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Review Article

Oligosaccharides and glycoconjugates of human milk could protect breast-fed infants against norovirus intestinal infections

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Abstract

Background: Noroviruses are, after rotaviruses, the most common cause of nonbacterial intestinal infections in children. It has been observed a lower incidence of norovirus gastroenteritis in breast-fed compared to formula-fed infants. Effective vaccines against noroviruses are not yet available, enhancing the interest of the protective mechanisms elicited by human milk. Human milk contains a wide range of agents that may play a role in the protection of breast-fed babies against different conditions. There is an increasing interest regarding the role of oligosaccharides and glycoconjugates of human milk as bioactive agents that could protect against norovirus intestinal infections in children.

Aim: The aim of our study was to review the current knowledge regarding the involvement of oligosaccharides and glycoconjugates in the protective properties that human milk elicits against norovirus intestinal infections.

Methods: Scientific literature was analyzed using the pubmed.gov web browser. No restrictions to the year of publications were included. The terms searched were norovirus, oligosaccharides and glycoconjugates of human milk.

Results: An outstanding role has been attributed to oligosaccharides and glycoconjugates of human milk in the defense against norovirus. Oligosaccharides and glycoconjugates of human milk show a structural resemblance to histo-blood group antigens that act as receptors of noroviruses in enterocytes. Therefore, they could act as decoy receptors and interfere the binding of noroviruses to their receptors, critical step to allow their infectivity.

Conclusions: Human milk constitutes an unmatched supply of essential protective substances for the infant. Oligosaccharides and glycoconjugates of human milk could be the agents responsible for the protection of breast-fed infants against norovirus intestinal infections.

ABBREVIATIONS

VLPs: Virus-Like Particles; HBGAs: Histo-Blood Group Antigens; Gal: D-Galactose; GlcNAc: N-acetylglucosamine; Fuc: Fucose; NeuAc: N-acetylneuraminic; Le: Lewis; 2'-FL: 2'-Fucosyllactose; 3-FL: 3-Fucosyllactose; LD: Lacto Difucotetraose; **LNT**: Lacto-N-tetraose; **LNnT**: Lacto-N-neotetraose; LNF-I: Lacto-N-Fucopentaose I; LNF-II: Lacto-N-Fucopentaose II; LND-I: Lacto-N-Difucohexaose I; LND-II: Lacto-N-Difucohexaose II; 3'-SL: 3'-Syalillactose; 6'-SL: 6'-Syalillactose; FUT: Fucosyltransferase; HMOs: Human Milk Oligosaccharides; Se: Secretor; ELISA: Enzyme-Linked Immunosorbent Assay; PGM: Porcine Gastric Mucin; SPRi: Surface Plasmon Resonance Imaging

INTRODUCTION

Noroviruses, after rotaviruses, are the most common etiology of viral intestinal infections in infants worldwide, [1] with GII.4 being the predominant genotype [2]. The relative frequency of noroviruses is increasing due to the widespread implementation of rotavirus vaccines in many countries [3]. The immune response against norovirus infections radically differs from that of other enteric viruses. Humans show a high susceptibility to norovirus infections, triggering a rapid and intense production of IgM and IgG antibodies in > 80% of exposed individuals [4] and conferring a strong, but short, highly genotype-specific protection [5,6]. The production of recombinant norovirus virus-like particles (VLPs) comprises the most promising strategy for the development of safe and effective vaccines to prevent norovirus infections [7,8]. Before obtaining a suitable vaccine for infants, however, there are still many challenges to be solved. First, it is necessary to determine the relationship between immunity and protection, [9] understand the brief duration of the humoral immune response

[4] and achieve the induction of cross-protection [10] against different antigenic and genetic types of noroviruses [11]. Clinical trials have been performed in young adults with preexisting antibodies. Whether this immunologic priming is important for immunization with VLPs will need to be assessed [7]. Therefore, other approaches to prevent norovirus infections in infants must be taken into consideration.

