

# Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides

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*Human milk is a rich source of oligosaccharides. Acidic oligosaccharides, such as sialyllactose (SL), contain sialic acid (SA) residues. In human milk, approximately 73% of SA is bound to oligosaccharides, whereas only 3% is present in free form. Oligosaccharides are highly resistant to hydrolysis in the gastrointestinal tract. Only a small portion of the available oligosaccharides in breast milk is absorbed in the neonatal small intestine. SL and sialylated oligosaccharides are thought to have significant health benefits for the neonate, because of their roles in supporting resistance to pathogens, gut maturation, immune function, and cognitive development. The need for SA to allow proper development during the neonatal period is thought to exceed the endogenous synthesis. Therefore, these structures are important nutrients for the neonate. Based on the potential benefits, SL and sialylated oligosaccharides may be interesting components for application in infant nutrition. Once the hurdle of limited availability of these oligosaccharides has been overcome, their functionality can be explored in more detail, and supplementation of infant formula may become feasible.*

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## INTRODUCTION

Human milk is often referred to as the optimal source of nutrition for the first few months of human life, as it provides all the necessary nutrients for normal growth and development. In addition, human milk also contains components that may provide benefits for the infant beyond traditional nutrients.<sup>1</sup> Human milk is associated with the reduction of incidence and severity of infections and related morbidity and mortality in infancy.<sup>2</sup> It contains growth factors and immunological components that are well balanced with respect to the specific needs of the neonate during its developmental stages. Other advantages of human milk include possible enhancement of cognitive development, prevention of obesity and hypertension, and support of immune maturation associated with prevention of allergies and insulin-dependent diabetes mellitus.<sup>3,4</sup>

Human milk oligosaccharides (HMOs) are an important fraction in human milk, consisting of neutral and acidic oligosaccharides. A broad range of functions have been attributed to HMOs, both locally in the gut lumen and systemically after absorption. Locally, HMOs can act as a component of human milk, supporting innate immunity by preventing attachment of potential pathogens to the intestinal lining. Furthermore, they can function as a prebiotic, which is defined as selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.<sup>5-8</sup>

Neutral oligosaccharides, which include galacto-oligosaccharides and fructo-oligosaccharides, are already used in fortified infant formula. Studies to investigate their effects on health showed, among other things, modulation of microbial composition, with stimulation of bifidobacteria and lactobacilli, and a reduction in the

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incidence of atopic dermatitis in infants.<sup>9,10</sup> Acidic oligosaccharides, such as sialyllactose (SL), have not been added to infant formula to date. Since they, too, may contribute important health benefits, they have recently received increased attention. Because of their potential beneficial effects, it is relevant to gain an overview of the functional role of the different types of oligosaccharides. Several papers have reviewed the benefits of HMOs in general (including, but not limited to, those cited here<sup>11–14</sup>). This review specifically focuses on the current state of knowledge on the functional role and underlying mechanisms of the acidic oligosaccharides SL and other dietary sialylated milk oligosaccharides. These oligosaccharides are important nutrients for, e.g., supporting resistance against pathogens, gut maturation, and cognitive development in humans, and in particular in infants.

## COMPOSITION OF HUMAN MILK OLIGOSACCHARIDES

Unique among mammalian milks, human milk contains a tremendous diversity of oligosaccharide structures.<sup>15</sup> More than 100 different HMOs have been identified, which can be separated into neutral and acidic fractions, mainly depending on the presence of one or more residues of, respectively, fucose or sialic acid (SA).<sup>11,13,16</sup> After lactose and lipids, HMOs are the third most predominant component in human milk.<sup>17,18</sup> HMO molecules are synthesized in the mammary glands, starting with lactose at the reducing end.<sup>11</sup> The core molecule is generally characterized by repetitive attachment of galactose (Gal) and *N*-acetylglucosamine (GlcNAc) via  $\beta$ -glycosidic linkage to lactose. Whereas the structure of the core molecule already gives rise to a wide range of different molecules, the structural and chemical variety becomes even greater because of  $\alpha$ -glycosidic linkages of fucose (neutral oligosaccharides) and/or SA (acidic oligosaccharides) to the respective core molecules.<sup>12</sup> As a result, approximately 50–70% and 10–30% of HMOs are fucosylated or sialylated, respectively, and less than 10% are neither fucosylated nor sialylated.<sup>19</sup> The exact composition of fucosylated and sialylated oligosaccharides depends, among other things, on the secretor status of the mother, which is closely associated with the blood group status.<sup>20</sup> Although the relative quantities of oligosaccharides differ, both fucosylated and sialylated oligosaccharides can be present in milk from a variety of mammals.<sup>15</sup> Until recently, it was thought that bovine milk mainly contains sialylated oligosaccharides but lacks fucosylated oligosaccharides.<sup>21</sup> Recent studies using sensitive and advanced mass spectrometric technology showed that fucosylated oligosaccharides, such as 2'- and 3'-fucosyllactose, can be detected in bovine milk.<sup>22,23</sup> However, from the total pool of oligosaccharides, less

