, the concentration of oligosaccharides in bovine milk is several orders of magnitudes lower. Bovine milk does not contain DSLNT, which might explain why HMF reduced NEC risk and bovine milk-based products were ineffective.

DSLNT protects rats from NEC at a concentration of 300 μM, which is within the physiological range reported for human milk.[3738] DSLNT concentrations in milk from mothers with term infants are higher during the first 3–5 days post-partum (~600–1500 μM) and drop within the first 2–3 weeks (~500–800 μM),[38] a timeframe that coincides with the occurrence of NEC in most preterm infants. Whether DSLNT concentrations in the milk from mothers with preterm infants are comparable to those of term infants needs to be elucidated. The inter- and intra-personal variations in the DSLNT concentration of human milk may provide one explanation for why some breast-fed infants still develop NEC. Some mother's milk may simply not contain sufficient amounts of DSLNT to protect the infant from NEC. If this is true, the concentration of DSLNT in the mother's milk may be a non-invasive biomarker to identify breast-fed infants at risk of developing NEC.

Acknowledgments

The authors are grateful to Drs Kim Barrett, Lars Eckmann, Jeffrey Esko, Jae Kim and Ajit Varki at the University of California–San Diego, for many valuable discussions that helped guide this project. They also thank Lauren Hsiao at the University of California–San Diego for technical assistance with the isolation of pooled HMO, Robbin Newlin and Gia Garci at the Histology Core of the Sanford-Burnham Institute for Medical Research (La Jolla, California, USA), for their excellent work embedding, sectioning and H&E staining of rat ileum specimens, and Dr

Larry Wang at the Children's Hospital (Los Angeles, California, USA), for reviewing and scoring the ileum sections.

Funding The study was funded by NIH grants K99/R00 DK078668 (LB), R03 HD059717S1 (LB), R21 AI083612 (AVG) and R01 AI014032 (HRF) and in part by Abbott Nutrition. The funding sources were not involved in the study design, the collection, analysis and interpretation of data, the writing of the report, or the decision to submit the paper for publication.

References

1. Uauy RD, Fanaroff AA, Korones SB, et al. Necrotising enterocolitis in very low birth weight infants: biodemographic and clinical correlates. National Institute of Child Health and Human Development Neonatal Research Network. J Pediatr. 1991; 119:630–8. [PubMed: 1919897]
2. Neu J, Walker WA. Necrotizing enterocolitis. N Engl J Med. 2011; 364:255–64. [PubMed: 21247316]
3. Holman RC, Stoll BJ, Clarke MJ, et al. The epidemiology of necrotizing enterocolitis infant mortality in the United States. Am J Public Health. 1997; 87:2026–31. [PubMed: 9431297]
4. Holman RC, Stoll BJ, Curns AT, et al. Necrotising enterocolitis hospitalisations among neonates in the United States. Paediatr Perinat Epidemiol. 2006; 20:498–506. [PubMed: 17052286]
5. Rees CM, Pierro A, Eaton S. Neurodevelopmental outcomes of neonates with medically and surgically treated necrotizing enterocolitis. Arch Dis Child Fetal Neonatal Ed. 2007; 92:F193–8. [PubMed: 16984980]
6. Dicken BJ, Sergi C, Rescorla FJ, et al. Medical management of motility disorders in patients with intestinal failure: a focus on necrotizing enterocolitis, gastroschisis, and intestinal atresia. J Pediatr Surg. 2011; 46:1618–30. [PubMed: 21843732]
7. Clark, Gordon P, Walker WM, et al. Characteristics of patients who die of necrotizing enterocolitis. J Perinatol. Published Online First: 19 May 2011. 10.1038/jp.2011.65
8. Blakely ML, Lally KP, McDonald S, et al. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD Neonatal Research Network. Ann Surg. 2005; 241:984–9. discussion 989–94. [PubMed: 15912048]
9. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet. 1990; 336:1519–23. [PubMed: 1979363]
10. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr. 2010; 15:562–7. e1. [PubMed: 20036378]
11. Sisk PM, Lovelady CA, Dillard RG, et al. Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. J Perinatol. 2007; 27:428–33. [PubMed: 17443195]
12. Schanler RJ, Shulman RJ, Lau C. Feeding strategies for premature infants: beneficial outcomes of feeding fortified human milk versus preterm formula. Pediatrics. 1999; 103:1150–7. [PubMed: 10353922]
13. Schanler RJ, Lau C, Hurst NM, et al. Randomized trial of donor human milk versus preterm formula as substitutes for mothers' own milk in the feeding of extremely premature infants. Pediatrics. 2005; 116:400–6. [PubMed: 16061595]
14. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr. 2005; 25:37–58. [PubMed: 16011458]
15. Kunz C, Rudloff S, Baier W, et al. Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr. 2000; 20:699–722. [PubMed: 10940350]
16. Bode L. Human milk oligosaccharides: prebiotics and beyond. Nutr Rev. 2009; 67 (Suppl 2):S183–91. [PubMed: 19906222]
17. Bode L. Recent advances on structure, metabolism, and function of human milk oligosaccharides. J Nutr. 2006; 136:2127–30. [PubMed: 16857829]
18. Wu S, Tao N, German JB, et al. Development of an annotated library of neutral human milk oligosaccharides. J Proteome Res. 2010; 9:4138–51. [PubMed: 20578730]

