Norovirus, causative agent of winter vomiting disease, exploits several histo-blood group glycans for adhesion

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To my mother Monika

Abstract

Norovirus is recognized as the major cause of outbreaks of gastroenteritis world-wide, yet no vaccines or drugs are available for prevention or treatment of the virus infection. Challenge studies and binding studies using virus-like particles (VLPs) have suggested susceptibility to norovirus infection to be associated with secretor status. This thesis supports this idea by demonstrating that among 105 Swedish blood donors, non-secretors had significantly lower plasma titers of norovirus genogroup (G) II.4 specific IgG antibodies than secretors ($p<0.0001$). However, some non-secretors had high antibody titers, indicating that secretor independent strains also exist.

In lack of *in vitro* cultivation methods, VLPs were used to characterize the glycan binding characteristics of different norovirus strains. VLPs from the Chron1 (GII.3) and the Dijon (GII.4) strain recognized saliva samples from secretors, but not from non-secretors. Using neoglycoproteins, the two VLPs were shown to recognize sialyl Lewis x and the structural analogues sialyl diLewis x and sialylated type 2 in addition to secretor gene dependent glycans. In contrast, VLPs from the Norwalk (GI.1) strain only recognized secretor gene dependent glycans. In inhibition experiments, the sialyl Lewis x conjugate could completely block binding of the Chron1 and Dijon VLP to saliva samples.

In search for receptor glycoconjugates, human norovirus VLPs were for the first time demonstrated to bind to glycosphingolipids. Using a chromatogram binding assay, radiolabeled Norwalk VLPs were shown to recognize both type 1 and type 2 chain glycosphingolipids terminated with blood group A and H, but not B epitopes. Quartz crystal microbalance with dissipation (QCM-D) monitoring was used to characterize VLP binding to glycosphingolipids in supported lipid bilayers. The Norwalk and the Dijon VLP bound to bilayers containing H type 1, but not to those containing Lewis a glycosphingolipids. In support of multivalency, both VLPs showed a threshold concentration of H type 1 below which no binding was observed.

To conclude, this thesis describes a wide variety of histo-blood group glycoconjugates recognized by human noroviruses, suggesting novel approaches for design of glycomimetics for norovirus anti-adhesion therapy.

Keywords: norovirus, glycobiology, virus-like particle, *FUT2*, ABO(H) histo-blood group antigen, sialyl Lewis x, neoglycoprotein, glycosphingolipid, QCM-D, supported lipid bilayer

List of Publications

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

I. Larsson, M. M., **Rydell, G. E. P.**, Grahn, A., Rodriguez-Diaz, J., Åkerlind, B., Hutson, A. M., Estes, M. K., Larson, G. and Svensson, L., (2006) Antibody prevalence and titer to norovirus (genogroup II) correlate with secretor *(FUT2)* but not with ABO phenotype or Lewis *(FUT3)* genotype, *The Journal of Infectious Diseases, 194(10), pages 1422-7*

II. **Rydell, G. E.**, Nilsson, J., Rodriguez-Diaz, J., Ruvoën-Clouet N., Svensson, L., Le Pendu, J. and Larson, G., (2009) Human noroviruses recognize sialyl Lewis x neoglycoprotein, *Glycobiology 19(3), pages 309-20*

III. Nilsson, J., **Rydell, G. E**., Le Pendu, J. and Larson, G., (2009) Norwalk virus-like particles bind specifically to A, H and difucosylated Lewis but not to B histo-blood group active glycosphingolipids, *Glycoconjugate Journal, Apr 23 DOI 10.1007/s10719-009-9237-x*

IV. **Rydell, G. E.**, Dahlin, A. B., Höök, F. and Larson, G., QCM-D studies of human norovirus VLPs binding to glycosphingolipids in supported lipid bilayers reveal strain specific characteristics, *Manuscript*

Contents

Abbreviations

ATE	*N*-succinimidyl-3-tributylstannyl benzoate
BSA	bovine serum albumin
CBA	chromatogram binding assay
CCR5	Chemokine receptor 5
Cer	ceramide
ELISA	Enzyme-linked immunosorbent assay
Fuc	fucose
FucT	fucosyltransferase
FUT	fucosyltransferase gene
G	genogroup
Gal	galactose
GalNAc	*N*-acetylgalactosamine
Gb_3	Globotriosylceramide (Galα4Galβ4GlcβCer)
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GSL	glycosphingolipid
HSA	human serum albumin
HOV	Houston virus
ICAM-1	Intracellular adhesion molecule 1
Le	Lewis
MD	molecular dynamics
Neu5Ac	N-acetylneuraminic acid
nt	nucleotide
NV	Norwalk virus
QCM-D	Quartz crystal microbalance with dissipation
PAA	polyacrylamide
PSGL-1	P-selectin glycoprotein ligand 1
RHDV	Rabbit hemorrhagic disease virus
RT-PCR	Reverse transcription-polymerase chain reaction
Se	secretor
VA	Virginia Beach
VLP	virus-like particle

1. Introduction

Infectious gastroenteritis is a common illness (Lopman *et al.* 2002). Early on, bacteria were revealed as a cause of the disease, yet to demonstrate viruses as etiological agents was more difficult (Kapikian 2000). The introduction of molecular biology methods for viral diagnosis in the 1990s enabled a large proportion of the cases of non-bacterial gastroenteritis to be associated with norovirus (Atmar & Estes 2001).

Today, norovirus is considered to cause about half of all outbreaks of gastroenteritis world-wide (Patel *et al.* 2009). Outbreaks of gastroenteritis in hospitals and other closed settings is a large economical problem (Hansen *et al.* 2007). In developing countries norovirus has been estimated to cause up to 200.000 deaths yearly in children of <5 years of age (Patel *et al.* 2008). For reasons not fully understood, the number of reported norovirus outbreaks has increased considerably since the emergence of a new virus variant in 2002 (Koopmans 2008). Subsequently, large epidemics caused by novel norovirus strains have appeared world-wide approximately every other year (Donaldson *et al.* 2008).

The identification of ABO(H) histo-blood group glycans as potential receptors for norovirus, opened a route to design of glycomimetics for anti-adhesion therapy (Le Pendu *et al.* 2006). Currently, no drugs or vaccines for the virus are available. The aim of this thesis is to determine the glycan binding characteristics of various norovirus strains to facilitate design of attachment inhibitors.

1.1. Norovirus

1.1.1. History

The winter vomiting disease was first described by Zahorsky in 1929 as “an illness characterized by the sudden onset of self-limited vomiting and diarrhea that typically peaked during the colder months” (Zahorsky 1929). The association of the disease to a virus was demonstrated through a series of challenge studies during the 1940s and 1950s (Kapikian 2000). These studies excluded bacteria as etiologic agents since the disease could be transmitted by fecal samples passed through filters with pore sizes too small to allow passage of bacteria. A number of subsequent challenge studies in a series proved the agent to multiply within the host, excluding toxins. However, efforts to identify the virus using the normal tissue-culture virology approach were not successful. Instead, using immunoelectron microscopy, the identification of viruses in stool specimens from gastroenteritis patients was achieved in 1972 (Kapikian *et al.* 1972). The association between the virus and the disease was established by demonstrating that antibodies in convalescent-phase, but not in prechallenge-phase serum reacted with the virus particles. The virus was named Norwalk virus after the specimens used for the identification of the virus, derived from an outbreak that occurred in a school in Norwalk, Ohio in 1968 (Adler & Zickl 1969). The virus genus was first called Norwalk-like viruses, but the name was later changed to “small round structured virus” and finally in 2002 settled to norovirus by the international committee on taxonomy of viruses (ICTVdB 2004). The cloning of the Norwalk virus genome in 1990 allowed the virus to be characterized as a calicivirus (Jiang *et al.* 1990).

1.1.2. Clinical manifestations

Acute infections

Norovirus infectious manifestations are characterized by a sudden onset of vomiting and diarrhea. Other common symptoms are nausea, abdominal pain, abdominal cramps, anorexia, malaise and low-grade fever. Challenge studies with the Norwalk virus have demonstrated that the predominant symptom may vary (Atmar & Estes 2006). Thus, some individuals only suffer from vomiting whereas others only have diarrhea. Vomiting is relatively more common in persons >1 year of age, while children <1 year more often develop diarrhea (Patel *et al.* 2009). The incubation period is usually between 24 and 48 hours and the symptoms usually last for 12-72 h (Estes *et al.* 2006). Excretion of norovirus was traditionally thought to end within 4 days after infection (Atmar & Estes 2006). However, studies using more sensitive detection methods (RT-PCR), have suggested that virus may be found in stool for more than three weeks after infection (Atmar *et al.* 2008, Rockx *et al.* 2002). Moreover, in one of these studies norovirus could be detected in stools even before the onset of symptoms (Atmar *et al.* 2008). Norovirus infections are generally self-limiting and need no extra treatment than water, glucose and electrolyte substitution. Necrotizing enterocolitis has been associated with NoV infection in a neonatal intensive care unit (Turcios-Ruiz *et al.* 2008). Mortality due to dehydration associated with norovirus infection has been described for elderly patients (Harris *et al.* 2008).

Chronic infections

A number of studies have reported chronic norovirus infection in immunocompromised patients (Gallimore *et al.* 2004b, Kaufman *et al.* 2005, Nilsson *et al.* 2003, Siebenga *et al.* 2008, Westhoff *et al.* 2009). One of these studies describes a prolonged infection in an heart transplant recipient in Sweden (Nilsson *et al.* 2003). After the initial infection, the acute symptoms for this patient turned into diarrhea only. However, neither treatment with breast milk nor immunoglobulin, administrated first orally and later intravenously reduced the diarrhea or the virus excretion, as determined by RT-PCR and electron microscopy. Not even reduction of the immunosuppression (azathioprine and cyclosporine) had any effect on the symptoms or on viral excretion.