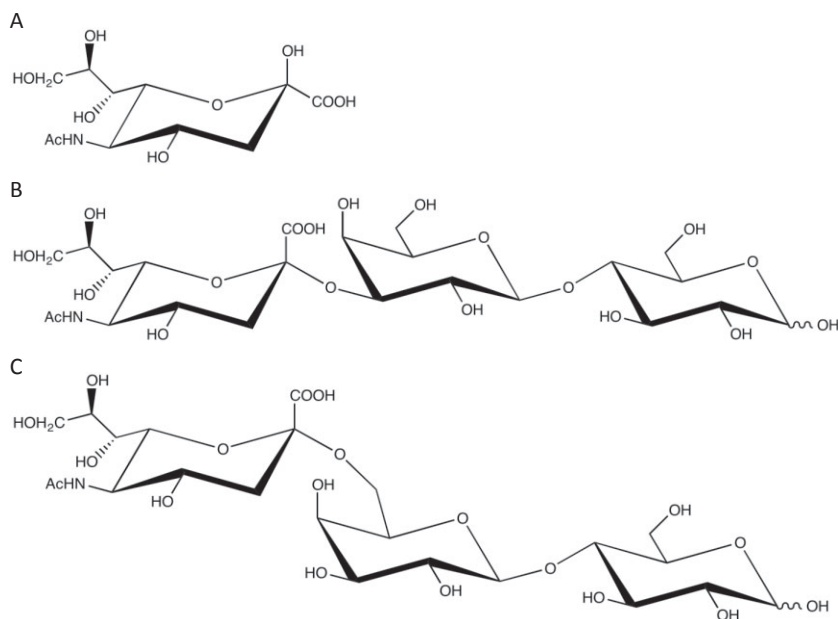
than 1% were fucosylated, indicating that the levels of fucosylated oligosaccharides are very low, whereas almost 70% were sialylated.<sup>21,23</sup> This may explain why less advanced methodologies sometimes fail to detect bovine fucosylated oligosaccharides.

SA is the generic term for the family of *N*- and *O*-substituted derivatives of neuraminic acid, a 9-carbon acidic sugar molecule. More than 50 naturally occurring derivatives of SA have been described.<sup>24</sup> *N*-acetylneuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid, abbreviated as Neu5Ac or NANA) is the most widespread form and usually the only form found in humans (Figure 1). SA rarely occurs free in nature but terminates oligosaccharide side chains at the nonreducing end of Gal or *N*-acetylgalactosamine (GalNAc) of, for example, mucins, glycoproteins, and glycolipids and therefore mainly functions in cell membranes.<sup>25</sup> The trisaccharide SL consists of lactose at the reducing terminus and an SA residue at the nonreducing end via an  $\alpha$ -2,3 binding or  $\alpha$ -2,6 binding, resulting in, respectively, 3'-SL and 6'-SL (Figure 1).<sup>24</sup>

## HUMAN MILK VERSUS BOVINE MILK

Human milk and bovine milk differ in composition with respect to the amount and type of glycoproteins and oligosaccharides. Human  $\kappa$ -casein, the major glycoprotein in human milk, contains more carbohydrate by weight (40–60%) than bovine  $\kappa$ -casein (10%).<sup>26</sup> Furthermore, the oligosaccharide moiety of human  $\kappa$ -casein is rich in SA compared with that of bovine  $\kappa$ -casein. Human milk is also a rich source of a large variety of free oligosaccharides, not only in colostrum (13.3–23 g/L) but also in mature milk (3.5–14 g/L).<sup>13,18,27–29</sup> Since the exact composition of the oligosaccharide fraction in human milk is variable, data reported in the literature vary strongly; however, the oligosaccharide content of bovine milk is reported to be lower (Table 1).<sup>40</sup> The most abundant sialylated oligosaccharides in human milk are 6'-SL and 3'-SL, disialyllactose-*N*-tetraose (DSLNT), and sialyllacto-*N*-tetraose (LNT). Whereas 6'-SL is the major SL in human milk, 3'-SL is predominant in bovine milk.<sup>32</sup>

Since concentrations of oligosaccharides in bovine milk are much lower than those in human milk, unsupplemented infant formulas derived from bovine milk have a much lower content of glycoproteins and oligosaccharides. Most (69–82%) of the SA in human milk is bound to oligosaccharides. A smaller fraction (15–28%) is bound to glycoproteins, whereas only 2–3% is present in free form.<sup>33,34</sup> Commercial infant formulas have been shown to contain a much lower SA content, and the SA is bound primarily to glycoproteins.<sup>33</sup>



**Figure 1** Structure of (A) *N*-acetylneuraminic acid (Neu5Ac or NANA), the predominant sialic acid found in mammalian cells, and (B) 3'-sialyllactose and (C) 6'-sialyllactose, the most abundant sialylated oligosaccharides in human milk.

### SIALIC ACID AND SIALYLATED MILK OLIGOSACCHARIDES DURING PREGNANCY AND LACTATION

All mammals, including humans, can synthesize SA *de novo*. However, it is speculated that, during the neonatal suckling period, *de novo* SA production is insufficient to meet the needs of all tissues in the rapidly developing newborn and that SA is a conditionally essential nutrient for the suckling neonate.<sup>24,33</sup>

Pregnancy is associated with an increase in concentration of SA in maternal saliva and plasma. SA in saliva increases from 50 mg/L at 10 weeks of gestation to up to 150 mg/L at 20–40 weeks of gestation, corresponding to a period of rapid accumulation of SA in the fetal brain.<sup>24</sup> This suggests that the mother synthesizes much of the SA, which crosses the placenta to contribute to fetal brain growth in the third trimester.<sup>24,46</sup> This fits with observations in mothers of preterm infants. Breast milk from these mothers has 13–23% more SA than milk from mothers of full-term infants at most lactation stages.<sup>33,47</sup> This may reflect the increased plasma and saliva SA levels observed in mothers during pregnancy, which, under normal conditions, contribute to growth and development of the unborn child. Maternal dietary intake may influence the concentrations of sialylated glycoconjugates in human plasma, breast milk, and other secretions and tissues, but to date it is unknown how these are related.<sup>24</sup>

Both the amount and the composition of oligosaccharides in human milk vary with the infant's gestation, the genetic makeup of the mother, the duration of lactation, and even diurnally. Total HMO content and the concentrations of SA (bound to oligosaccharides, proteins, and fat) are both high in milk during early lactation but decrease toward weaning.<sup>48</sup> SA content of human milk decreases from 4.85 mmol/L on day 3–5 postpartum to 1 mmol/L at 4–8 months (corresponding to 1,500 mg/L and 300 mg/L, respectively).<sup>33</sup>