19. Wu S, Grimm R, German JB, et al. Annotation and structural analysis of sialylated human milk oligosaccharides. J Proteome Res. 2011; 10:856–68. [PubMed: 21133381]
20. Stahl B, Thurl S, Zeng J, et al. Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. Anal Biochem. 1994; 223:218–26. [PubMed: 7887467]
21. Kobata A. Structures and application of oligosaccharides in human milk. Proc Jpn Acad Ser B Phys Biol Sci. 2010; 86:731–47.
22. Thurl S, Henker J, Siegel M, et al. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. Glycoconj J. 1997; 14:795–9. [PubMed: 9511984]
23. Blank D, Gebhardt S, Maass K, et al. High-throughput mass finger printing and Lewis blood group assignment of human milk oligosaccharides. Anal Bioanal Chem. 2011; 401:2495–510. [PubMed: 21898157]
24. Bode L, Kunz C, Muhly-Reinholz M, et al. Inhibition of monocyte, lymphocyte, and neutrophil adhesion to endothelial cells by human milk oligosaccharides. Thromb Haemost. 2004; 92:1402–10. [PubMed: 15583750]
25. Bode L, Rudloff S, Kunz C, et al. Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil beta 2 integrin expression. J Leukoc Biol. 2004; 76:820–6. [PubMed: 15240751]
26. Barlow B, Santulli TV. Importance of multiple episodes of hypoxia or cold stress on the development of enterocolitis in an animal model. Surgery. 1975; 77:687–90. [PubMed: 1173200]
27. Nadler EP, Dickinson E, Knisely A, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. J Surg Res. 2000; 92:71–7. [PubMed: 10864485]
28. Upperman JS, Potoka D, Grishin A, et al. Mechanisms of nitric oxide-mediated intestinal barrier failure in necrotizing enterocolitis. Semin Pediatr Surg. 2005; 14:159–66. [PubMed: 16084403]
29. Guner YS, Franklin AL, Chokshi NK, et al. P-glycoprotein induction by breast milk attenuates intestinal inflammation in experimental necrotizing enterocolitis. Lab Invest. 2011; 91:1668–79. [PubMed: 21788941]
30. Stefanutti G, Lister P, Smith VV, et al. P-selectin expression, neutrophil infiltration, and histologic injury in neonates with necrotizing enterocolitis. J Pediatr Surg. 2005; 40:942–7. discussion 947–8. [PubMed: 15991175]
31. Becker DJ, Lowe JB. Leukocyte adhesion deficiency type II. Biochim Biophys Acta. 1999; 1455:193–204. [PubMed: 10571012]
32. Luhn K, Wild MK, Eckhardt M, et al. The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter. Nat Genet. 2001; 28:69–72. [PubMed: 11326279]
33. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. Nat Rev Immunol. 2007; 7:255–66. [PubMed: 17380156]
34. Koliwer-Brandl H, Siegert N, Umnus K, et al. Lectin inhibition assay for the analysis of bioactive milk sialoglycoconjugates. Int Dairy J. 2011; 21:413–20.
35. Sodhi C, Richardson W, Gribar S, et al. The development of animal models for the study of necrotizing enterocolitis. Dis Model Mech. 2008; 1:94–8. [PubMed: 19048070]
36. Ganapathy V, Hay JW, Kim JH. Costs of necrotizing enterocolitis and cost-effectiveness of exclusively human milk-based products in feeding extremely premature infants. Breastfeed Med. Published Online First: 30 June 2011. 10.1089/bfm.2011.0002
37. Leo F, Asakuma S, Fukuda K, et al. Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of Samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography. Biosci Biotechnol Biochem. 2010; 74:298–303. [PubMed: 20139617]
38. Bao Y, Zhu L, Newburg DS. Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. Anal Biochem. 2007; 370:206–14. [PubMed: 17761135]