The lower incidence of intestinal infections in breast-fed compared with formula-fed infants suggests that human milk contains protective components [12]. Among the effectors of this property, there are different agents such as IgA antibodies, probiotic factors, lactoferrin, lysozime, mucin, lactadherin, certain anti-inflammatory, antioxidant and immuno modulating agents, oligosaccharides and glycoconjugates of breast milk. Histo-blood group antigens (HBGAs) have been described as constitutional factors involved in susceptibility and resistance mechanisms against noroviruses. These are complex carbohydrates present in the protruding part of glycoproteins or glycolipids on the surface of enterocytes. They are also present as free or conjugated oligosaccharides in human milk and saliva [13]. Human milk oligosaccharides and glycoconjugates could act as decoy receptors avoiding norovirus infections, as described in studies *in vitro* and *in vivo* for rotaviruses [14,15]. Nevertheless, specific HBGAs responsible for this function in noroviruses need further understanding.

Oligosaccharides and glycoconjugates of human milk

Glycans are present in human milk as either free oligosaccharides or glycoconjugates, such as glycoproteins, glycopeptides, glycolipids, glycosaminoglycans or mucins [16]. In the last years, oligosaccharides and glycoconjugates have gained an increasing interest as agents of human milk with protective properties. By means of their ability to inhibit the binding of the pathogens to their specific receptors in intestinal and bronchial host cells they comprise an, up to this moment, underestimated mechanism that may offer an efficacious protection to the lactating infant.

Structural diversity

Most of human milk oligosaccharides (HMOs) are structures that result of the addition of monosaccharides to the molecule of lactose by specific glycosyltransferases of the mammary gland [17]. The combination of D-galactose (Gal) and *N*-acetylglucosamine (GlcNAc) by means of three types of terminal bindings of L-fucose (Fuc): alpha 1, 2, alpha 1, 3 and alpha 1, 4; and two types of syalic acid and *N*-acetylneuraminic acid (NeuAc): alpha 2, 3 and alpha 2,6; generates a wide variety of components. They mainly coincide in the content of lactose in its reductive terminal region and fucose or syalic acid in the non-reductive terminal region. Based on its chemical composition, HMO could be classified in: (a) basic oligosaccharides: that represent the fundamental structures for the synthesis of more complex oligosaccharides comprised by Gal and GlcNAc; (b) fucosyl-oligosaccharides: that result from the addition of this basic structure to one or more molecules of Fuc, (c) sialyl-oligosaccharides: that result of the addition of the basic structure to one or more molecules of NeuAc and (d) sialylfucosyl-oligosaccharides: that contain both Fuc and NeuAc [18]. Glycoconjugates could associate this basic structure to a protein (glycoproteins or glycopeptides), lipid (glycolipids) or carbohydrate (glycosaminoglycans or mucins).

An interesting finding was that some of these oligosaccharides showed haptenic activities of blood group determinants. 2′-FL and LNF-I showed haptenic activity of H determinant, LND-I showed activity of Lewis b (Le^b) determinant, and LNF-II showed activity of Lewis a (Le^a) determinant. These findings of HMOs significantly contributed to the elucidation of the structures of H and Le blood group determinants [19]. In 1967, Grollman and Ginsburg found an interesting fact 2′-FL was not detected in the milk samples obtained from individuals of non-secretor blood type. Non-secretor individuals express ABO blood types on the surface of their erythrocytes according to their genetic background of ABO locus, but not in the glycoproteins secreted from the epithelial cells of mucous glands. In order to extend this interesting finding further, Kobata et al. devised a new technique to fingerprint the oligosaccharides by using small amount of milk samples. Fourteen milk oligosaccharides were successfully fractionated by using approximately 10 ml of milk samples (Table 1). They were: 2 and 3-Fucosyllactose (2' and 3-FL), Lactodifucotetraose (LD), Lacto-N-tetraose (LNT), Lacto-N-neotetraose (LNnT), Lacto-N-fucopentaose I and II (LNF-I and II), Lacto-N-difucohexaose I and II (LND-I and II), 3' and 6'-Syalillactose (3' and 6'-SL), LST-a, b and c. By analyzing around 50 human milk samples, three

Table 1: Structures of human milk oligosaccharides found until 1965.