The natural oligosaccharide profile in humans is related to the individual's ABH and Lewis blood group and secretor status and, in women, is reflected in the SA content of breast milk. Milk of women with blood group (H+)Le(a+b+) contains the highest levels of sialylated oligosaccharide, whereas milk of women with blood group ABH(–)Le(a+b–) contains the lowest levels.<sup>49</sup> Whether this blood-group-associated variation in the levels of HMOs affects a child's predisposition to certain infectious diseases or brain development and function *in vivo* is largely unknown at present,<sup>14,50</sup> but there are studies indicating that it is relevant and should be considered when dietary intervention studies are designed.<sup>7</sup> SA appears to be one of the most variable fractions of human milk, as this acidic component can vary by a factor 3 between mothers at the same stage of lactation.<sup>14,46</sup> After delivery, total NANA and 6'-SL concentrations in human milk decrease with time, whereas the concentration of 3'-SL remains fairly stable.<sup>17,28,51</sup>

**Table 1 Total and sialylated oligosaccharides in human milk, bovine milk, and infant formulas based on cow's milk.**

Oligosaccharides	Human colostrum (<7 days)	Mature human milk (30–120 days)	Bovine colostrum	Mature bovine milk	Infant formulas
Total oligosaccharides <sup>27–31</sup>	13,300–23,000 mg/L	3,500–14,000 mg/L	~1,000 mg/L	300–500 mg/L	400–8,000 mg/L <sup>a</sup>
Sialylated (acidic) oligosaccharides <sup>6,9,14,18,30,32–39</sup>	1,000–3,300 mg/L	135–2,150 mg/L	230–1,500 mg/L	10–195 mg/L	14–288 mg/L
6'-sialyllactose <sup>18,32,39–43</sup>	250–1,300 mg/L	170–500 mg/L	30–243 mg/L	17–88 mg/L	3.8–4.6 mg/L
3'-sialyllactose <sup>18,20,32,39–45</sup>	90–350 mg/L	76–300 mg/L	354–1,250 mg/L	30–119 mg/L	17–19 mg/L

<sup>a</sup> Products include formulas supplemented with galacto-oligosaccharides and/or fructo-oligosaccharides.

## DIGESTION OF SIALYLATED OLIGOSACCHARIDES IN THE UPPER GASTROINTESTINAL TRACT

It is generally accepted that most of the oligosaccharides resist the pH of the stomach in infants; they are resistant to enzymatic hydrolysis in the small intestine and are thus largely undigested and unabsorbed.<sup>52</sup> Therefore, it is likely that most oligosaccharides will pass through the intestinal tract and enter the colon intact.<sup>52,53</sup>

Indeed, in a model mimicking the physiological pH of the gastric fluid of the infant's stomach, Gnoth et al.<sup>54</sup> demonstrated that acidic oligosaccharides, including SL, show minor changes in their structure. Furthermore, it has been demonstrated that a mixture of SL and sialyllactitol is not hydrolyzed during retention in the stomach.<sup>55</sup>

In vitro experiments have shown that milk oligosaccharides, including their acidic fraction, are also highly resistant to hydrolysis by human salivary, pig pancreatic, and intestinal brush border enzymes.<sup>54,56</sup> This is because most enzymes present in the gastrointestinal tract are not capable of cleaving fucose, *N*-acetylneuraminic acid (Neu5Ac), or *N*-acetylglucosamine (GlcNAc) from oligosaccharides and glycoconjugates.<sup>54</sup> Although SL is highly resistant to hydrolysis by most enzymes in the intestinal mucosa of humans and rats, SL is hydrolyzed to some extent by mucosal sialidases. The intestinal mucosal sialidase activity seems to correlate with milk SA content, at least in the suckling period of rats, mice, rabbits, cats, and guinea pigs.<sup>57</sup> It has been shown in infant rats that sialidases are not located in the brush border, and are not secreted, but are probably of lysosomal origin.<sup>31</sup> Therefore, the SL of rat milk can be hydrolyzed only if absorbed by the enterocytes. Upon hydrolysis of SL, SA may be released as a nutrient for neonatal tissue and organ development. Whether SL hydrolysis occurs in a similar way in human infants is not known.

Still, the majority of HMOs seem to reach the colon, where they are available for fermentation by the microbiota, and as much as 40–50% may pass unaltered into the feces.<sup>17,58–60</sup>

## ABSORPTION OF SIALYLLACTOSE AND SIALYLATED MILK OLIGOSACCHARIDES

A small fraction of milk oligosaccharides, including SL, is absorbed (partly intact) by the paracellular route, transported via blood, and excreted in urine.<sup>61</sup> In vitro experiments using Caco-2 intestinal epithelial cell lines showed that acidic oligosaccharides, including 6'-SL, pass via the nonspecific paracellular route only, whereas neutral oligosaccharides can pass by either the paracellular or the (receptor-mediated) transcellular route.<sup>61</sup> Moreover, dietary SA in free form can be taken up by the distal small