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- to 10-fold lower risk of developing NEC than formula-fed infants.
- Biomarkers to identify at-risk infants do not exist.
- Options to treat NEC are limited and include cessation of enteral feeding, antibiotic therapy, and in severe cases surgical removal of the necrotic intestine, all of which are accompanied by devastating long-term complications.

What are the new findings?

- 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.
- Galacto-oligosaccharides, currently used to supplement formula with HMO-like oligosaccha-rides, have no effect on NEC in neonatal rats.

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

- Feeding with mother's milk or donor milk might provide the preterm infant with protective DSLNT.
- Low DSLNT concentrations in the mother's milk might become a non-invasive biomarker to identify breast-fed infants at risk of developing NEC.
- Supplementing infant formula with DSLNT might protect the formula-fed infant from NEC.

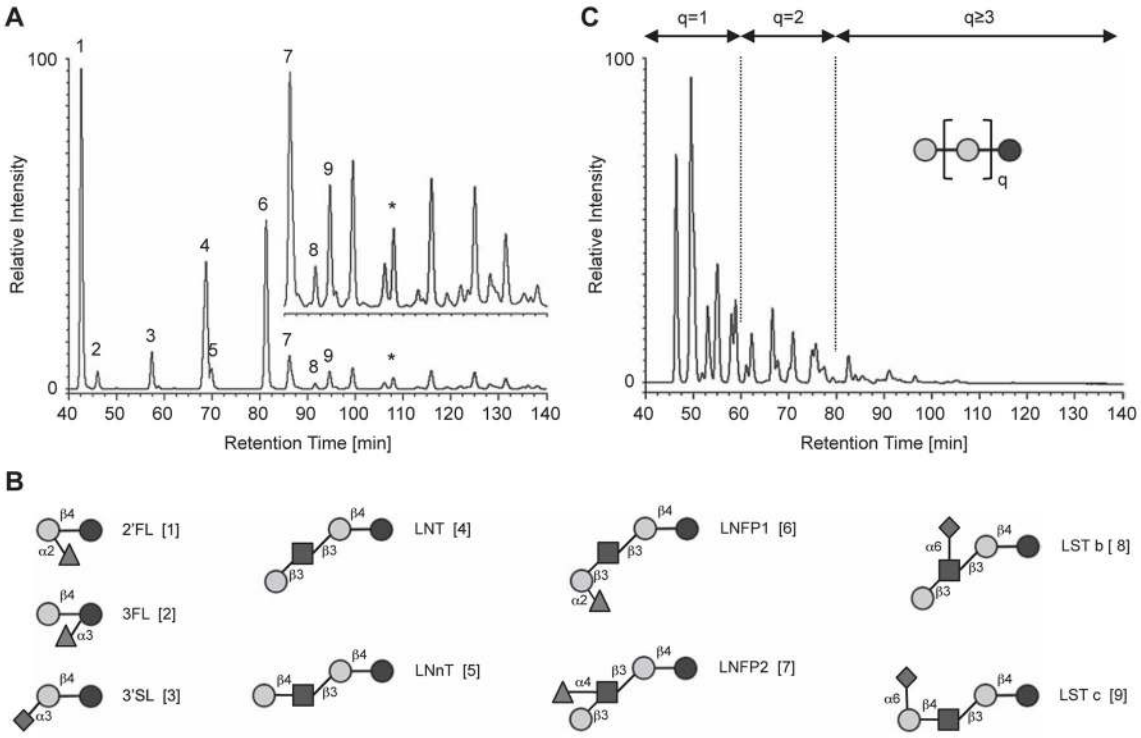

Figure 1.