Unusual clinical presentations

A few reports of severe symptoms associated with norovirus infection may be found in the literature. One of these describes an outbreak among British soldiers in Afghanistan (Brown *et al.* 2002). In this outbreak, four patients displayed symptoms such as headache, neck stiffness, light sensitivity and confusion requiring emergency assistance. Furthermore, for one of the patients, disseminated intravascular coagulation was observed. In another study norovirus was detected by real-time quantitative RT-PCR in stools, serum and cerebrospinal fluid in a 23-month girl with altered consciousness suggesting norovirus-associated encephalopathy (Ito *et al.* 2006).

Asymptomatic infections

Early volunteer studies revealed that some individuals were asymptomatically infected when challenged with the Norwalk virus (Hutson *et al.* 2004). These individuals developed Norwalk virus-specific antibody response and shed virus, but did not show any

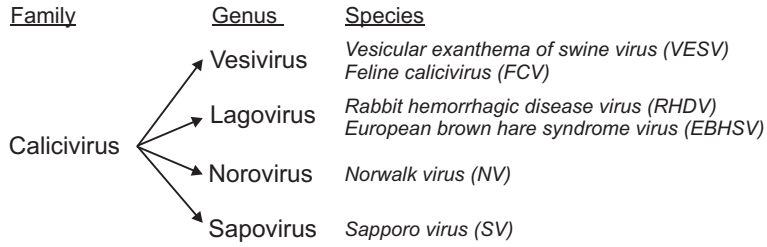


Figure 1
The genus and species of the calicivirus family. See also Figure 2 for norovirus heterogeneity.

symptoms of disease. Such infections have also been identified in outbreaks caused by other norovirus strains (Gallimore *et al.* 2004a, Ozawa *et al.* 2007).

1.1.3. Classification

Norovirus, together with sapovirus represent the human caliciviruses (Fig. 1). The prevalence of sapovirus, also causing gastroenteritis, is hard to estimate since few studies of the virus have been conducted. However, infections of young children seem more frequent than of adults (Hansman *et al.* 2007). The other two members of the calicivirus family, lago- and vesivirus, infect animals and include rabbit hemorrhagic disease virus (RHDV) and feline calicivirus (FCV).

Historically, the classification of noroviruses was based on cross-challenge studies in volunteers (Wyatt *et al.* 1974) and analysis of antisera cross-reactivity by immunoelectron microscopy (Lewis *et al.* 1995). In lack of a cell culture system, the current classification is based on sequence similarities in the capsid protein (Zheng *et al.* 2006). Currently, five genogroups (G) are recognized (Fig. 2). The genogroups GI, GII, and GIV contain human strains, whereas GIII infect cattle and GV contains murine strains (Scipioni *et al.* 2008). Porcine strains are found in GII and recently a lion (Martella *et al.* 2007) and a dog (Martella *et al.* 2008) strain have been identified in GIV. The difference in amino acid sequence of the major capsid protein is as much as 43% between isolates within the same genogroup and up to 61% between isolates from different genogroups. The strains may be further classified into genetic clusters within each genogroup. Today 31 such clusters have been identified (Fig. 2) (Wang *et al.* 2005, Zheng *et al.* 2006). The difference in amino acid sequence of the major capsid protein between strains in the same genocluster is up to 14% (Zheng *et al.* 2006).

1.1.4. Laboratory diagnosis

Before the introduction of molecular biology methods, norovirus diagnosis was based on immunoelectron microscopy. This method was time consuming. Consequently, the impact of norovirus was underestimated. The standard assay to diagnose a norovirus infection today is RT-PCR using fecal samples (Atmar & Estes 2001, 2006). Because of the extensive sequence diversity, no single primer pair can detect all strains of norovirus. However, >90% of all strains can be detected using separate primer pairs for GI and GII. The time of the analysis can be reduced by using real-time RT-PCR (Atmar & Estes 2006).

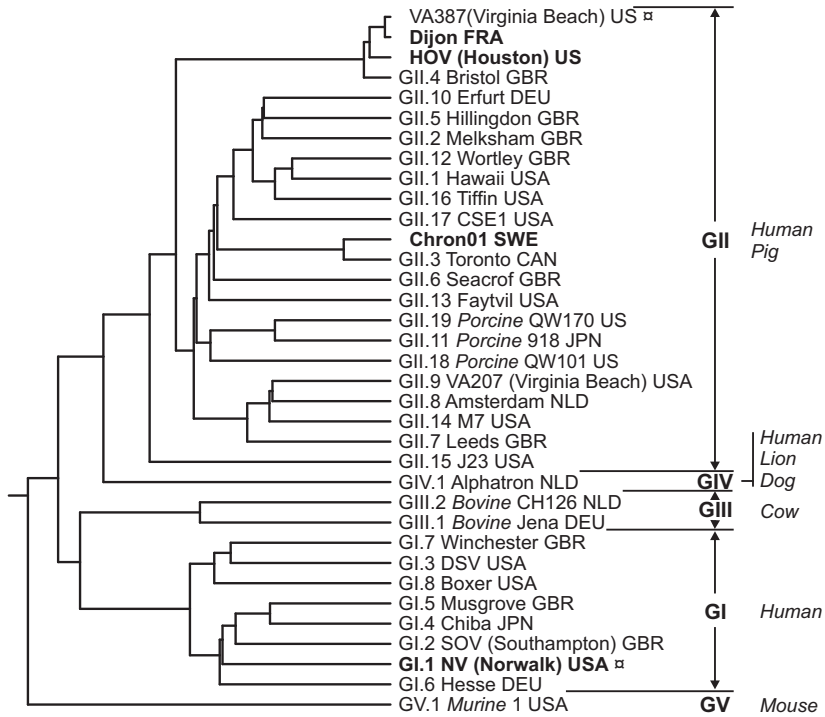


Figure 2
Phylogenetic analysis of the complete major capsid protein amino acid sequence of the norovirus strains studied in this thesis (bold), the suggested type strains for each genocluster (Wang et al. 2005, Zheng et al. 2006) and strains with the structure of the capsid protein determined (¤). Name and country of isolation are given for each strain. For the type strains genoclusters are indicated. Host species other than humans are indicated in italic together with the strain name. In addition, the host species for each genogroup is indicated. The full length sequence of the major capsid proteins were aligned using ClustalW2 with default settings on the European Bioinformatics Institute server (Larkin et al. 2007). The tree was constructed from the alignment using the Evolutionary Trace server (Trace Suite II, University of Cambridge) (Innis et al. 2000).

Enzyme-linked immunosorbent assays (ELISAs) for norovirus detection are also available (Atmar & Estes 2006). These typically have poor sensitivity (40-60%) but similar specificity (94-96%) compared to RT-PCR (Gray *et al.* 2007, Patel *et al.* 2009). The low sensitivity is associated with the high antigenic diversity of norovirus strains. Because of the high specificity ELISAs may be useful for diagnosing norovirus in outbreak investigations where several specimens are available (Patel *et al.* 2009).

1.1.5. Epidemiology

Norovirus is considered to cause about half of all outbreaks of gastroenteritis and 75-90% of all outbreaks of non-bacterial gastroenteritis in developed countries (Atmar & Estes 2006, Fankhauser *et al.* 2002, Lopman *et al.* 2003). The impact of norovirus in developing countries has been less well studied but recent estimations suggest that it is large enough to state that norovirus causes about half of all outbreaks of gastroenteritis world-wide

(Atmar & Estes 2006, Patel *et al.* 2009, Patel *et al.* 2008). It has been estimated that norovirus cause 23 million infections, 50 000 hospitalizations and 300 deaths per year in the USA (Mead *et al.* 1999).

The number of norovirus cases identified has clearly increased during the last years. The importance of the introduction of detection assays with increased sensitivity for this increase is hard to determine (Widdowson *et al.* 2005). However, the emergence of a new GII.4 variant in the winter of 2002 has been well established (Lopman *et al.* 2004, Widdowson *et al.* 2004). The new variant displaced other strains and caused a large epidemic. Subsequently, novel GII.4 subgroups have developed, causing new epidemics over the world (Bucardo *et al.* 2008, Kroneman *et al.* 2008, Okada *et al.* 2005, Patel *et al.* 2008), including Sweden (Johansen *et al.* 2008). During these years, GII.4 strains have caused more than half of all reported norovirus outbreaks (Donaldson *et al.* 2008, Kroneman *et al.* 2008, Patel *et al.* 2008).

Seasonality

As the name suggests, the winter vomiting disease is more common during wintertime, even though it is diagnosed year-round (Fig. 3).The reason for the seasonality is unclear, but it has been postulated that it is due to a combination of climatic conditions that favor the survival of the virus and an increased likelihood of person-to-person and food-borne transmission caused by social behavior (Lopman *et al.* 2004). It has been suggested that the seasonality is largely caused by GII strains, whereas GI strains are more evenly spread over the year (Nordgren *et al.* 2008).

Transmission

Noroviruses are primarily transmitted through the fecal-oral route, usually either by consumption of contaminated food or water or by direct person-to person contact (Atmar & Estes 2006). The virus is also spread by infectious vomit (Said *et al.* 2008). Outbreaks have been associated with raspberries, oysters, salads, sandwiches and bakery products (Bresee *et al.* 2002). Oysters are particularly interesting vehicles as Norwalk virus-like particles (VLPs) have been demonstrated to bind specifically to oyster tissues (Le

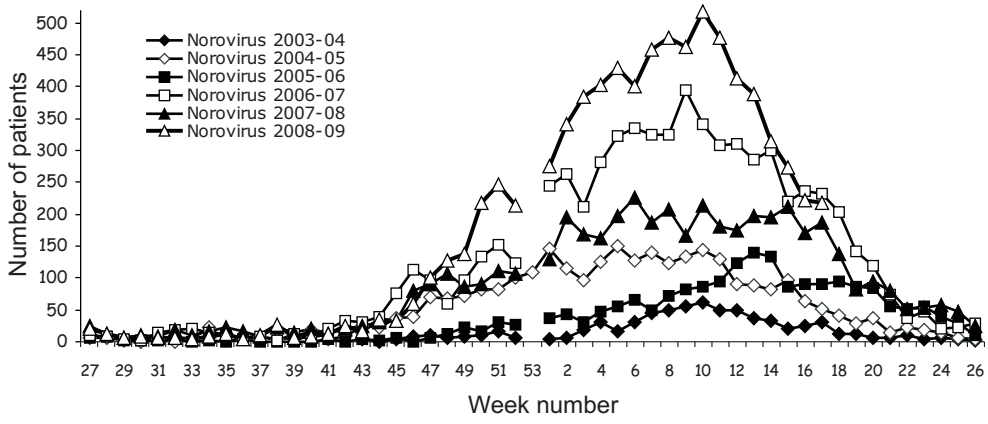


Figure 3
Number of norovirus cases reported to the Swedish Institute for Infectious Disease Control from the seasons 2003-2009 per week. Data obtained from Hedlund (2009).