intestine by plasma membrane endocytosis/pinocytosis. A recent study in rats showed that, from the HMOs fed to rat pups, only 3'-SL was absorbed and detected in serum and urine.<sup>62</sup> This is in contrast to findings in human studies, where the HMOs detected in urine of infants are more diverse.<sup>63</sup> This may be related to the fact that the oligosaccharides in human milk are more diverse than those in rat milk, which contains mainly 3'-SL.<sup>15</sup> In fasted mice, it was shown that 50% of orally administered (<sup>14</sup>C-labelled) SL was excreted unchanged in urine within 24 hours.<sup>48,55</sup> Furthermore, after 24 hours, only 1% of the labelled SL was still detectable in the body, indicating that only a minor fraction is metabolized upon absorption.<sup>55</sup> In infant urine, HMOs are detectable in small amounts, in a range of 50–500 mg/day, which corresponds to less than 10% of the daily HMO intake.<sup>63,64</sup> This suggests that a larger fraction of the HMOs is absorbed in humans than in mice. HMOs are also detectable in the urine of the mother (500–800 mg/day).<sup>65</sup> The excretion of oligosaccharides in the mother's urine during lactation suggests that the oligosaccharides synthesized by the mother not only enter the breast milk but also become available systemically as well, as reflected by urinary excretion. This has been suggested to protect the mother against urinary tract infections.<sup>64</sup> In a human study, oral ingestion of <sup>13</sup>C-labeled galactose by a lactating woman resulted in 7% of the label ending up in the milk in the form of lactose and neutral oligosaccharides after 16 hours. After breastfeeding, about 1% of the label derived from this labeled human milk was detectable in the urine of the breastfed child.<sup>66</sup>

SA is detectable in various organs, including the brain,<sup>24</sup> and is found in human milk, plasma, and urine. Moreover, SA is present in many other body fluids, including saliva, gastric juice, and tears, in the form of glycoproteins or as terminal sugars of oligosaccharide chains of mucins.<sup>25</sup> Both the bound and free SA content of saliva is higher in breastfed infants than in bottle-fed infants.<sup>14,67</sup> This suggests that sialylated oligosaccharides present in breast milk may act as a source of SA for the newborn.

## POTENTIAL EFFECTS OF SIALYLACTOSE AND SIALYLATED OLIGOSACCHARIDES

This section provides an overview of the potential health effects and mechanisms of SL and sialylated oligosaccharides. Figure 2 summarizes this overview.

### Development of the microbiota

The gut microbiota during early infancy is relatively simple but highly dynamic. Incidental exposures (e.g., method of delivery, maternal microbiota, breastfeeding versus formula, environmental exposure) play a major role

in colonization of the neonatal gut.<sup>68</sup> HMOs are able to shape the composition of neonatal gastrointestinal microbial communities.<sup>48</sup> Different HMOs are differently fermented by gut microbiota, as was recently shown for breastfed and formula-fed infants.<sup>69</sup> Among the main products resulting from fermentation of oligosaccharides by bacteria present in the gut are short-chain fatty acids (SCFA). These are produced in varying amounts, depending on the diet and on the composition of the intestinal microbiota.<sup>70</sup>

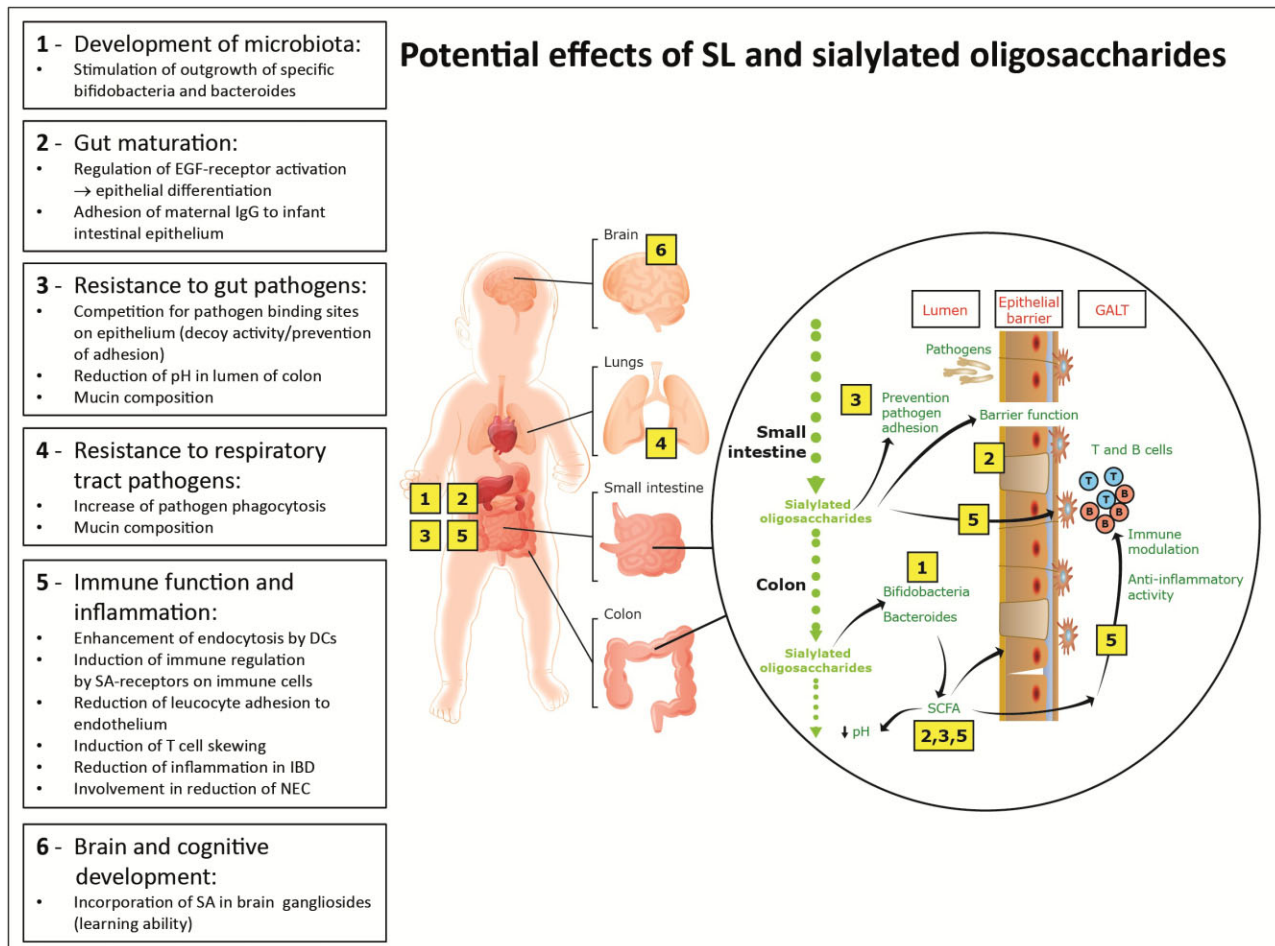
It is well accepted that the gut microbiota and SCFA play a key role in the maturation of the intestine and act as a barrier against pathogens. SCFA also influence nutrient absorption, host metabolism (amino acid, lipid, antioxidant and drug), and immune development and response.<sup>71</sup> In addition, they are considered anti-diarrheal agents, since the uptake of SCFA coincides with the absorption of sodium and water.<sup>72</sup> Furthermore, SCFA have been associated with a reduced risk of diseases, including cardiovascular disease, cancer, and inflammatory bowel disease.<sup>73</sup>