Human milk oligosaccharides (HMO) and galacto-oligosaccharides (GOS) are structurally different. (A) Fluorescence high-performance liquid chromatography (HPLC-FL) chromatogram of 2AB-labelled HMO isolated from pooled human milk. Most common HMO are annotated and listed in panel B. *Disialyllacto-N-tetraose (DSLNT), which was later identified as the necrotising enterocolitis-protective HMO. (B) Schematic representation of the most common oligosaccharides found in the isolated pooled HMO. Numbers in brackets correspond to the annotated peaks in panel A. 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP1, lacto-N-fucopentaose 1; LNFP2, lacto-N-fucopentaose 2; LSTb, sialyllacto-N-tetraose b; LSTc, sialyllacto-N-tetraose c. Monosaccharide key: dark circle, glucose (Glc); light circle, galactose (Gal); square, N-acetyl-glucosamine (GlcNAc); triangle, fucose (Fuc); diamond, N-acetyl-neuraminic acid (NeuAc). (C) HPLC-FL chromatogram of Vivinal GOS. Peak clusters represent structural isomers of oligosaccharides with the same degree of polymerisation and depend on the number of galactose residues per GOS molecule. Comparison of the HMO and GOS chromatograms confirmed a clear difference in the structural composition.

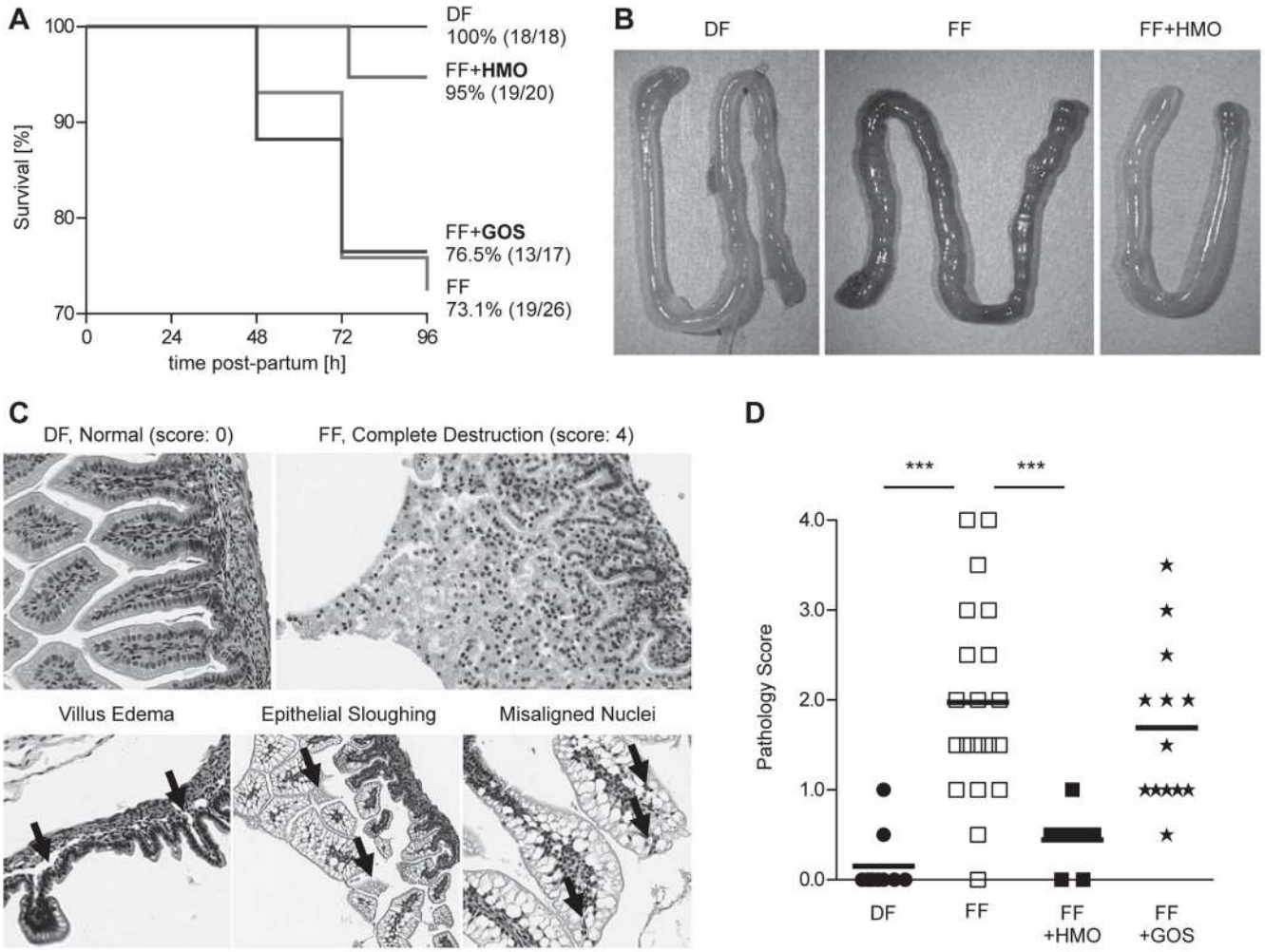

Figure 2.

Pooled human milk oligosaccharides (HMO), but not galacto-oligosaccharides (GOS) improve survival and reduce necrotising enterocolitis (NEC) in neonatal rats. (A) Survival of neonatal rats within the first 96 h post-partum. DF, dam-fed; FF, formula-fed; FF+HMO, fed