2'-Fucosyllactose (2'-FL)	Galβ1-4Glc 2 | Fucα1
3-Fucosyllactose (3-FL)	Galβ1-4Glc 3 | Fucα1
Lactodifucotetraose (LD)	Galβ1-4Glc 2 3 | | Fucα1 Fucα1
Lacto-N-tetraose (LNT)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-N-neotetraose (LNnT)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose I (LNF-I)	Galβ1-3GlcNacβ1-3Galβ1-4Glc 2 | Fucα1
Lacto-N-fucopentaose II (LNF II)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 | Fucα1
Lacto-N-difucohexaose I (LND-I)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 2 4 | | Fucα1 Fucα1
Lacto-Ndifucohexaose II (LND-II)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 3 | | Fucα1 Fucα1
3'-Siallylactose (3'-SL)	Galβ1-4Glc 3 | Neu5 Acα2

6'-Siallylactose (6'-SL)	Galβ1-4Glc 6 | Neu5Acα2
LST-a	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 | Neu5Acα2
LST-b	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 | Neu5Acα2
LST-c	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 6 | Neu5Acα2

types of oligosaccharide profiles were found to occur in human milk. Approximately 80% of human milk contained all fourteen oligosaccharides. Approximately 15% of human milk lacked four oligosaccharides: 2′-FL, LD, LNF-I, and LND-I, and remaining 5% lacked three oligosaccharides: LNF-II, LND-I, and LND-II. Important evidence was that all mothers whose milk gave the second profile were non-secretors, and those who gave the third profile were all Le negative, lacking both Le^a and Le^b antigens. The four oligosaccharides: 2′-FL, LD, LNF-I, and LND-I, which were missing in the milk of non-secretor individuals, contain the Fucα1-2Gal group in common. Namely, the secretory organs of non-secretor individuals probably lack the fucosyl transferase (FUT) responsible for the formation of the disaccharide group. The three oligosaccharides: LNF-II, LND-I, and LND-II, which were missing in Le negative individuals, all contain the Fucα1-4GlcNAc group, indicating that these mothers may lack another FUT responsible for the formation of the Fucα1-4GlcNAc group [20]. These estimations were proven by enzymatic studies [21,22].

Therefore, the addition of the residues of fucose depends of the action of FUTs in a genetically determined process: (a) α 1, 2-fucosyltransferase (FUT2) is present in 80% of the Caucasian individuals from northern Europe and America, the so-called secretor (Se) individuals. Milk from Se women is characterized by the presence of 2-Fucosyllactose (Fucα1, 2Galβ1, 4Glc), lacto-*N*-fucopentaose I (Fucα1, 2Galβ1, 3GlcNAcβ1, 3Galβ1, 4Glc) and more complex oligosaccharides, (b) fucosyltranferase 3 (FUT3) is present in approximately 90% of the population, it binds Fuc residues by α 1,4 bindings to GlcNAc residues from chains type 1. In milk from non-secretor women that own the FUT3 gene, the main fucosylated oligosaccharide is the lacto-*N*-fucopentaose II (Galβ1, 3[Fucα1,4] GlcNAcβ1, 3Galβ1, 4Glc). In the case that both genes, FUT2 and FUT3 are present, the most common oligosaccharide would be lacto-*N*-difucohexaose I (Fucα1, 2Galβ1, 3[Fucα1, 4] GlcNAcβ1, 3Galβ1, 4Glc) [23]. Apart from FUTs, several sialyltransferases could bind NeuAc in several positions to oligosaccharides of human milk [24] (Table 2).

Otherwise, blood types A and B antigenic determinants are formed by adding GalNAc residue and Gal residue to H antigenic determinant, respectively (Table 3). Although oligosaccharides containing blood type A and B antigenic determinants were not included among the milk oligosaccharides listed in Table (1) an alpha-N-acetylgalacosaminyltransferase (A-enzyme) and an alpha-galactosyltransferase (B-enzyme) were found to occur in the milk of blood type A and B individuals, respectively. These enzymes catalyze the addition of an alpha-GalNAc residue and an alpha-Gal residue to the C-3 position of the Gal residue of the Fucα1-2Gal group, respectively. GalNAc,1-3(Fuc,1-2)Gal-1-4Glc and GalNAc,1-3(Fuc,1-2)Gal-1-3GlcNAc-1-3Gal-1-4Glc, which were formed respectively from 2′-FL and LNF-I by the catalysis of partially purified A enzyme, showed haptenic activity of blood type A [20].