In vitro, bifidobacteria and *Bacteroides* strains are able to grow on HMOs as the sole carbon source.<sup>74,75</sup> Recently, evaluation of the growth and metabolism of various microbiota species in the presence of fucosylated or sialylated HMOs showed that specific species preferentially digest specific HMOs.<sup>76,77</sup> This may subsequently protect the newborn against infection by pathogens<sup>78,79</sup> and has been associated with the development and maturation of the immune system.<sup>80</sup> Bifidobacteria and *Bifidobacterium longum* strains often predominate the colonic microbiota of exclusively breastfed infants.<sup>81</sup> Of the three subspecies of *B. longum* (*infantis*, *longum*, and *suis*), only *B. longum* subsp. *infantis* grows robustly on HMOs.<sup>82</sup> It has two sialidases that predominantly cleave  $\alpha$ -2,6 linkages.<sup>74,83</sup> SL has been shown to support not only the growth of *B. longum* subsp. *infantis* but also its adhesion to intestinal epithelial cells, which may be involved in intestinal colonization.<sup>84</sup> Moreover, *Bifidobacterium infantis*, a bifidobacterium present mainly in infants, preferentially consumes small HMOs and possesses fucosidase and sialidase activities not present in several other bifidobacteria strains.<sup>74,82</sup> A recent in vitro study showed that *Bifidobacterium bifidum* expresses an *exo*- $\alpha$ -sialidase that is capable of liberating SA from sialo-oligosaccharides, gangliosides, mucins, and glycoproteins.<sup>85</sup> In contrast, bifidobacteria that are present mainly in adults, such as *B. longum* subsp. *longum* and *Bifidobacterium adolescentis*, are not able to ferment SAs.<sup>74</sup>

### Gut maturation

In early life, maternal antibodies are important for protection of the infant against infections. Maternal immu-





**Figure 2 Potential effects and mechanisms of SL and sialylated oligosaccharides.**

**Abbreviations:** DCs, dendritic cells; EGF, epidermal growth factor; GALT, gut-associated lymphoid tissue; IBD, inflammatory bowel disease; IgG, immunoglobulin G; NEC, necrotizing enterocolitis; SA, sialic acid; SCFA, short-chain fatty acids; SL, sialyllactose.

noglobulin G (IgG) antibodies are taken up in the intestine via specific receptors, such as FcRn.<sup>86</sup> In rat neonates, SA on the surface of the intestinal microvilli has been shown to enhance the binding of IgG antibodies to the epithelium.<sup>87</sup> Towards the time of weaning, after which maternal IgG is no longer available, the expression of SA decreased, explained by a decrease in  $\alpha$ -2,6-sialyltransferase activity.<sup>88,89</sup>

Recent in vitro studies suggest that acidic milk oligosaccharides may inhibit intestinal epithelial cell proliferation and induce cell differentiation.<sup>90,91</sup> This effect is mediated via interaction of the oligosaccharides with carbohydrate moieties on the epidermal growth factor (EGF) receptor, thereby regulating activation of this receptor.<sup>91</sup> In addition to oligosaccharides, breast milk is also an important source of EGF in neonates.<sup>92</sup> Via modulation of EGF receptor signaling, EGF and oligosaccharides from breast milk may promote intestinal maturation in early life.

### Resistance to gut pathogens

Diarrheal disease is the second leading cause of death in children under 5 years of age and is responsible for killing 1.4 million children every year, mainly in developing countries.<sup>93</sup> During the first years of life, most enteric infections and diarrheal diseases may be attributed to infection with enteric pathogens, including *Escherichia coli* (enterotoxigenic *E. coli* and enteropathogenic *E. coli*), rotavirus, *Campylobacter*, and *Salmonella*.<sup>68,94</sup> Rates of morbidity and mortality due to diarrhea are lower in breastfed infants than in formula-fed infants. This has been attributed primarily to the secretory antibodies and prebiotic factors in human milk.<sup>7</sup> The ability of HMOs to protect against infectious agents may result, in part, from the effects of HMOs on the gut microbiota, but it is thought to be due primarily to their inhibitory (decoy) effect on pathogen binding to host cells in the small intestine.<sup>7</sup>

SA, present as glycoconjugates on bacterial surfaces as well as on host cell membranes, has multiple substitutional binding sites. This enables specific binding to adjacent molecules through distinct glycosidic linkages.<sup>95</sup> By competing for these binding sites, SA components, including 3'-SL and 6'-SL, can prevent or reduce in vitro adhesion of pathogens such as *Salmonella*,<sup>96–98</sup> various *E. coli* strains (S-fimbriated strains in particular),<sup>96,98–101</sup> *Vibrio cholerae*, *Helicobacter pylori*,<sup>98,102–105</sup> *Campylobacter jejuni*,<sup>98,106</sup> and rotavirus.<sup>107,108</sup>