Guyader *et al.* 2006). The secondary attack rate in outbreaks may exceed 30%, causing large outbreaks, especially in closed settings such as hospitals, retirement centers and cruise ships (Atmar & Estes 2006, Said *et al.* 2008). Together with influenza viruses, norovirus has been identified as the most common reason for closure of hospital wards (Hansen *et al.* 2007). Strains belonging to GII.4 are especially common amongst outbreaks in closed settings (Said *et al.* 2008).

The spread of norovirus is facilitated by the prolonged duration of virus shedding, which may last for weeks and for chronically infected patients several years. Transmission following recovery from symptomatic infection has been demonstrated (Patterson *et al.* 1993). If in addition, some individuals may get asymptomatically infected (section 1.1.2), the impact of asymptomatic virus shedding on virus transmission may be assumed to be large.

Norovirus is extremely infectious. Based on challenge studies with the Norwalk virus, the probability of infection with a single virus particle has been estimated to be close to 50% among genetically susceptible individuals (Teunis *et al.* 2008). The spread of the virus is further facilitated by its high environmental stability. Norovirus seems highly resistant to alcohol and quaternary ammonium compounds (Bresee *et al.* 2002). The suggested method for disinfection is cleaning with detergent followed by household bleach at 5000 ppm (Said *et al.* 2008).

1.1.6. Propagation in cell culture

The study of noroviruses has been hampered by the lack of *in vitro* cultivation systems. The recently discovered murine norovirus grows in cell-culture (section 1.1.9), but despite extensive efforts (Duizer *et al.* 2004) human norovirus has not yet been replicated productively in cultured cells. In one study, limited replication of human noroviruses was achieved using a 3D organoid model derived from a human intestinal epithelium cell-line (Straub *et al.* 2007). In another study, viral RNA transfected into human hepatoma cells (Huh-7) was shown to be replicated and also subsequently released from the cells together with viral particles (Guix *et al.* 2007). The latter study suggests that viral RNA is infectious in cultured cells, and that the block to *in vitro* cultivation occurs at the stage of receptor and/or co-receptor binding and/or uncoating.

1.1.7. Virus structure and genome

The norovirus genome is a positive-sense single stranded RNA with an approximate length of 7.5 kb, organized into 3 open reading frames (ORFs) (Fig. 4). ORF1 encodes a non-structural polyprotein, which is cleaved by the viral protease, 3CLpro, into at least six proteins (Hardy 2005). Beginning at the N-terminal of the polyprotein these are: p48, which has been proposed to interfere with cellular trafficking; the nucleoside triphosphatase, NTPase; p22, which has an unknown function but shows some similarity to the picornavirus 3A protein involved in membrane localization of replication complexes; VPg, which is covalently linked to genomic and subgenomic RNA and proposed to interact with translation initiation factors (Chaudhry *et al.* 2006); 3CLpro, the viral protease; and finally RdRp, the RNA-dependent RNA polymerase (Hardy 2005). ORF2 encodes the major capsid protein and ORF3 encodes a minor structural protein (Glass *et al.* 2000, Jiang *et al.* 1992).

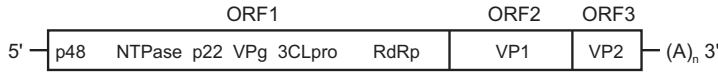


Figure 4
Norovirus genome organization (Hardy 2005).

The virus capsid and virus-like particles

The lack of success in growing noroviruses in cell culture has greatly impaired the study of the biological properties of the virus (Duizer *et al.* 2004). Therefore, the observation that virus-like particles (VLPs) could be formed by expressing the capsid protein in insect cells, transfected with a recombinant baculovirus, was of great importance (Jiang *et al.* 1992). Furthermore, the VLPs were shown to be morphologically and antigenically similar to authentic virions (Green *et al.* 1993, Jiang *et al.* 1992). Subsequently, VLPs have also been produced in venezuelan equine encephalitis virus replicon vectors in mammalian cells (Baric *et al.* 2002). The three dimensional structure of the Norwalk VLP has been determined by X-ray crystallography at a resolution of 3.4 Å (Prasad *et al.* 1999). The virus capsid has a T = 3 icosahedral symmetry, which means that it may be modeled from 60 identical equilateral triangles each consisting of 3 copies of the capsid protein (Fig. 5). The X-ray structure demonstrated that the major capsid protein folds into two domains, the S and P domains. The S domain forms the interior shell of the virus capsid, while the P domain forms dimers extending from the shell in arch like structures. Subsequent morphogenesis studies have revealed that the S domain is required for assembly of the capsid while intermolecular contacts between the dimeric P domains increase the stability of the capsid (Bertolotti-Ciarlet *et al.* 2002). The P domain is further divided into the P1 and P2 domains with the latter being the most exterior one (Prasad *et al.* 1999). By expressing only the P domain of the capsid protein, P dimers (Tan *et al.* 2004b) and P particles (Tan *et al.* 2008a, Tan & Jiang 2005b) may be formed. P particles and P domains show similar binding patterns compared to the corresponding VLPs and may, in contrast to VLPs, be produced in *E. coli* or yeast. Interestingly, a large amount of soluble P domains are found in stool specimens of norovirus infected patients, even though it is not known whether these form P particles or not (Hardy *et al.* 1995).

Cryo-EM studies of VLPs from the Grimsby strain (GII.4) have suggested that the overall structure is similar to the structure of the Norwalk virus (Chen *et al.* 2004). The identified differences were mainly located to the P2 domain and the relative orientation between the S and P domains. However, a recent cryo-electron microscopy study of the murine norovirus has suggested that the P domains in that strain is rotated as much as 40 degrees compared to the structure of the Norwalk virus (Katpally *et al.* 2008).

In addition to the major capsid protein, one or two copies of the minor capsid protein can be found in each virion (Hardy 2005). The minor capsid protein is also included in some VLPs. The function of this protein is unknown, but it has been proposed to function in RNA genome packaging (Glass *et al.* 2000) and for increasing the expression and the stability of the major capsid protein (Bertolotti-Ciarlet *et al.* 2003).

1.1.8. Pathogenesis

The target cell for human norovirus has not been unequivocally identified, but it is assumed that the virus replicates in the upper intestinal tract. Thus, biopsies of the

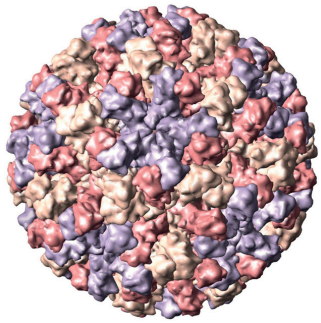


Figure 5
Structure of the Norwalk virus capsids. The diameter of the capsid is approximately 38 nm. The coordinates were adopted from Prasad et al. (1999) and the image was constructed using the Chimera package (Pettersen et al. 2004).

jejunum from volunteers challenged with the Norwalk (GI.1) or Hawaii (GII.1) virus have been demonstrated to exhibit histopathologic lesions (Agus *et al.* 1973, Schreiber *et al.* 1973, 1974). These studies revealed reversible broadening and blunting of the jejunal villi whereas the mucosa remained histologically intact. Also infiltration with mononuclear cells in the epithelium and cytoplasmic vacuolization was observed. Furthermore, the studies showed that infection was accompanied by transient fat and D-xylose malabsorption. Jejunal lesions have also been identified in individuals who did not develop symptomatic disease after virus challenge (Schreiber *et al.* 1973, 1974). Studies of biopsies from a more recent challenge study have confirmed and extended the results from the old challenge studies (Troeger *et al.* 2008). In the latter study, the infiltrating mononuclear cells were identified as CD8+ lymphocytes belonging to a unique T-cell population interspersed between epithelial cells both in the small and the large intestine. The study also suggested that these T-cells may be involved in the morphological alterations of the villi induced by the virus.

So far, it has not been possible to visualize virus particles in biopsies from norovirus challenge studies. However, a study of Norwalk VLPs binding to tissue sections of the gastroduodenal junction demonstrated binding mainly at the villi level and weaker binding at the crypt level (Marionneau *et al.* 2002).

1.1.9. Animal models

Murine norovirus

The discovery of the murine norovirus (Karst *et al.* 2003) and the subsequent propagation of the virus in cell culture (Wobus *et al.* 2004) were major breakthroughs for the norovirus field (Scipioni *et al.* 2008, Wobus *et al.* 2006). The murine norovirus was identified in immunocompromised mice lacking the recombination-activating gene 2 (RAG2) as well as signal transducer and activator of transcription 1 (STAT1) (RAG2/STAT1$^{-/-}$). The animals sporadically succumbed to a systemic disease (Karst *et al.* 2003). However, subsequent studies have demonstrated that around 30% of mice in research facilities throughout the United States, Canada and Europe have antibodies against murine norovirus in serum, suggesting it is possibly the most prevalent of all endemic viruses in research mice (Henderson 2008, Pritchett-Corning *et al.* 2009). At least one article describing a direct influence of murine norovirus on research investigations has been published (Lencioni *et al.* 2008).