As described already, structural study of human milk oligosaccharides get started by the interest of their relation to blood group antigens. The development of research as introduced so far and the possibility of important functions of these oligosaccharides aroused the interest of many researchers, and the structures of remaining larger oligosaccharides are being investigated. Currently, there is believed to be hundreds and even thousands of oligosaccharides in human milk, since new molecules are continuously being recognized.

Local effects

Different effects have been attributed to HMOs (prebiotic,

Table 2: Several human milk oligosaccharides with NeuAc.

Sialyl-LNF-II (S-LNF-II)	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 | Fucα1
Sialyl-LNF-I (S-LNF-I)	Neu5Acα2 | 6 Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Disialyl-LNT (DS-LNT)	Neu5Acα2 | 6 Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Disialyl-LNF-II (DS-LNF-II)	Neu5Acα2 | 6 Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 | Fucα1
Disialyl-LNF-V (DS-LNF-V)	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 | Fucα1

Table 3: Structures of ABO blood group antigenic determinants.

H Antigenic determinant	Galβ1-3/4GlcNAcβ1- 2 | Fucα1
A Antigenic determinant	GalNAcα1-3Galβ1-3/4GlcNAcβ1- 2 | Fucα1
B Antigenic determinant	Galα1-3Galβ1-3/4GlcNAcβ1- 2 | Fucα1

immune modulator, nutrition for brain development and antiadhesive antimicrobials). We will focus on the antiadhesive properties of these molecules.

HMOs' expression is especially heterogenous. Heterogeneity of glycan expression in milk implies highly variable protection from disease. However, milks that protect poorly against one pathogen may protect more strongly against another, depending on the overall HMO's expression pattern [25]. The HMO composition mirrors blood group characteristics, which depend on the expression of certain glycosyltransferases. Four milk groups can be assigned based on the Se and Le blood group system, which is determined by the activity of two gene loci encoding for the α1-2-fucoslyltransferase FUT2 (encoded by the Se gene) and the α1-3/4-fucosyltransferase FUT3 (encoded by the Le gene). However, this explanation is an oversimplification of HMOs complexity. For example, FUT2 and FUT3 compete for some of the same substrates and the levels of enzyme expressions and activities create a continuum of HMO profiles throughout the population. Even the milk of Le-negative non-secretor women that express neither FUT2 nor FUT3 contains fucosylated HMO like 3-FL or LNFP III, suggesting that other Se- and Le-independent FUTs (FUT4, 5, 6, 7 or 9) may be involved. In addition, α1-2-fucosylated HMOs have been found in the milk of non-secretor women toward the end of lactation, and it has been suggested that FUT1 may also participate in HMO fucosylation [26].

Oligosaccharides and some glycoconjugates of human milk are synthesized by the same type of glycosyltransferases responsible for the synthesis of the glycans of the cell surfaces: the HBGAs. HBGAs are used by most of the intestinal and respiratory pathogens for the identification and binding to target cells as a critical step for their infectivity. Due to the structural analogy between both molecules, it was stated that HMO could competitively act with cell receptors preventing the binding of certain pathogens and thus avoiding the development of infection. This would imply, in the case of intestinal pathogens, the inhibition of their ability to bind to enterocytes and the protection of the lactating infant from certain intestinal infections and, in the case of respiratory pathogens, the blockade of their binding to bronchial cells and thus the lung infection [27].

Considerable research work has targeted the hypothesis that oligosaccharides and glycoconjugates of human milk could act as decoy receptors that compete with the binding of certain bacterial and viral pathogens, and even certain toxins, to their specific receptors in the intestinal tract and the bronchial tree. In 1983 Parkkinen et al., demonstrated that the salivated oligosaccharides of human milk were able to inhibit the activity of the binding of the strains of *Escherichia coli* that caused neonatal meningitis and sepsis [28]. This is related with the specificity of binding of the *S* fimbria of certain strains of *Escherichia coli* and the sialylated galactosides. It could be speculated that human milk with high content of these epitopes could be responsible of a lower rate of infections by *Escherichia coli* in breastfed *vs*. formula fed infants [29]. Approximately, 70% of the cases of otitis media in neonates are caused by infections by *Streptococcus pneumoniae* and *Haemophillus influenzae.* In 1986, Andersson et al., demonstrated that the adhesion of these microorganisms to specific carbohydrate structures of the pharyngeal or oral epithelial cells were inhibited by human milk [30].