Infection studies in mice showed anti-infective effects of SA components against *H. pylori* (SA)<sup>109</sup> and rotavirus (sialyl lipids, SA).<sup>7,53,108,110,111</sup> SL was shown to prevent cholera toxin binding and its sequelae in rabbit ileal loops.<sup>112,113</sup> This was shown to be specific to SL and was not mediated by SA or lactose.<sup>112</sup> Furthermore, SL promoted eradication of *H. pylori* in rhesus monkeys.<sup>114</sup> Recently, a mixture of SA and SL was shown to reduce rotavirus binding and infection in vitro, whereas both acidic and neutral HMOs were able to reduce rotavirus replication in situ in pigs.<sup>115</sup>

A study in infants in Mexico showed that lactadherin levels in breast milk were associated with protection against rotavirus in infants.<sup>110</sup> Lactadherin contains an N-linked carbohydrate moiety that includes SA.<sup>110</sup> The inhibitory effect of lactadherin on rotavirus replication in vitro and in experimental rotaviral-induced gastroenteritis depended on the presence of SA.<sup>111</sup> In a study with a small number of adult human subjects infected with *H. pylori* (a pathogen occurring in the stomach), 1-day oral treatment with 3'-SL was ineffective in reducing the number of *H. pylori*.<sup>116</sup> Supplementation with 3'-SL for 4 weeks in a larger trial was also ineffective.<sup>117</sup> Furthermore, oral supplementation with 3'-SL (doses of 1–5 g per day) for several weeks did not change Lewis antigen expression of *H. pylori* strains isolated from human gastric mucosa.<sup>118</sup> The lack of a protective effect of SL against *H. pylori* infection may be due to the strong mucus layer in the stomach, which covers the bacteria and prevents access of SL to the pathogen. To date, the effect of SL on gut infections in humans has been studied only for *H. pylori* infection, and not for other gut infections.

So far, evidence of the protective effect of SL and sialylated oligosaccharides against gut infections has been obtained mainly from in vitro studies, since data from animal models and humans is limited.

### Resistance to respiratory tract pathogens

Acute lower respiratory tract infection (pneumonia), which in children is due mostly to bacterial infection, is one of the leading causes of child mortality in developing countries.<sup>119</sup> *Streptococcus pneumoniae* is a leading cause of bacterial pneumonia, meningitis, and sepsis

in children worldwide.<sup>120</sup> Viral lower respiratory tract infections are mild and self-limiting in most cases. Worldwide, the respiratory syncytial virus is by far the most common cause of viral lower respiratory tract infections in infants and young children, followed by influenza viruses.<sup>121</sup>

Exclusive breastfeeding for 3 or more months is associated with a lower risk of hospital admission for respiratory tract infections in the first 6 months of life.<sup>122</sup> This effect might be attributable to HMOs.<sup>6</sup> Absorption of milk oligosaccharides by breastfed infants may provide an adequate source of SA, which can be incorporated in, for example, mucins in the respiratory tract. The influenza virus initiates infection by attachment to host cells, which is followed by endocytosis and fusion of the viral and endosomal membranes. Attachment is mediated by the interaction of the viral surface glycoprotein hemagglutinin, with host cell surface receptors containing sialylated oligosaccharides.<sup>123</sup> An extracellular agent that resembles the host receptors may inhibit this binding. Indeed, intranasal inoculation of an SA bound to a polymeric compound (6-sialyl-N-acetylglucosamine) reduced disease symptoms and decreased mortality in influenza-infected mice.<sup>123</sup> In contrast, another study showed that intranasally administered soluble 3'-SL worsened influenza infection outcomes, as it decreased mouse survival and increased the lung inflammatory response.<sup>124</sup> This was shown to be due primarily to interference with phagocytosis of infected lung cells. To limit influenza infection, virus-infected cells become apoptotic and are phagocytosed by macrophages. Phagocytosis depends on desialylation of sugar moieties on the macrophage cell membrane by viral neuraminidase. The data suggested that SL interfered with this desialylation and thereby reduced phagocytosis, leading to reduced elimination of the virus.<sup>125</sup> The discrepancy between these studies may be explained by the fact that the effect of SA compounds on viral respiratory tract infection is dependent on the type of compound provided. The interaction between viral hemagglutinin and SA on host cells is a low-affinity binding. By involving multiple binding sites, the overall multivalent binding provides a high-avidity binding. Therefore, polymeric SA compounds may be more effective than monovalent compounds. Indeed, a large study in children aged 10–24 months who had been given intranasal monovalent 3'-sialyllacto-N-neotetraose found no effect on either nasopharyngeal carriage of *Haemophilus influenzae* or prevention of otitis media infections.<sup>126</sup> In contrast, polymeric SL was shown to inhibit adenovirus binding and infection of human corneal epithelial cells in vitro. This effect was stronger with increasing multivalency.<sup>127</sup> Furthermore, a recent study that used 3'-sialyllacto-N-neotetraose in liposomes, thereby providing polyvalent binding sites, showed that it

is efficient in preventing infection *in vitro* in cell lines and *in vivo* in a mouse model.<sup>128</sup>

In human milk, most SA-containing compounds are monomeric (such as 3'-SL and 6'-SL). Some dimeric (oligosaccharides such as disialyllacto-*N*-tetraose or disialyllacto-*N*-fucopentaose, or glycolipids such as disialogangliosides) or trimeric (e.g., trisialyllacto-*N*-hexaose) compounds have also been identified.<sup>13,129</sup> However, little is known about the difference in the potential of these mono-, di- or trimeric compounds to prevent infection. Glycoproteins with up to 18 SA residues have been detected in human milk. One of these was further characterized as CD36. CD36 as present in breast milk was shown to contain at least 18 SA residues per molecule. In contrast, CD36, when present in blood platelets, was not polysialylated, suggesting that the multiple SA residues in CD36 are specific for CD36 in milk.<sup>130</sup> This glycoprotein was suggested to be important for reduction of pathogen invasion, but supporting data are missing.