formula with HMO (10 mg/ml); FF+GOS, fed formula with GOS (8 mg/ml). (B) Macroscopic evaluation of rat intestines at 96 h post-partum. Compared to DF (left) and FF +HMO (right) animals, the intestines of FF animals (centre) were darker with patchy necrosis and evidence of haemorrhagic intestine as well as intramural gas cysts (*Pneumatosis intestinalis*). (C) Microscopic evaluation of H&E-stained rat ileum sections. Based on the presence or absence of histological anomalies (three examples are shown in the bottom panel), ileum sections were graded from 0 (normal) to 4 (complete destruction). (D) Ileum pathology scores at 96 h post-partum. Each intervention was tested in a total of 10–20 animals in three independent experiments. Each symbol represents the pathology score for an individual animal. Horizontal lines represent mean pathology scores. ***p<0.001.

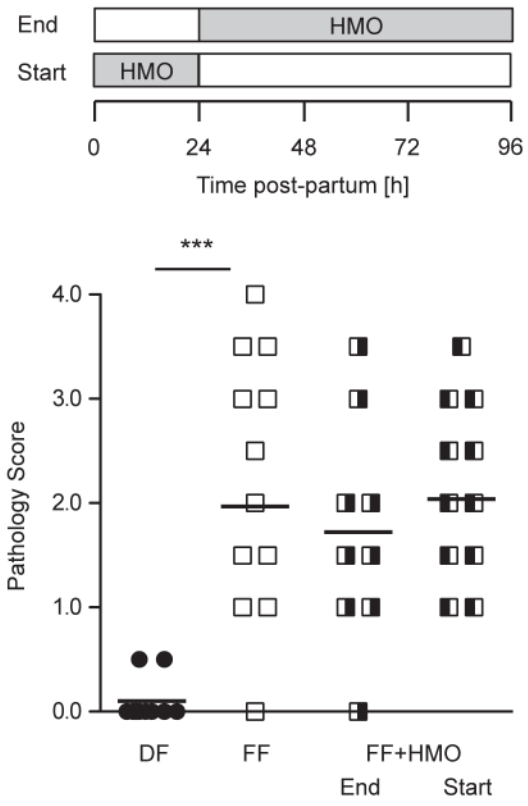

Figure 3.

Exposure to human milk oligosaccharides (HMO) in the first 24 h post-partum is required, but not sufficient to reduce necrotising enterocolitis. Neonatal rats were dam-fed (DF), fed HMO-free formula for the entire first 96 h post-partum (FF), fed HMO-free formula for the first 24 h and then switched to HMO-containing formula (10 mg/ml) for the remaining 72 h (FF+HMO End), or fed HMO-containing formula for the first 24 h and then switched to HMO-free formula (FF+HMO Start). Each intervention was tested in a total of 9–12 animals in two independent experiments. ***$p<0.001$.