Immunocompromised mice infected with murine norovirus develop a systemic disease with signs of encephalitis, vasculitis in cerebral vessels, pneumonia and hepatitis whereas infection in wild-type mice seems to be asymptomatic (Henderson 2008, Wobus *et al.* 2006). An interesting parallel is the severe human norovirus infections observed in immunocompromised patients (section 1.1.2). Studies in wild type mice have shown that the infection is established in the proximal small intestine and that the virus subsequently spreads to other organs such as the liver, lungs, lymph nodes and spleen (Henderson 2008). The virus is shed in stool, resulting in fecal-oral transmission (Manuel *et al.* 2008). In cell culture, murine norovirus has a tropism for cells of the hematopoietic lineage, specifically macrophages and dendritic cells (Wobus *et al.* 2004).

Human norovirus in gnotobic pigs and calves

Porcine noroviruses form the distinct genoclusters GII.11, GII.18 and GII.19. So far, no human noroviruses have been classified into these clusters (Scipioni *et al.* 2008). However, recent studies have demonstrated that gnotobic pigs can be experimentally infected with human GII.4 strains (Cheetham *et al.* 2006, Souza *et al.* 2007). In the first of these studies, 74% of the inoculated pigs developed mild diarrhea, even though only 44% secreted detectable amounts of viral RNA in feces (Cheetham *et al.* 2006). In the second study, it was demonstrated that GII.4 norovirus induced a Th1 like immune response (Souza *et al.* 2007). Such a response was recently demonstrated also for gnotobiotic calves infected with the same virus strain (Souza *et al.* 2008). Finally, VLP binding studies on paraffin-embedded intestinal tissues from gnotobiotic pigs have suggested that the virus adheres to carbohydrate structures related to the glycans suggested as receptors for human norovirus (Cheetham *et al.* 2007).

Zoonosis

The demonstration of cross-infection of human norovirus in pigs and calves raises concerns about risk for zoonosis. Co-infections of animal and human norovirus possess a risk for genetic mixing as recombination seems to be common for noroviruses (Bull *et al.* 2007). In this context, a recent study reporting detection of GII.4 strains in livestock pig stool samples and also in a retail meat sample, is worrying (Mattison *et al.* 2007).

1.1.10. Immunity

Most of the information regarding immunity to norovirus infections has been obtained in challenge studies. The interpretation of the early studies is complicated by the absence of information regarding secretor status. Furthermore, the viral dose administered in most studies must be considered extremely high, as the infectivity of a single Norwalk virus particle recently has been estimated to be close to 50% (Teunis *et al.* 2008). Nevertheless, early challenge studies have demonstrated the existence of short term immunity (Dolin *et al.* 1972, Parrino *et al.* 1977, Wyatt *et al.* 1974). Furthermore, a cross-challenge study using the Norwalk (GI.1) and Hawaii (GII.1) strains suggested the immunity to be strain or genogroup specific (Wyatt *et al.* 1974). This was illustrated by the finding that volunteers, who fell ill following a virus challenge were usually shown to be protected when being re-challenged with the same virus strain 6-14 weeks later. However, when re-challenged with the other strain they fell ill again.

The results from the challenge studies regarding long-term immunity are conflicting (Donaldson *et al.* 2008). One study demonstrated that when volunteers who initially fell

ill were re-challenged with the same inoculum of Norwalk virus after 2-4 years they became symptomatically infected again (Parrino *et al.* 1977). In a more recent challenge study 44% of the genetically susceptible individuals were not infected, indicating the presence of long-term immunity (Lindesmith *et al.* 2003). Furthermore, early longitudinal studies suggested that serum antibody titers were associated with protection of children against norovirus infection (Black *et al.* 1982, Ryder *et al.* 1985). However, pre-challenge antibody titers could not be correlated to susceptibility in challenge studies (Johnson *et al.* 1990).

Antibody cross-reactivity

A number of studies have demonstrated antibody cross-reactivity between different strains of norovirus, especially within each genogroup (Hale *et al.* 1998, Lindesmith *et al.* 2005, Rockx *et al.* 2005a, Rockx *et al.* 2005b). The cross-reactivity of blocking antibodies seem to be more restricted (Harrington *et al.* 2002b, Rockx *et al.* 2005a, Rockx *et al.* 2005b).

Until recently, the information regarding antibody cross-reactivity within genogroups was limited. However, in a recent study VLPs representing different GII.4 subgroups that have caused time-ordered global epidemics, were constructed (Lindesmith *et al.* 2008). Thereby the antigenic evolution of this genocluster could be studied (Cannon *et al.* 2009, Lindesmith *et al.* 2008). By measuring titers of IgG antibodies towards the different VLPs in antisera from mice immunized by each VLP, serological differences between the VLPs could be identified (Lindesmith *et al.* 2008). Similarly, differences could be identified using pre- and post-epidemic human anti-sera (Cannon *et al.* 2009, Lindesmith *et al.* 2008). Importantly, pre-epidemic anti-sera were shown to poorly recognize post-epidemic VLPs (Cannon *et al.* 2009, Lindesmith *et al.* 2008).

These two studies suggest that GII.4 norovirus evolves by so called epochal evolution (Donaldson *et al.* 2008). Other studies have proposed a similar evolutionary process based on bioinformatics analysis (Allen *et al.* 2008, Siebenga *et al.* 2007). Epochal evolution means that periods of stasis (epochs), under which the genetic diversity grows, are followed by sudden changes in phenotype and emergence of novel epidemic strains. Only a subset of the genetic variation observed accounts for the change in fitness (Donaldson *et al.* 2008). An epochal evolution has been suggested also for the influenza virus (van Nimwegen 2006). For this virus the process of antigenic drift is even faster, as it seems to evade herd immunity on a yearly basis.

Chronic human norovirus infections represent *in vivo* models to study the effects and localization of viral mutations over time. From the Swedish patient with a chronic GII.3 infection described in section 1.1.2 viral sequences were obtained once a month during one year. Eleven amino acid mutations accumulated in the major capsid protein could be detected (Nilsson *et al.* 2003). Notably, 8 of these mutations occurred in the outermost domain (P2) of the capsid protein, indicating an immune driven selection. Later, 3 of the 11 amino acids were identified to achieve accumulated mutations in a similar study of three other patients with chronic GII.3 infections (Siebenga *et al.* 2008). In a recent study, the virus in the Swedish patient was demonstrated to evolve as a quasispecies population. Capsid sequences isolated at the same time point tended to cluster together in a phylogenetic analysis (Carlsson *et al.* 2009b). The glycan binding pattern of a VLP constructed from the first viral isolate from this patient is described in Paper II.

1.2. Glycobiology

Glycans are, together with nucleic acids, proteins and lipids, the fundamental macromolecules of all living cells (Marth 2008). In comparison to nucleic acids and proteins, glycans have an enormous information coding capacity per monomeric unit in the polymer. This is mainly because glycans, in contrast to the other two polymer classes, have a variability of linkage positions, anomeric configuration and the ability to form branched structures (Gabius 2008). These variabilities make glycans ideal for high-density information storage. This stored information is mainly decoded by glycan binding proteins, e.g. glycosyl-transferases, -hydrolases and lectins.

Glycan-protein interactions are involved in a wide range of biological functions. These include protein maturation and turnover, cell adhesion and trafficking as well as receptor binding and activation (Marth & Grewal 2008). The extensive use of glycans as small ligands for proteins is probably not explained solely by their coding capacity. Of relevance is probably also the fact that glycans have few energetically favoured conformations. Even though carbohydrates are often considered to be flexible molecules, they are generally more rigid than peptides of the same weight. Thus, it has been proposed that the entropic cost of locking a ligand in a protein binding pocket is lower for a glycan compared to a similar sized peptide (Gabius 2008). Another important property of protein-carbohydrate interactions is the ability to fine-tune interactions by multivalency (section 1.3.4).

1.2.1. Glycan biosynthesis

In contrast to proteins, glycans are secondary gene products. Thus, glycans are not directly encoded by the genome, but instead produced by the sequential action of glycosyltransferases and other glycan-processing enzymes (Ohtsubo & Marth 2006). Consequently, glycan synthesis is not template driven, but instead, in each cell, determined by the availability and specificity of the enzymes involved and the competition between them. Also the availability of substrates and acceptor structures for the different enzymes affects the biosynthesis. The regulation of the glycan-processing enzymes is complex (Murrell *et al.* 2004) and, in addition to cell and tissue specific expression, at least some of them exist in different splice forms (Grahn *et al.* 2002, Grahn & Larson 2001, Russo *et al.* 1990). Glycosyltransferases may be found both as membrane bound and as soluble proteins (Hart *et al.* 2007). Furthermore, chaperones and other activator proteins are essential for activity and correct localization of some glycosyltransferases (Ju & Cummings 2002, Wu *et al.* 2004).

1.2.2. Glycoconjugates

Glycans may occur as free saccharides but are usually covalently linked to either proteins or lipids. The protein or lipid in such glycoconjugates is denoted the aglycone and may be directly involved in interactions with proteins together with the glycan (Cummings & Esko 2008). In addition the aglycone may influence protein interactions indirectly by affecting the conformation or the availability of the glycan (Lingwood 1996).

Glycoproteins

In glycoproteins, the glycans are usually linked either to the side-chain nitrogen of an aspargine (N-linked) or to the side-chain oxygen of a serine or threonine residue

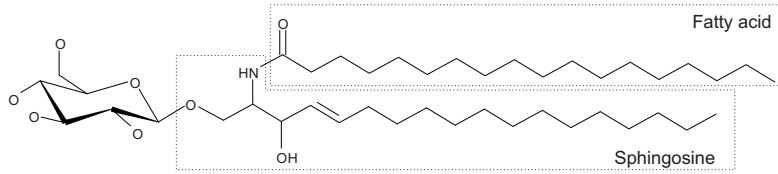


Figure 6
Structure of a glucosylceramide. The ceramide shown consists of sphingosine (dihydroxy 18:1) and stearic (18:0) fatty acid.