Overall, *in vivo* human evidence of the positive effect of oral SL and sialylated oligosaccharides on resistance against respiratory infections is limited. The data suggest that polyvalent compounds containing SA are more effective than monovalent structures, which may explain discrepancies between studies. Only intranasal administration has been studied, which is a completely different approach from the potential application of these compounds in infant formulas. So far, no studies have been carried out in young infants. Furthermore, viral receptor binding is highly dependent on the virus strain, host species, and tissue, making extrapolation of research findings difficult.<sup>131-133</sup>

### Immune function and inflammation

SA also plays an important role in immune function and regulation. Inflammatory diseases can develop in early infancy. Prevention or reduction of early inflammation may prevent the subsequent development of disease. 3'-SL has anti-inflammatory properties, shown by the reduced mRNA levels of proinflammatory cytokines, such as IL-8 and TNF- $\alpha$ , in Caco-2 cells. This effect was mediated via enhancement of the expression of peptidoglycan recognition protein 3 (PGlyRP3).<sup>134</sup> PGlyRP3 is a pathogen recognition receptor, shown previously to regulate inflammatory responses *in vitro*.<sup>135</sup>

Prebiotic oligosaccharides can be transported across the epithelium, as shown *in vitro*, which suggests that they can reach the immune cells that are present in the epithelial layer.<sup>136</sup> Direct *in vitro* exposure of peripheral blood mononuclear cells from pigs to various HMOs was shown to alter proliferation and to increase IL-10 production.<sup>137</sup> Direct exposure of human naïve cord blood mononuclear cells to acidic oligosaccharides was shown to skew the

cytokine response towards production of IL-10 and interferon- $\gamma$  (IFN- $\gamma$ ), without induction of interleukin (IL)-13, which is considered a Th1-polarized regulatory immune response that may suppress the induction of Th2 responses associated with allergy.<sup>136</sup> Indeed, when peripheral blood mononuclear cells from peanut-allergic patients were stimulated with peanut allergen in the presence of these oligosaccharides, IFN- $\gamma$  increased and IL-4 decreased, which is a more Th1-skewed response.<sup>136</sup> These cytokine profiles suggest that oligosaccharides may be able to reverse an imbalanced immune response towards a balanced response, at least *in vitro*.

SA is important for the dampening of immune responses via IgG. IgG can have either pro- or anti-inflammatory effects. The anti-inflammatory effects have been shown to be mediated via sialylation of IgG. Sialylated IgG binds to inhibitory rather than activating IgG receptors on dendritic cells (DCs), which results in downregulation of the immune response.<sup>138</sup>

SA is also present on the surface of monocytes and DCs as part of glycan structures and seems to be involved in the regulation of endocytosis and immune activation. During DC maturation, the SA composition on the cell surface changes, resulting in an enhanced capacity for bacterial endocytosis and induction of a proinflammatory T-cell response.<sup>139,140</sup> Early T-cell activation has been shown to result in increased SA content on the T-cell surface.<sup>141</sup> This surface SA is involved in the interaction of T cells with antigen-presenting cells (e.g., DCs, B cells), which express specific receptors for SA, such as SA-binding immunoglobulin-like lectins (Siglecs).<sup>142</sup> Many Siglecs have inhibitory effects on immune cells and are therefore believed to be important for immune regulation.<sup>143</sup>

Together, these data show that SA is an important building block for adequate immune function. This underlines the importance of adequate SA availability in infancy, when proper development of the immune system is important for the prevention of diseases or to combat infections.

In humans, inflammatory bowel disease (Crohn's disease and ulcerative colitis) develops mainly in adulthood (75% of the patients) but can also begin during childhood or adolescence (up to 25%).<sup>144</sup> Fuhrer et al.<sup>145</sup> showed that exposure of infant mice to 3'-SL increased susceptibility to colitis at adult age, whereas exposure to 6'-SL did not. This was associated with a change in intestinal bacterial colonization and not with altered immune maturation.<sup>145,146</sup> The proinflammatory effect of 3'-SL in this colitis model involved direct stimulation of DCs in the mesenteric lymph nodes via Toll-like receptor 4, resulting in the expansion of Th1 and Th17 cells and production of proinflammatory cytokines.<sup>147</sup> In another study, however, breast milk was suggested to have a pro-



tective effect against the development of early-onset pediatric inflammatory bowel disease.<sup>148</sup> Although the mechanism of this effect is not clear, it may suggest that either different compounds in breast milk are involved, or that early exposure to SL has different effects on inflammatory processes in infancy and adulthood. Furthermore, differences in the relative amounts of 3'-SL and 6'-SL in breast milk of different species (humans or animals) may play a role.

Preterm delivery is known to increase the risk of the development of necrotizing enterocolitis (NEC) in infants. It has been shown that the level of various milk oligosaccharides is more diverse in mothers who delivered preterm and that these levels do not normalize during lactation. This may be related to the enhanced susceptibility of the preterm neonate to NEC.<sup>149</sup> In a rat model of NEC, sialylated oligosaccharides were able to prevent NEC and reduce the pathology. SA was required for this effect.<sup>150</sup> Remarkably, only one specific dimeric SA structure, disialyllacto-*N*-tetraose, and not sialylated oligosaccharides containing one, three, or four SA groups, was effective, indicating that the exact structure is important for the protective effect.<sup>150</sup>

Together, these studies show that *in vivo* evidence of the ability of sialylated oligosaccharides to intervene in inflammatory diseases or to actively modulate immune responses is still limited, and mechanisms underlying the regulatory action of oligosaccharides remain largely unknown.