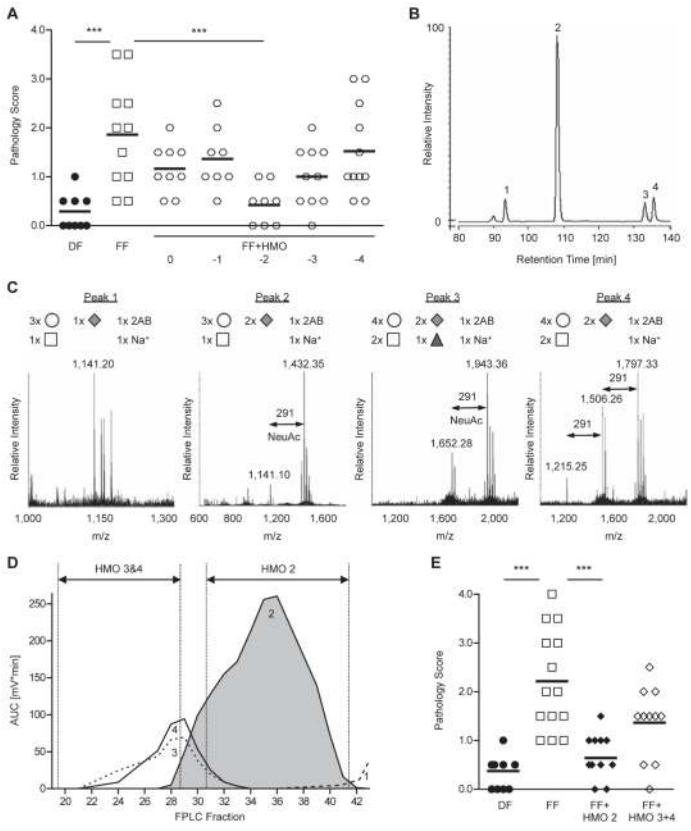

Figure 4.

A single, disialylated human milk oligosaccharide (HMO) reduces necrotising enterocolitis. (A) Ileum pathology scores in response to adding charge-fractionated HMO to formula. Anion exchange chromatography was used to fractionate pooled HMO by charge based on whether HMO contained no (0), one (−1), two (−2), three (−3) or four (−4) sialic acid residues. The −2 charged HMO fraction, containing oligosaccharides with two sialic acids (two negative charges) had the most pronounced effect. (B) HPLC-FL chromatogram of −2 charged HMO fraction. (C) MALDI-TOF mass spectra and potential composition of the four major HMO peaks in the −2 charged HMO fraction. The predicted number of hexoses (circles), hexosamines (square), N-acetylneuramic acid (NeuAc, diamond) and fucose (triangle) per molecule are listed above each mass spectrum. Loss of NeuAc during analysis reduces the mass by 291 Da. (D) Fast protein liquid chromatography (FPLC) with a gel exclusion column was used to separate the four major HMO peaks in the −2 charged HMO fraction by size. FPLC fractions containing mostly HMO peak 2 were pooled together (HMO 2). HMO peaks 3 and 4 could not be separated by gel exclusion and were pooled in one fraction (HMO 3+4). (E) Ileum pathology scores in response to adding size-fractionated HMO to formula. Each intervention was tested in a total of 11–14 animals in two independent experiments. ***p<0.001.

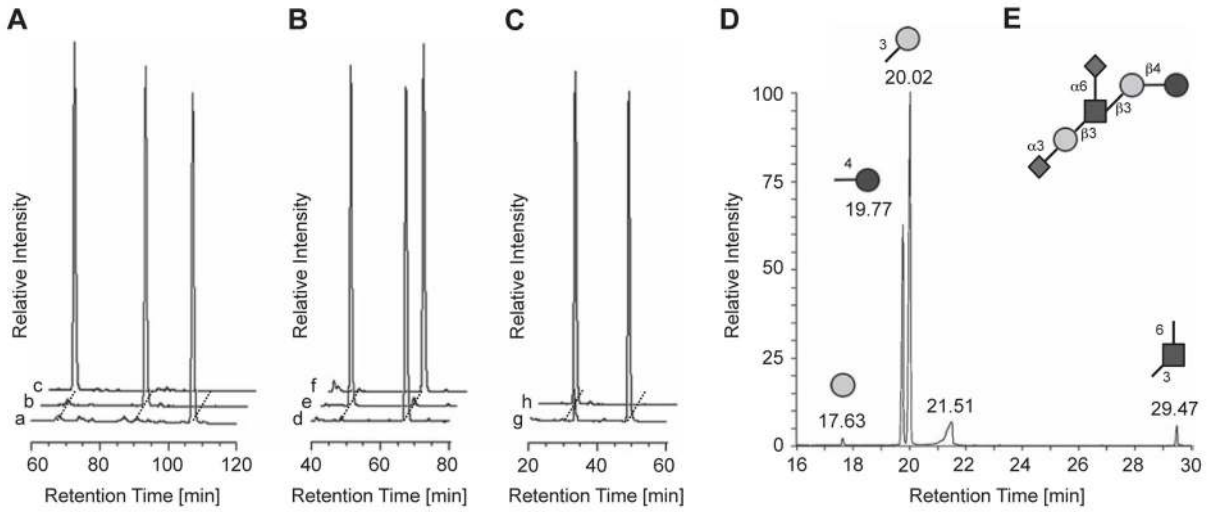

Figure 5.