(O-linked) (Brockhausen *et al.* 2008, Stanley *et al.* 2008). Of these classes, N-linked glycans are the most studied because of their essential functions in protein folding and quality control in the secretory pathway. Aspargines linked to N-glycans are commonly found in the consensus sequence Asn-X-Ser/Thr. O-linked glycans are typically found densely packed in mucin domains, where they contribute to the hydration of the mucus as well as to the protection of the underlying tissue. In analogy with N-glycans, O-glycans may function in signaling, as exemplified by the O-fucose and O-glucose linked glycans on the Notch receptor (Acar *et al.* 2008, Stahl *et al.* 2008). Glycans contribute to the structural properties of glycoproteins and do in many cases protect the polypeptide backbone from proteases. Many receptors on the cell surface are glycosylated. The glycans are important for the localization of these proteins. In addition, the halftime of circulating serum glycoproteins depends on the glycans since receptors in the liver internalize glycoproteins with terminal Gal or GalNAc residues (Grewal *et al.* 2008).

Proteoglycans

Proteoglycans are a special group of glycoproteins carrying long repetitive linear polysaccharides linked to serine side chains. The polysaccharides may constitute up to 95% of the weight of proteoglycans and thus dominate the chemical properties of the conjugate. Proteoglycans are important components of the extracellular matrix and provide a hydrated gel resistant to compressive force. Further, proteoglycans are important in many biological signaling processes as co-receptors for a number of cytokines, chemokines and growth factors (Esko *et al.* 2008).

Glycosphingolipids

Glycosphingolipids (GSLs) are glycolipids based on ceramide (Fig. 6). The ceramide component consists of a fatty acid in amide linkage to the amino alcohol sphingosine. The length and the number of double bonds of both the sphingosine and the fatty acid may vary. Despite these variations, GSLs are usually classified based on their glycans. The species-, tissue- and cell-specific distribution suggests that GSLs play important roles. Accordingly, mice lacking all of the complex GSLs, as a result of a knockout of the glucosylceramide synthase, die as embryos (Yamashita *et al.* 1999). Mice lacking more distal glycosyltransferases show milder phenotypes (Degroote *et al.* 2004). On the cellular level the GSLs seem to play important roles for the organization of the plasma membrane and appear to be important also for the sorting of proteins. GSLs associate with cholesterol, sphingomyelin, and other sphingolipids to form micro domains called lipid-rafts. Studies suggest that GSLs are not essential for the formation of these detergent resistant domains, but rather vital for specific functions fulfilled by the domains

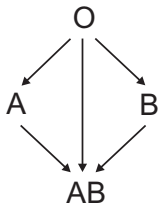


Figure 7
Blood transfusion may be performed across the ABO blood groups in the directions indicated by the arrows. Blood group O individuals are universal donors whereas blood group AB individuals are universal receivers. This chart is often referred to as the Landsteiner rule.

(Degroote *et al.* 2004). It has been shown that GSLs are involved in cell signaling interactions with lectins (Kopitz *et al.* 1998, Schnaar *et al.* 1998). Cell signaling also involves GSL-GSL interactions (Hakomori 2004). Moreover, GSLs can modulate signaling by interaction with key transmembrane receptors (Degroote *et al.* 2004).

1.2.3. The ABO(H) and Lewis histo-blood group systems

The ABO blood group system was discovered by Karl Landsteiner over a century ago and the carbohydrate basis of the antigens involved was described by Morgan half a century later (Morgan 1950). In the ABO system, individuals are classified by blood group A, B, AB or O (neither A nor B) depending on which antigen(s) are displayed on their erythrocytes. Since natural IgM antibodies are produced against antigens not present (non-self), blood transfusions can only be performed in the directions illustrated in Figure 7. The ABO antigens are also widely distributed in other tissues and thus these antigens are often referred to as histo-blood group antigens (Clausen & Hakomori 1989).

The ABO blood group system is closely related to the Lewis system as the antigens of both systems may be present on the same carbohydrate chains and overlap structurally as well as biosynthetically. The antigens are mainly found on four different carbohydrate chains, designated type 1-4 (Table 1).

Biosynthesis

The biosynthesis of the ABO(H) and Lewis histo-blood group structures is illustrated in Figure 8. The principles for the synthesis of the A, B and H antigens are applicable for all chain types, whereas the synthesis of the Lewis antigens, containing α1,3- or α1,4-linked fucose, is restricted to the type 1 and 2 chains.

The H antigens

The biosynthesis of the ABO antigens starts with the addition of an α1,2-linked fucose to the terminal galactose on either of the 4 precursor chains, to form the H epitope (Fig. 8). The human genome encodes two functional α1,2-fucosyltransferases, denoted FucT-I and

Table 1 Carbohydrate chains carrying ABO(H) and Lewis histo-blood groups (Clausen et al. 1985b, Marionneau et al. 2001, Ravn & Dabelsteen 2000)

Name	Structure	Found on
Type 1	Galβ1,3GlcNAcβ1-R	N-,O-glycoproteins, GSLs of the lactoseries
Type 2	Galβ1,4GlcNAcβ1-R	N-,O-glycoproteins, GSLs of the neolactoseries
Type 3	Galβ1,3GalNAcα1-R	O-glycoproteins (core 1), GSLs as elongated blood group A series
Type 4	Galβ1,3GalNAcβ1-R	GSLs of globo and ganglioseries

GSLs = glycosphingolipids

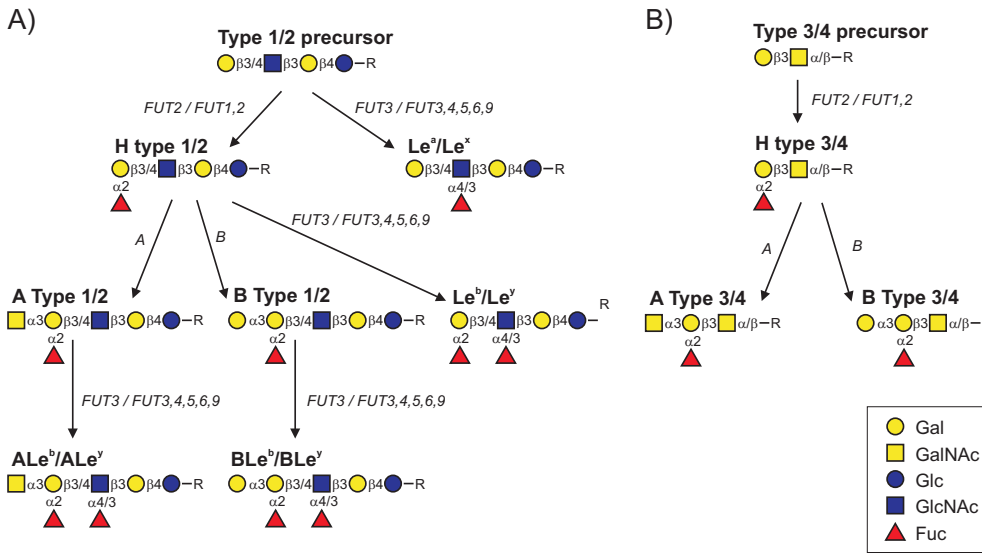


Figure 8
Biosynthetic pathways of the ABO(H) and Lewis antigens on the type 1/2 (A) and 3/4 (B) chains. Antigen names (bold), glycosyltransferases (italic) and linkages of the different chains are, when differing between the chains in each figure, separated by a diagonal in the format indicated by the antigen names.

FucT-II and encoded by the *FUT1* and *FUT2* genes, respectively (Kelly *et al.* 1995, Rajan *et al.* 1989). These enzymes differ in their acceptor specificities and expression profiles and both of them are polymorphic in the human population. Historically, *FUT2* was assumed to be a regulatory gene controlling the expression of *FUT1* in secretions. It was thus controversial when Oriol and co-workers in 1981 suggested (Oriol *et al.* 1981) and later demonstrated (Le Pendu *et al.* 1985) the existence of two distinct human α1,2-fucosyltransferases.

The distribution of the H antigens in human tissues in relation to expression of *FUT1* and *FUT2* has been reviewed (Mollicone *et al.* 1995, Oriol *et al.* 1986, Ravn & Dabelsteen 2000). The FucT-I is considered to be active exclusively, or at least predominantly, towards the type 2 chain whereas the type 1 and type 3 chains are the typical acceptors for FucT-II. However, FucT-II is active also towards the type 2 chain (Ravn & Dabelsteen 2000). A recent study of breast cancer stem cells suggests that both FucT-I and FucT-II may synthesize H type 4 (Chang *et al.* 2008). Ravn and Dabelsteen suggest that the degree of cell differentiation affects the expression of FucT-I and FucT-II (Ravn & Dabelsteen 2000). Undifferentiated cells tend to express *FUT1*, whereas more differentiated cells tend to express *FUT2*. The expression of *FUT1* in bone marrow erythropoietic cells is clearly analogous with this. A supporting observation is that the α1,2-fucosylation of type 2 chain structures in the mucus cells of the pyloric and Brunner's glands is independent of *FUT2* (Mollicone *et al.* 1985).

As the H epitope is the acceptor for the blood group A and B transferases, the biosynthesis of the A and B antigens requires the expression of a functional α1,2-fucosyltransferase. Thus, the biosynthesis of the A and B epitopes on the type 1

chain is strictly dependent on the expression of a functional *FUT2* gene, whereas the biosynthesis of the same epitopes on the type 2 chain may be dependent on either *FUT1* or *FUT2*. However, since these transferases show different expression profiles, the expression of H antigens on erythrocytes and vascular endothelium is dependent on *FUT1*, whereas the expression of the antigens on most epithelial cells and in mucosal secretions, is dependent on *FUT2* (Mollicone *et al.* 1995, Ravn & Dabelsteen 2000).