### **Brain and cognitive development**

The highest amount of SA in the body is found in the brain's grey matter, where SA is present in gangliosides and glycoproteins. Sialoglycopeptides are highly concentrated in the synaptosomal fraction and may have a role in cell-to-cell communication. It has even been postulated that SA is the actual receptor for neurotransmitters in the central nervous system. All human brain gangliosides contain NANA as SA.<sup>46</sup>

SA seems to be able to pass the blood-brain barrier.<sup>46,151,152</sup> Breastfed babies have 32% more ganglioside-bound SA and 22% more protein-bound SA in brain tissue when compared with formula-fed babies.<sup>46</sup> Intraperitoneally administered SA in rats resulted in accumulation in brain gangliosides, especially in the synaptosomal fraction.<sup>46,153–157</sup>

Many studies, including meta-analyses, have shown that breastfed infants have biologically significantly higher intelligence scores and better learning abilities, also when results are corrected for socioeconomic confounders.<sup>158</sup> This effect of breastfeeding has not been directly attributed to SA or sialylated oligosaccharides. However, strikingly, several diseases of the brain such as

retardation, psychosis in schizophrenia, brain dysfunction in phenylketonuria, and Alzheimer's disease have been shown to be associated with lower SA content of the brain and/or brain gangliosides.<sup>46</sup> Evidence of potential causal mechanisms related to these observations to date comes from animal studies.

A recent study in rats showed that feeding dietary SA to pregnant and lactating rats but not to their litters did not affect total SA content in the cortex of the rat pups.<sup>159</sup> Another study showed that, when SA was fed to both the mothers and the pups, cortical ganglioside SA content did increase.<sup>160</sup> In addition, SA feeding in aged rats normalized brain SA levels displayed on gangliosides to the levels measured in young rats.<sup>161</sup> Furthermore, in a swimming learning test, rats that had been fed SL or galactosylated SA showed improved learning ability, which was associated with increased SA and ganglioside content of the brain.<sup>162</sup> Dietary SA supplementation in developing piglets exposed to active learning tests resulted in increased learning and memory function, increased sialylated brain proteins, and increased levels of mRNA expression of uridine-diphospho-*N*-acetylglucosamine-2-epimerase, a key enzyme in the biosynthetic pathway of SA, in brain and liver.<sup>24,163</sup> In addition, in rats, feeding SA during pregnancy and lactation improved the recognition index of rat pups.<sup>159</sup> Together, these animal studies suggest that oral SL or SA administration increases brain SA content and can improve learning function. Whether brain dysfunction in later life is related to a shortage of SA or sialylated oligosaccharides in early life remains a matter of speculation. No behavioral studies in humans are yet available to resolve the question.<sup>164</sup>

### **APPLICATION OF OLIGOSACCHARIDES TO FORTIFY INFANT FORMULAS**

Because of the known beneficial effects of HMOs, many attempts have been made to develop infant formulas that beneficially stimulate gut microbial colonization. One of the strategies used to achieve this is the supplementation of prebiotics to infant formula.<sup>165</sup> Examples of prebiotic oligosaccharides that are currently used in infant formulas are galacto-oligosaccharides and fructo-oligosaccharides, which are both examples of neutral oligosaccharides. Several studies in infants have shown that consumption of these structures can exert beneficial effects such as the promotion of a bacterial microbiota dominated by bifidobacteria. However, galacto-oligosaccharides and fructo-oligosaccharides are not sialylated or fucosylated, while the carboxyl group of SA in acidic oligosaccharides such as SL introduces a negative charge that is critical to some of the benefits of HMOs.<sup>12,166</sup> Food ingredients containing SA, for example as part of the milk-derived caseinoglycomacropeptide or

milk fat globule membrane, are on the market. However, in these products, SA is mainly protein bound, rather than being part of oligosaccharides. Because of the important biological functions of SA, and considering that most of the effects of HMOs are highly structure specific, routes are being explored to develop HMO structures, such as SL and other sialylated oligosaccharides, for application in infant formula.<sup>1,167</sup> A number of methods for the development of SL and sialylated oligosaccharides have been studied, among which are isolation and microbial and enzymatic methods.<sup>167–169</sup> As soon as larger amounts of sialylated oligosaccharides become available, human studies can be started to confirm whether SL and sialylated oligosaccharides indeed contribute to the various health aspects described in this review. With the availability of larger amounts of these structures, supplementation of infant formula can become feasible as well.

## CONCLUSION

There is high interest in exploring the functionalities of HMOs, including sialylated oligosaccharides. Sialylated oligosaccharides, including SL, undoubtedly play an important role in a diverse range of health aspects. The most promising health targets for these structures are resistance to infectious disease (especially intestinal infectious disease), the immune function, and neonatal brain development. In addition, they may have effects on gut microbiota, gut maturation, and inflammation. Many initial studies that have addressed these health outcomes show positive effects and pave the way for follow-up research.

So far, evidence of beneficial effects of sialylated oligosaccharides is limited mainly to in vitro and animal studies. While there is a strong need for substantiation of health effects in humans, current research progression is hampered by overall limited availability of (purified) sialylated oligosaccharides, including SL. For similar reasons, these oligosaccharides are currently not used for application in infant formula. SA can be synthesized by most mammals, including humans, but external supplies (e.g., via consumption of sialylated oligosaccharides) are likely needed during periods of high demand, such as neonatal development. Therefore, if further evidence can be generated for the health effects in human infants, sialylated oligosaccharides may be an interesting component for fortification of infant formula in the near future.

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