It is possible that certain oligosaccharides and glycoconjugates of human milk could also display the property to interfere with the binding of certain virus to epithelial targets. A good example of this would be Influenza A, B and C viruses that recognize NeuAc α2, 6Lac and NeuAcα2, 3Lac, both principal components of human milk. With regard to intestinal viruses, human milk has demonstrated a protective property against infections by rotavirus, first cause of gastroenteritis in children [31]. Furthermore, it has been recently described that rotavirus recognize HBGAs and oligosaccharides of human milk that interfere between the viral particles and the receptor have been identified [32].Yu et al. stated that although sialic acid has been thought to be important as a surface receptor for RVs, their studies indicated that sialic acid is not required for binding of glycans to individual VP8* domains. Remarkably, each VP8* recognized specific glycan determinants within a unique subset of related glycan structures where specificity differences arise from subtle differences in glycan structures [33].

Liu et al. described that VP8* of a P [11] RV (N155) recognized the infant saliva but not adult's. This fact did not correlate with the ABO, Se and Le HBGAs but with the binding of the lectin Lycopersicon esculentum that is known to recognize the oligomers of Nacetyllactosamine (LacNAc), a precursor of human HBGAs. These results suggested that the poly-LacNAc could serve as an age-specific receptor for P [11] RVs and well explained the epidemiology that P [11] RVs mainly infect neonates and young children. [34] Otherwise, one of the most plausible settings where oligosaccharides could exert a protective action would be preventing from the intestinal infection by norovirus in lactating infants [35].

Protection against norovirus intestinal infections

There is increasing evidence regarding the protective activity of the oligosaccharides and glycoconjugates of human milk against the intestinal infections by norovirus in breastfed infants. This hypothesis starts from the observation that a group of individuals show a natural resistance against the infections by these viruses: the non-secretor individuals. They lack of the FUT2 gene that encodes for the α1, 2-fucosyltransferase. This led to the hypothesis that the epitope α1, 2-Fuc (antigen H type 1) is essential for the binding of norovirus to enterocytes [35]. Accordingly the antigen H type 1 is recognized as the main receptor of Norwalk virus (the prototype of norovirus) in the enterocytes. However, there are other receptors that bind specifically to different strains of norovirus making the interaction of norovirus with the host of a high level of complexity.

The different genetic endowment of the individuals establishes the type of glycans that they express in the surface of their enterocytes and are present in their biological fluids, such as human milk and saliva. It is the case of non-secretor individuals that lack of the gene FUT2 that encodes for α 1, 2-Fuc epitopes. As virus Norwalk requires this receptor for their internalization, these individuals would not be susceptible to this virus. Moreover, the specificities of binding between other strains of norovirus and HBGAs have been described. In consequence, certain genes

might determine the profile of susceptibility or resistance of each individual to the different strains of norovirus.

Therefore, in the case that the genetic endowment and thus the profile of HBGAs on the enterocytes make an infant susceptible to a specific strain of norovirus, HMOs would offer a chance to avoid the intestinal infection. Due to the resemblance between HMOs and the receptors in enterocytes (HBGAs), oligosaccharides could act as decoy receptors preventing the binding of norovirus to their receptors on intestinal cells and thus avoiding the infection. In other words, HMOs could be effectors of the non-specific immunity of human milk against norovirus intestinal infections, as the mother could confer a protection to her child by breastfeeding [36].