The necrotising enterocolitis-protective human milk oligosaccharide (HMO) is disialyllacto-N-tetraose (DSLNT). (A) Linkage specific neuraminidase treatment shows the presence of one α2–3- and one α2–6-linked N-acetyl-neuraminic acid (NeuAc). Fluorescence high-performance liquid chromatography (HPLC-FL) chromatogram a: protective HMO 2; b: HMO 2 after treatment with α2–3-specific neuraminidase; c: HMO 2 after treatment with linkage promiscuous neuramidase. (B) The underlying HMO backbone has a type I structure (Galβ1–3GlcNAc). HPLC-FL chromatogram d: asialo-HMO 2 (after treatment with α2–3/6 neuraminidase, product c); e: asialo-HMO 2 after treatment with β1–3-specific galactosidase; f: asialo-HMO 2 after treatment with β1–4-specific galactosidase. (C) The subterminal sugar in the HMO backbone is N-acetyl-glucosamine (GlcNAc). HPLC-FL chromatogram g: asialo-agalacto-HMO 2 (after treatment with α2–3/6 neuraminidase and β1–3 galactosidase, product e); h: asialo-agalacto-HMO 2 after treatment with GlcNAcase. (D) Gas chromatography mass spectrum (GC–MS) of partially methylated alditol acetate (PMAA) derivatives of HMO 2. (E) Schematic representation of DSLNT based on the results from sequential exoglycosidase digestion and GC–MS PMAA linkage analysis.

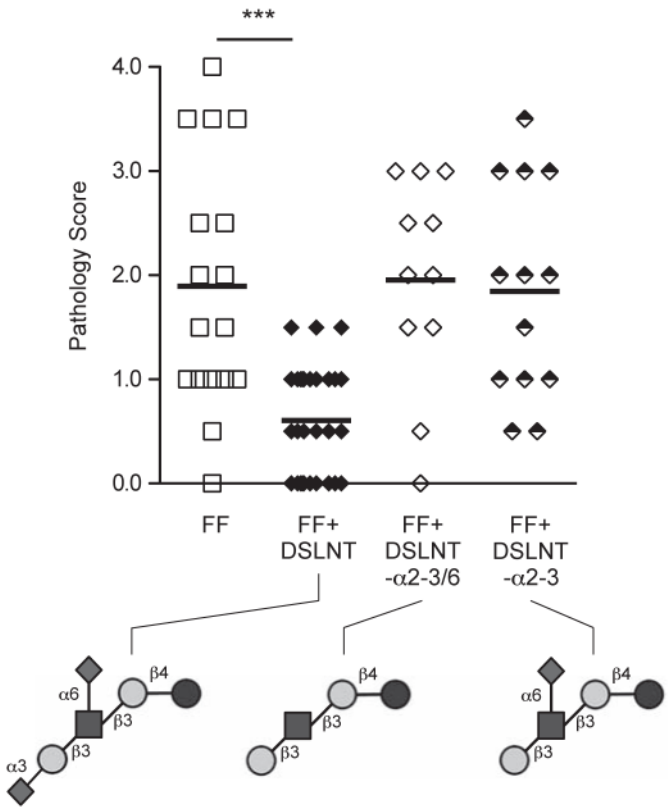

Figure 6.

Sialic acid is required for the necrotising enterocolitis (NEC) protective effects of disialyllacto-N-tetraose (DSLNT). Ileum pathology scores in response to adding DSLNT or neuraminidase-treated DSLNT to formula. Commercially available DSLNT (300 μM) significantly reduced NEC pathology scores. Treatment with a linkage promiscuous neuraminidase (α2–3/6) or an α2–3-specific neuraminidase abolished the protective effects of DSLNT. Each intervention was tested in a total of 11–26 animals in three independent experiments. ***p<0.001.