About 20% of the Caucasian population have two non-functional *FUT2* alleles and consequently do not express any ABH blood group antigens on epithelial cells or in mucosal secretions. These individuals cannot have their ABO blood group determined from saliva and are denoted non-secretors (sese). In contrast, individuals with at least one functional *FUT2* allele are denoted secretors (Sese or SeSe). The most common and completely dominating (>99%) inactivating mutation of *FUT2* in the Caucasian population is G428A, which introduces a premature stop codon in the gene (Kelly *et al.* 1995). The mutation A385T causes a weakened enzyme activity and the so called weak secretor phenotype common in Asia (Henry *et al.* 1996b, a, Yu *et al.* 1995). An additional 50 inactivated an non-inactivated human *FUT2* alleles are listed in the blood group antigen gene mutation database (dbRBC) (Blumenfeld & Patnaik 2004).

Inactivating mutations have been found also in *FUT1*, even though these are much more rare than the mutations causing the non-secretor genotype (Koda *et al.* 2001). Many of the *FUT1* mutations are linked to inactivating mutations in *FUT2*, which is located close to *FUT1* on chromosome 19 (Fernandez-Mateos *et al.* 1998, Koda *et al.* 1997). Thus, homozygote carriers of inactivating mutations in *FUT1* often lack both FucT-I and FucT-II activity and are consequently devoid of all A, B and H antigens. This phenotype is denoted Bombay, after the place where it was first identified (Bhende *et al.* 1952). The mutation causing the original Bombay phenotype was T725G in *FUT1*, linked to a deletion mutation in *FUT2* (Fernandez-Mateos *et al.* 1998, Koda *et al.* 1997). Subsequently, other mutations causing the Bombay phenotype have been identified (Koda *et al.* 2001). Estimations suggest 1 in a million Europeans and 1 in 10 000 of Indian ancestry to be Bombay individuals (Blumenfeld & Patnaik 2004). Individuals with inactivating mutations in *FUT1*, but a functional *FUT2* gene are denoted to be of para-Bombay phenotype (Koda *et al.* 2001).

The A and B antigens

The glycosyltransferases encoded by the *ABO* gene are responsible for the addition of the terminal α1,3GalNAc/Gal resulting in the histo-blood group A and B epitopes (Fig 8). Blood group A individuals have at least one functional α1,3-GalNAc transferase (A) allele, whereas blood group B individuals have at least one functional α1,3-Gal transferase (B) allele. Blood group AB individuals have one A and one B allele, but may in rare cases instead carry a cis-AB allele with both α1,3GalNAc and α1,3Gal transferase activity (Yazer *et al.* 2006). Blood group O individuals are homozygous for alleles coding for proteins lacking enzymatic activity. The cloning of the *ABO* alleles showed that the typical A and B enzymes differ in only 4 out of 354 amino acids (Yamamoto *et al.* 1990). Subsequent studies have revealed that the *ABO* gene is highly polymorphic. More than 160 alleles are now described (Blumenfeld & Patnaik 2004). The most common subgroups are the A_1 and A_2 phenotypes constituting about 80% and 20% of the blood group A individuals, respectively. Individuals in the A_2 group have a less efficient

enzyme than those in the A_1 group. The most common A_2 allele is characterized by a single base deletion mutation creating an enzyme with 21 extra amino acids (Yamamoto *et al.* 1992). Because of the lower enzyme activity, erythrocytes of A_2 individuals display a lower number of A epitopes and a larger number of H epitopes compared to erythrocytes of A_1 individuals (Svensson *et al.* 2009). However, qualitative differences between the groups also exist. Both the A type 3 GSL (GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcβCer) (Clausen *et al.* 1985b) and the A type 4 (Globo A) GSL (GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4GlcβCer) (Clausen *et al.* 1984) have been reported to be restricted to A_1 individuals. However, recent experiments have suggested that when compensating for the lower amount of A epitopes on GSL extracts from A_2 erythrocytes, only the A type 4 structure remains unique for A_1 individuals (Svensson *et al.* 2009).

The Lewis antigens

The Lewis blood group system refers to the presence - or absence - of an α1,3- or α1,4-linked fucose on the subterminal GlcNAc on the type 1 and type 2 chains, in combination with the presence or absence of the H epitope, on the same glycan.

The addition of an α1,4-linked fucose to the type 1 chain precursor forms the Lewis a (Le^a) structure, whereas the addition of a similar fucose to the H type 1 structure forms the Lewis b (Le^b) structure (Fig. 8). In a similar manner the A type 1 and B type 1 structures can be transformed into ALe^b and BLe^b, respectively. The fucosyltransferase catalyzing all these reactions is encoded by the Lewis *FUT3* gene. Also the gene product of *FUT5* has been reported to have activity for the type 1 chain but since individuals with inactivating mutations in *FUT3* essentially lack Le^a and Le^b structures, the *FUT3* gene product is considered the main enzyme responsible for these differences (Marionneau *et al.* 2001). Homozygote carriers of inactive *FUT3* alleles are denoted Lewis negative (lele) and constitute about 5% of the Caucasian population (Mollison *et al.* 1993). The most common inactivating mutations in *FUT3* are T202C (Elmgren *et al.* 1997), G508A (Koda *et al.* 1993, Nishihara *et al.* 1994) and T1067A (Mollicone *et al.* 1994). Antibodies towards Le^a and Le^b are routinely used to phenotype for secretor status, as the expression of Le^b, but not Le^a, requires a functional *FUT2* gene. The phenotyping is generally performed on erythrocytes, even though these cells mainly display type 2 chain structures. The small amount of type 1 chain structures found on erythrocytes is associated with GSLs being adsorbed from plasma lipoproteins (Marcus & Cass 1969). Notably, the secretor status of Lewis negative individuals may not be determined using antibodies towards Le^a and Le^b, as those individuals do not express any of the antigens. Lewis positive secretors do not express detectable amounts of Le^a on erythrocytes. However, Lewis positive weak secretors display both Le^a and Le^b on erythrocytes (Henry *et al.* 1990).

The type 2 chain isomers of Le^a and Le^b are denoted Le^x and Le^y respectively (Fig. 8). The difucosylated blood group A and B antigens, ALe^y and BLe^y also exist. In contrast to the α1,4-fucose of the type 1 chain Lewis structures, a number of different fucosyltransferases may catalyze the addition of the α1,3-fucose, characterizing the type 2 chain Lewis structures (Marionneau *et al.* 2001).

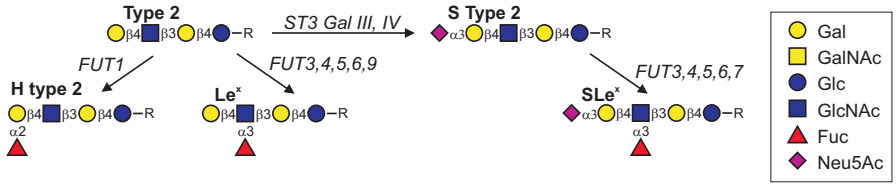


Figure 9
The biosynthetic pathway for sialyl Lewis x. Names of antigens are in bold and glycosyltransferase genes in italic.

Sialylated and sulfated antigens

The type 1 and type 2 precursors may be sialylated by α2,3-sialyltransferases to form sialylated type 1 and 2, respectively. These structures may be acceptors for α1,3/4-fucosyltransferases giving sialyl Lewis a (SLe^a) and sialyl Lewis x (SLe^x). In analogy with Le^a and Le^x, the synthesis of SLe^a requires a functional *FUT3* gene, whereas a number of α1,3-fucosyltransferases may catalyze the formation of SLe^x (Marionneau *et al.* 2001). Consequently, the lack of expression of SLe^x is very uncommon, suggesting an important function for the structure. The biosynthesis of SLe^x is illustrated in Figure 9.

The Lewis structures may also be modified by sulfation. The most common sulfate modifications are attached to carbon 3 or 6 of the terminal Gal residue or to carbon 6 of the sub-terminal GlcNAc residue. Examples of sulfated Lewis antigens are 3'-sulfo-Le^a, 3'-sulfo-Le^x, 6-sulfo-SLe^x, 6'-sulfo-SLe^x and 6,6'-bissulfo-SLe^x (Stanley & Cummings 2008).

Functions of the ABO(H) and Lewis histo-blood groups systems

Even though the structural basis of the ABO(H) histo blood group glycans has been known for a long time the functional role of the system has remained unclear (Greenwell 1997). However, for some of the less polymorphic structures, specific functions have been demonstrated. The blood group glycan with the most thoroughly characterized function is SLe^x.

Sialyl Lewis x

SLe^x is the minimal common ligand for the E-, P- and L-selectins (Lowe 2003). These are cell adhesion molecules with important functions for lymphocyte homing and leukocyte recruitment to sites of inflammation. In addition to SLe^x, the E-selectin recognizes SLe^a and VIM-2 (Neu5Acα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ-R) (Lowe 2003) as well as heparan sulfate (Varki 2007). For P- and L-selectin, the binding to SLe^x also involves adjacent sulfate groups. Thus, L-selectin shows optimal binding to 6-sulfo-SLe^x, whereas high affinity binding of P-selectin to the P-selectin glycoprotein ligand 1 (PSGL-1) requires the presence of specific sulfated tyrosine residues. The importance of SLe^x as a selectin ligand has been demonstrated in transgenic mice lacking various glycosyltransferases involved in SLe^x biosynthesis (Lowe 2003). In these studies FucT-VII was identified as the major fucosyltransferase involved in the biosynthesis of the glycans responsible for selectin binding. However, the phenotype was more severe in the double knockout mice, lacking both *FUT4* and *FUT7*. When it comes to humans, the situation is quantatively different. Neutrophils from an individual homozygous for the rare inactivating mutation G329A in *FUT7* show normal E- and P-selectin binding

activities, despite the lack of SLe^x expression (Bengtson *et al.* 2001, Bengtson *et al.* 2002). An increase in expression of VIM-2, caused by an upregulation of *FUT4*, was suggested to be responsible for the maintained selectin binding.