Mature milk and serum samples from 108 mothers were assessed for specific IgA to norovirus GII.4-2006b and for their blocking activity on the binding of norovirus VLPs to FUT positive and negative saliva [37]. The results of the blockade of milk on the binding of norovirus GII.4-2006b VLPs to saliva demonstrated that human milk exerts an *in vitro* protective activity against noroviruses. The differences found among non-secretor and Se saliva assays highlight the secretor status (FUT2-negative) of the receptor as a strong predictor of the ability of human milk to inhibit norovirus GII.4-2006b infections. Moreover, the inhibition activity of milk against norovirus infections remained for a wide age range with the same intensity did not vary between individuals of different races and was owned even by mothers that finished their gestations extremely premature. To determine whether specific immune components of human milk can interfere with the binding of norovirus GII.4-2006b VLPs to saliva, norovirus GII.4-2006b-specific IgA titers were determined in mature milk and detected in a higher proportion than described previously. Jiang et al. [12], found milk IgA antibodies against Norwalk virus (35%), VA386 strain (43%) and MOH strain (24%) in secretors, but did not in non-secretors. Also Makita et al. [38], detected IgA against norovirus in 31 (13%) of 239 breast milk samples collected from mothers in Chiba City (Japan) in 1994–1997. IgA to norovirus GII.4 was detected in only 5.9% of the samples, whereas a higher number contained IgA towards GII.6 genotype (11.3%). Differences in the prevalence of milk IgA antibodies to norovirus might be due to different rates of norovirus infections in particular geographic locations. Norovirus-specific IgA titers were found in a significantly lower number of human milk samples than in those that exerted an inhibition activity, suggesting that these antibodies are partly, but not completely, responsible for this effect and other milk components, such as oligosaccharides and glycocconjugates, could be involved in this effect. The specific binding patterns of norovirus variants to HBGAs and the individual variability of HMOs might be able to explain the differences observed among breast milk samples. There has been much evidence offered supporting the highest effect of human milk from Se mothers against norovirus infections. Human milk with a higher content of lacto-N-difucohexaose, a fucosylated oligosaccharide with Le^b as an epitope, is related to a lower incidence of Calicivirus diarrhea in breast-fed infants compared with formula-fed infants [13]. Jiang et al. [12], analyzed the binding inhibition of VA387, Norwalk, VA207 and MOH VLPs to specific receptors by breast milk samples, observing that milk from both Se and non-secretor mothers showed a blocking activity against the binding of VA207 VLPs to saliva, even though only samples from Se mothers interfered in the binding with other norovirus genotypes to saliva.

Over the past decade, GII.4 genotype has been the dominant cause of outbreaks worldwide. However, recent molecular epidemiological studies have speculated that GII.17 noroviruses might replace the GII.4 noroviruses, since their prevalence appears to be increasing worldwide. One possible reason for the recent increase in the prevalence in GII.17 noroviruses might be related to the change in their HBGAs binding profiles. The previous nonprevalent GII.17 noroviruses that appeared in 2014 and 2015 might bind a greater panel of HBGA types, and yet this genotype remains relatively rare. The reason for a particular norovirus to bind or not to bind HBGA is not so evident. Moreover, the corresponding consequence of the HBGA interaction or not interaction is not so clear. However, the HMO binding site on the GII.10 capside was recently discovered. Structural data show that HBGAs and HMOs bind in copious interactions. The fucose moieties of HBGAs and HMOs are always held in identical positions, whereas the other saccharide units are orientated slightly differently. The terminal saccharide units are usually held with only a few if any direct hydrogen bonds, suggesting a weaker interaction. In summary, the HBGA/HMO site on the GII capsid is a multipurpose binding pocket for norovirus [39].

Additionally, it has been described that two human milk oligosaccharides 2'-FL and 3-FL, could block norovirus from binding to surrogate HBGA samples. They bound at the equivalent pockets on the norovirus capsid using X-ray crystallography, therefore they structurally mimic HBGAs. These results suggest that 2-FL and 3-FL might act as naturally occurring decoys in humans. In this study, the ability of two HMOs (2'-FL and 3-FL) to block GII.10 norovirus VLPs from binding to HBGAs was analyzed. A slightly modified blocking enzyme-linked immunosorbent assay (ELISA) was developed using both porcine gastric mucin type III (PGM) and human saliva (A and B types). The PGM sample was confirmed to contain a mixture of A and H types using specific anti-HBGA monoclonal antibodies. The untreated VLPs were first examined for binding to PGM and saliva samples using a direct ELISA. The inhibition study was performed with an identical ELISA format, except that the VLPs were first mixed with serial diluted HMOs. 2'-FL and 3-FL were able to block GII.10 VLPs from binding to PGM, A-type saliva and B-type saliva in a mostly dose dependent manner. In order to better understand the 2'-FL and 3-FL binding interactions from a structural perspective, the X-ray crystal structures of the GII.10 P domains in complex with 2'-FL and 3-FL were solved. These data showed that the P domain is capable of binding HMOs and HBGAs in copious interactions, despite the fact that the fucoses were always held in identical positions. [40]

Another approach for the understanding of the relationship between the presence of specific structural motifs in the human glycan and its ability to inhibit binding by specific norovirus strains requires facile, accurate and miniaturized-binding assays. Toward this end a high-throughput biosensor platform was developed based on surface Plasmon resonance imaging (SPRi) of glycan microarrays. The SPRi was validated, and its utility

was tested, by measuring binding specificities between human milk glycan epitopes and the capsids of two common norovirus strains, VA387 and Norwalk. SPRi binding results established that the glycan motifs that bind norovirus capsids depend upon strain; VA387 capsid interacts with two neoglycoproteins, whereas Norwalk capsid binds to a different set of HMOS motifs in the form of both polyvalent neoglycoproteins and monovalent oligosaccharides. SPRi competitive binding assays further demonstrated that specificity.

Norovirus-binding glycans are able to inhibit norovirus capsid binding to their host receptors. A polyvalent neoglycoconjugate with clustered carbohydrate moieties is required for the inhibition of VA387 capsid binding to host receptor glycans, whereas both monovalent oligosaccharides and polyvalent neoglycoconjugates are able to inhibit Norwalk capsid binding to its host receptor [41].

Clinical efficacy of the protection

Provided that specific oligosaccharides and glycoconjugates have demonstrated their ability to inhibit certain pathogens in their evaluation *in vitro*, the demonstration of their clinical relevance is fundamental because it is possible that they might not be effective in the complex matrix of the intestinal content and even their effect quantitatively insignificant in the context of other protective components of human milk. The existence of a clinical correlation between the ingestion of certain oligosaccharides and the decreased risk of intestinal infections would support the statement that oligosaccharides could act as effectors of the non-specific immunity of human milk.

The variation of the expression of the glycans of human milk provides the chance to evaluate the effectiveness of these inhibitors of intestinal pathogens in the population. A prospective study followed 93 mother and child couples from their birth up to two year-old. The data of the infants' diet and the appearance of diarrhea weekly were registered. Additionally, a sample of human milk was collected in the first five weeks postpartum and the content of oligosaccharides was analyzed. Results showed that children that developed a diarrhea associated to the stable toxin of *Escherichia coli* were ingesting milk with higher content of oligosaccharides encoded by FUT3 compared to those encoded FUT2. Diarrhea by *Campylobacter spp*. occurred in a lower rate in those children whose mother's secreted milk containing a higher content of 2'-FL (a fucosyloligosaccharide with bindings α1, 2). Furthermore, the diarrhea by norovirus affected in a lesser extent those whose mothers produced milk with higher levels of lacto-*N*-difucohexaose I (LND-I) [42].

CONCLUSIONS

Noroviruses, after rotaviruses, are the most common etiology of viral intestinal infections in infants worldwide. Humans show a high susceptibility to norovirus infections, triggering an intense production of antibodies that confer a strong, but short, highly genotype-specific protection. Effective vaccines against noroviruses have not been achieved yet. Therefore, the finding of a protective tool against this virus in children is required.

Breast-fed infants have demonstrated a lower susceptibility to these viruses. Additionally, it has been demonstrated that human milk displays an *in vitro* protective activity against norovirus infections. This function does not correlate with the titer of specific antibodies to norovirus in human milk and serum samples, suggesting the involvement of other milk components such as oligosaccharides and glycoconjugates in this activity.

The structural resemblance of oligosaccharides and glycoconjugates of human milk and the HBGAs, receptors of these viruses in enterocytes, suggests that they might act as decoy receptors avoiding the binding of norovirus to intestinal cells. This would reduce viral infection and spread. The specific binding patterns of NoV variants to HBGAs and the individual variability of the human milk oligosaccharides might be able to explain the differences of susceptibility and resistance observed among humans. Recent studies have demonstrated the structural basis for the interaction between certain strains of norovirus and HBGAs and thus helped to understand their involvement in the protection of breast-fed babies against these agents. More studies are required in order to understand the interactions of other strains of these viruses and the changing affinity pattern overtime. Future research could direct the synthesis of HMO and their administration to populations at risk of enteric disease.

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