The ABO(H) histo blood group system

One theory about the function of the ABO(H) histo-blood group system, is that the diversity provides resistance to pathogens on the population level. This theory was proposed more than 50 years ago (Haldene 1949) and has later been extended (Gagneux & Varki 1999). The theory states that a pathogen, using a specific glycan as receptor, cannot completely eliminate a population if some individuals lack that specific glycan. The diversity in blood group distribution between different populations would thereby reflect different histories of infectious diseases. A second selection pressure comes from viruses. As they exploit the glycosylation machinery of the host cell, viruses would be expected to incorporate histo-blood group glycans during the propagation. Individuals with antibodies towards the incorporated glycans would be protected against infection with that specific virus as the antibodies would cause complement mediated lysis of the virus. The concept has been experimentally evaluated for membrane viruses. These may carry blood group glycans not only on viral proteins but also on host proteins and GSLs, incorporated into the viral membrane. Cell culture studies have shown that HIV (Neil *et al.* 2005), measles virus (Preece *et al.* 2002) and SARS (severe acute respiratory syndrome) coronavirus (Guillon *et al.* 2008) may carry ABO blood group structures. The selection pressure caused by viral incorporation of blood group antigens suggests blood group O individuals to be underrepresented among infected individuals. Epidemiological evidence for protection of blood group O individuals in viral outbreaks has been scarce. One study of a hospital outbreak of SARS coronavirus, however, reported blood group O individuals to have very low odds of infection compared to non-O individuals (Cheng *et al.* 2005, Guillon *et al.* 2008).

Experimental proof for the theory is hard to achieve as the diversity seen today reflects a long evolutionary process and many of the pathogens involved are not longer present. The introduction of the RHDV in new rabbit populations may however provide opportunities to study an ongoing evolution of glycan expression. RHDV causes a lethal systemic infection and seems to use H type 2 as receptor (Ruvoen-Clouet *et al.* 2000). Studies of buccal epithelial cells from wild rabbits have demonstrated that rabbits with an H type 2-negative phenotype occur more frequently in areas with a high impact of RHDV (Guillon *et al.* 2009).

1.3. Receptors and attachment factors

To infect a cell, a virus must first adhere to it (Helenius 2007, Smith & Helenius 2004). Whereas viruses are bound only by the attachment factors, the receptors additionally promote the entry of the virus into the cell. Many viruses use more than one receptor (Helenius 2007).

Receptors may promote viral entry through several distinct mechanisms (Helenius 2007). Firstly, receptors may induce conformational changes in the virus capsid thus allowing further interactions. Secondly, they may transmit signals through the plasma membrane that lead to viral uptake. Finally, receptors may guide the virus into various endocytic pathways. In practise, the distinction between attachment factors and receptors is

complicated. The importance of attachment factors for the viral infection should not be underestimated. The concentration of virus particles at the cell surface facilitated by attachment factors may be crucial for the subsequent receptor interactions. A large number of virus receptors have been identified in different molecular families. Among the most represented families are the IgG superfamily of transmembrane proteins, integrins and glycoconjugates (Helenius 2007).

1.3.1. Glycoconjugates

A large number of viruses interact with glycans (Karlsson 1995, Marsh & Helenius 2006, Olofsson & Bergstrom 2005). Interestingly, most of these glycans are negatively charged, including sulfated glycosaminoglycans and sialylated glycans. The reason for this charge preference is unclear, but few exceptions seem to exist (Olofsson & Bergstrom 2005).

Proteoglycans

Numerous viruses interact with sulfated proteoglycans (Olofsson & Bergstrom 2005). These molecules also interact with a large number of ligands for various cell surface receptors, and thus concentrate these ligands at the cell surface. By this process, proteoglycans are important for the formation of many morphogen gradients (Lander 2007). In a similar manner, for most viruses proteoglycans have been described as attachment factors rather than receptors.

Glycoproteins and glycosphingolipids

A large number of viruses interact with sialic acids, localized to either GSLs or to glycoproteins. Probably, the most known sialic acid binding virus is the influenza A virus (Esko & Sharon 2008). Human influenza A virus has a preference for sialic acid linked in α2,6-linkage whereas avian influenza A has a preference for sialic acid linked in a α2,3-linkage. This difference in binding specificity explains, to a large extent, the host tropism for these viruses (section 1.3.2). Despite years of studies, a specific glycoprotein or GSL responsible for the entry of influenza virus has not been identified. This illustrates the complexity of the entry mechanism. In fact, for most viruses shown to use glycans as receptors no specific glycoconjugate receptors has been described. A few examples of glycoconjugate receptors will be given below.

Glycoproteins

The glycoprotein α-dystroglycan is used as receptor by several viruses from the arenavirus family (Rojek & Kunz 2008). Cell culture studies of the lymphocytic choriomeningitis virus (LCMV) have suggested that the characteristic mannosyl O-glycans found on α-dystroglycan (Moore & Hewitt 2009) are important for the viral infection (Imperiali *et al.* 2005). However, subsequent studies in knockout mice have indicated that the dependence of these glycans is weak *in vivo* (Imperiali et al. 2008).

Glycosphingolipids

Several studies have shown that viruses from the polyomavirus family use gangliosides as receptors. Using a sucrose gradient flotation assay it was demonstrated that murine polyoma virus specifically recognized GD1a and GT1b whereas simian virus 40 recognized GM1a (see Fig. 10 for ganglioside structures) (Tsai *et al.* 2003). The study also showed that a C6 glioma rat cell line transfected with the GD3 synthase was poorly

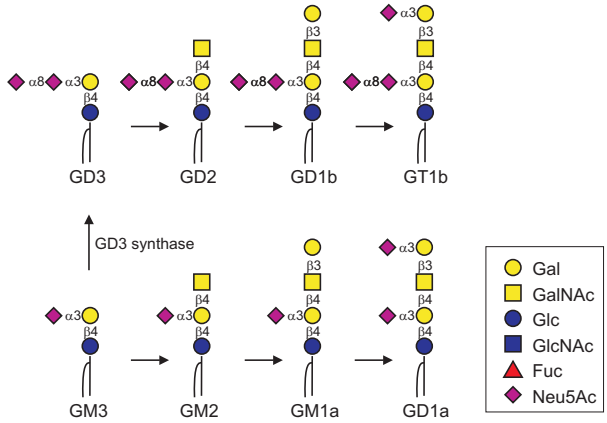


Figure 10 Structures and biosynthetic pathways for the gangliosides discussed in the text.

infected by both viruses compared to non-transfected cells, where the virus establishes a non-productive infection. As the GD3 synthase directs ganglioside synthesis away from GD1a and GM1a, the resistance of the transfected cells suggests these structures to be required for infection with the respective virus. Furthermore, the infection could be restored by addition of the respective gangliosides. Similar results were achieved in studies with murine polyomavirus VLPs and GM95 mouse cells, lacking the glucosylceramidetransferase gene (Smith *et al.* 2003). In addition, later studies with the GD3 synthase transfected C6 glioma rat cell line have suggested gangliosides to be receptors for the bovine adeno-associated virus from the dependovirus family (Schmidt & Chiorini 2006).

There is strong evidence that parvovirus B19 uses neutral GSLs as receptors. An early study showed that the virus agglutinated erythrocytes with the P_1 and P_2 but not the p phenotypes of blood group P (Brown *et al.* 1993). In contrast to the latter, the P_1 and P_2 phenotypes are characterized by the expression of globotria- and globotetraosylceramide (Gb_3, Galα4Galβ4GlcβCer; Gb_4, GalNAcβ3Galα4Galβ4GlcβCer). In addition, direct binding of virus capsids to Gb_4 was demonstrated using a thin layer chromatogram binding assay. A subsequent publication demonstrated that, in contrast to individuals with the P_1 or P_2 phenotype, none of 11 investigated individuals with the p phenotype of blood group P had detectable amounts of Parvovirus B19-specific IgG antibodies (Brown *et al.* 1994). These studies, together with the observation that Gb_3 is only synthesized as a GSL (Yang *et al.* 1994) strongly suggest the receptor for parvovirus B19 to be a GSL.

GSLs are also clearly involved in HIV pathogenesis even though the precise mechanism is not fully understood (Viard *et al.* 2004). Several studies have proposed galactosylceramide or sulfatide to function as receptors for HIV in CD4 negative cells such as neuroglial, colorectal epithelial and vaginal epithelial cells (Viard *et al.* 2004). In these studies, direct binding of virus to GSLs on thin layer plates and inhibition of infection of cells by antibodies directed against GSLs, were demonstrated. However, it is not clear whether these cells are infected by the virus *in vivo*.

Secondly, also Gb_3 and GM3 have been demonstrated to bind to HIV (Hammache *et al.* 1999). In addition, these GSLs have been suggested to facilitate virus fusion (Viard *et al.* 2004). However, cell culture studies with a glucosylceramide synthase inhibitor suggest

that the presence of Gb_3 correlate inversely to susceptibility to HIV infection (Ramkumar *et al.* 2009). A protective role for Gb_3 has also been suggested by studies of peripheral blood-derived mononuclear cells (PBMCs) from individuals with Fabry disease, who accumulate Gb_3, (Lund *et al.* 2005) and from individuals with different P blood groups (Lund *et al.* 2009). The role for Gb_3 in HIV pathogenesis is puzzling, as it seems to function as both a receptor and as a decoy receptor. A speculation about the cause of these contradictory results could be that Gb_3 has different functions in different membrane domains. In that case the cells studied would differ when it comes to the distribution of Gb_3 in the membrane.

GSLs are also receptors for a large number of bacterial toxins (Karlsson 1995). These toxins typically consist of a single A subunit and five B subunits and are thus designated AB_5 toxins. The B subunits are responsible for the attachment of the toxin. The toxic A subunit is subsequently delivered into the target cell. Cholera toxin is the most studied AB_5 toxin and was demonstrated to bind to GM1 already in 1973 (Holmgren *et al.* 1973). Another AB_5 toxin is the Shiga toxin which binds to Gb_3 (Lindberg *et al.* 1987). Recently, a model for the mechanism by which Gb_3 may function as a receptor for Shiga toxin was proposed (Romer *et al.* 2007). The toxin was demonstrated to induce invaginations in giant unilamellar vesicles containing phospholipids (DOPC), a fluorescent dye, cholesterol and Gb_3. The authors proposed that the interactions between clusters of toxins and Gb_3 induced an asymmetric stress in the membrane, which caused the invaginations. In analogy with this, the degree of saturation of the fatty acid of Gb_3 was demonstrated to be crucial for the process.

Interestingly, many of the viruses exploiting GSLs as receptors are non-enveloped. It could be assumed that the rigid organization of the receptor binding sites in a protein capsid provides better opportunities for induction of tension in the host cell plasma membrane, than the mobile organisation of glycan binding proteins observed in lipid envelopes.

1.3.2. Receptors, species and viral tropism

Recent RNAi screens have indicated that at least HIV, West Nile and dengue virus each are dependent on hundreds of host proteins for successful infection (Brass *et al.* 2008, Krishnan *et al.* 2008, Zhou *et al.* 2008). Still, the distribution of receptors is a prime determinant of tropism for many viruses (Arnberg 2009, Marsh & Helenius 2006, Webby *et al.* 2004). Human influenza A virus has a preference for sialic acid linked in α2,6 conformation to galactose whereas avian influenza A has a preference for those in a α2,3-linkage. To be able to infect humans efficiently, an avian influenza virus has to adopt to bind to α2,6-linked sialic acid, as the target cells in the human upper respiratory tract express very little α2,3-linked sialic acid (Matrosovich *et al.* 2004). In recent years about 200 humans have died from infections with avian H5N1 influenza viruses. Fortunately so far very limited human to human transmission of these viruses has been observed (Abdel-Ghafar *et al.* 2008). The infections seem to have been located in the lower respiratory tract of the infected individuals, where sialic acid in α2,3-linkage is comparatively widely distributed (Shinya *et al.* 2006, van Riel *et al.* 2006). It has been suggested that virus from the lower airways is not as efficiently spread by sneezing and coughing as virus from the upper airways (Shinya *et al.* 2006).

The importance of the presence of receptors for host tropism is also illustrated by the successful development of a mouse model for rhinovirus infection (Bartlett *et al.* 2008). This was achieved by replacing the extracellular domain of the receptor, intracellular adhesion molecule 1 (ICAM-1), with the human counterpart. Another example is Hepatitis C virus, which has been hard to cultivate in cell culture. However, after the recent identification of occludin as an additional receptor for the virus, all cells investigated, expressing all the the four known receptors, were susceptible to infection with the virus (Pietschmann 2009, Ploss *et al.* 2009).

1.3.3. Natural decoy receptors

Most epithelial cells in the human body are covered by a mucus layer, acting as a physical barrier protecting the underlying cells from microbes, dehydration and physical as well as chemical injury. Major components of the mucus layer are the mucins, a family of high molecular weight glycoproteins (Linden *et al.* 2008, Thornton *et al.* 2008). O-glycans typically comprise more than 70% of the mass of mucins and are essential for the gel properties of the mucus layer. In addition these glycans function as decoy receptors trapping various microbes (Linden *et al.* 2008). The mucus layer is not static but moves and thus transports the trapped microbes away from the underlying tissue.

1.3.4. Multivalency

Protein-carbohydrate interactions typically show a low affinity with Kd values in the low mM range (Collins & Paulson 2004). However, these interactions are often strengthened by multivalency, which may result in an essentially irreversible binding (Mammen *et al.* 1998). Many lectins are multivalent. In addition, many of the monovalent lectins are membrane associated and may thus be multivalently presented on the cell surface (Varki *et al.* 2008). On the glycan side of the interaction multivalency may be achieved in several different ways (Gabius 2008). Firstly, branching may be used to present several copies of an epitope on the same glycan. Secondly, one protein may present several glycans containing the epitope, as exemplified by mucins. Thirdly, different glycoconjugates may be clustered in spatial vicinity, as, for instance, GSLs in lipid rafts. The arrangement of multiple copies of a few capsid proteins forming a viral capsid is optimal for multivalent interactions. Thus, it is not surprising that most non-enveloped viruses rely on multivalency for cell surface attachment (Helenius 2007, Mammen *et al.* 1998). Furthermore, membrane viruses typically have multiple copies of the receptor binding proteins in the membrane (Helenius 2007, Mammen *et al.* 1998). Thus, multivalency is a characteristic for viral attachment, and it is not restricted to glycan binding viruses (Helenius 2007).

The concept of multivalency has been extensively described (Mammen *et al.* 1998). According to the review article, the increase in avidity of a multivalent compared to a monovalent binding is due to a decrease in the rate of dissociation (k_{off}), rather than an increase in the rate of association (k_{on}). This can be explained by assuming that if one binding in a multivalent interaction is dissociated the other bonds keep the dissociated binding partners in close proximity, thus increasing the probability of re-binding. In contrast, the thermodynamic cost for the first binding in the multivalent interaction is approximately the same as the cost of the analogous monovalent interaction, giving the similar k_{on}-values. However, the advantages of multivalency are not restricted to binding

Table 2 The strongest associations identified between resistance to infectious diseases and common gene polymorphisms according to Hill (2006).

Infectious agent	Gene/protein
Plasmodium falciparum malaria	Haemoglobin (sickle cell disease)
Plasmodium vivax malaria	Duffy antigen receptor for chemokines (*DARC*)
Malaria	Erythrocyte membrane band 3
HIV-1	Chemokine receptor 5 (*CCR5*)
Creutzfeldt-Jacob prion disease	Prion protein (*PRPN*)
Norovirus	*FUT2*

strength. In the review article, a number of characteristics of multivalent interactions are described that are not present in monovalent interactions. The ones most applicable to virus carbohydrate interactions, apart from achieving very tight binding from ligands with low surface area, are; grading biological responses; creating new interactions by mixing, matching and multiplying existing ones; achieving large contacts between biological surfaces; inducing changes in the morphologies of large structures; inducing changes in the distribution of molecules in a membrane and signalling through oligomerization. These characteristics may be used by viruses in different steps in the entry into the host cell. Thus, the dependence on receptor valency for binding to one receptor, several co-receptors or attachment factors may be used to identify and adhere to the target cell. Likewise, induction of changes in the morphology of the membrane or signalling achieved through oligomerization of receptors may be used to promote entry into the cell.

1.3.5. Modulation of glycan presentation

In biological systems, glycans are often used to fine tune responses (Raman *et al.* 2005, Taylor & Drickamer 2007). Glycan structures may be modulated by other mechanisms than multivalency. A recent study has demonstrated that the addition of the core fucose and the bisecting GlcNAc affects the conformation or presentation of terminal epitopes on N-glycans (Andre *et al.* 2007). In addition, these modifications were demonstrated to affect the glycan biorecognition, both in lectin binding studies and in studies of neoglycoprotein serum half time in mice.

1.4. Host genetics and infectious diseases

Recent advances in genomics have improved the capability to track down genes involved in diseases (Frazer *et al.* 2007). Consequently, a number of large scale genome-wide association studies have identified novel genes involved in complex diseases such as diabetes (Manolio *et al.* 2008). A few such large scale genome-wide association studies have also been conducted for infectious diseases, providing expectations for the future (Burgner *et al.* 2009, Fellay *et al.* 2007).

The six strongest associations between resistance to infectious disease and common gene polymorphisms (minor allele frequency>0.01) identified so far are listed in Table 2 (Hill 2006). The association between secretor status and norovirus infection is one of these associations. The list also includes two other associations considered to be linked to receptor deficiency (*DARC* and *CCR5*). Notably, both *FUT2* and *DARC* are related to blood groups.

For a number of other pathogens, weaker associations between blood groups and susceptibility to infection have been identified (Moulds & Moulds 2000). One example is the resistance of individuals with the rare p phenotype of blood group P to infection with parvovirus B19 (section 1.3.1). Another example is the association between blood group O and severe cholera infection, even though the mechanism behind this association is not yet understood (Glass *et al.* 1985, Hill 2006).

1.4.1. Secretor status

Secretor status has been linked to susceptibility to infection with a number of pathogens in addition to norovirus. Among the best characterized is the protection of secretors from urinary tract infections caused by *E. coli* (Ilver *et al.* 2003, Le Pendu *et al.* 2006). Early studies revealed that *E. coli* recognized GSLs carrying the epitope Galα4Gal, characteristic for the globoseries (Bock *et al.* 1985). Later, two extended globoseries GSLs (Neu5Acα3Galβ3GalNAcβ3Galα4Galβ4GlcβCer and Neu5Acα3Galβ3(Neu5Acα6)GalNAcβ3Galα4Galβ4GlcβCer), were shown to be especially good binders (Stapleton *et al.* 1998). The basis for the association between susceptibility to urinary tract infection and secretor status seems to be that these two glycolipids are specific for non-secretors. In secretors, the biosynthesis is instead directed towards Globo H (H type 4) (Fucα2Galβ3GalNAcβ3Galα4Galβ4GlcβCer), which is a weaker binder (Ilver *et al.* 2003). Non-secretors have also been suggested to be predisposed to infections with *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*, even though the basis for these associations has not been resolved (Le Pendu *et al.* 2006). A recent study has suggested that children with the Le^{a+b-} phenotype have increased susceptibility to diarrhea caused by enterotoxigenic *E. coli* expressing colonization factor I group fimbriae (Ahmed *et al.* 2009).

In contrast to the mentioned bacterial infections, non-secretors are underrepresented among patients suffering from infections with influenza viruses A and B, rhinoviruses, respiratory syncytial virus and echoviruses (Raza *et al.* 1991). In addition, two studies have suggested a lower risk for non-secretors of getting infected with HIV-1 (Ali *et al.* 2000, Blackwell *et al.* 1991). In contrast, one study has reported the opposite association, or more precisely a lower Le^{a-b+} phenotype frequency among HIV infected patients compared to healthy blood donors (Puissant *et al.* 2005). Furthermore, a recent study has identified an association between *FUT2* genotype and HIV-1 progression, with non-secretors being overrepresented among long-term non-progressors compared to progressors (Kindberg *et al.* 2006).

Many studies have investigated associations between *H. pylori* infection and secretor status, as the BabA adhesin recognizes secretor gene dependent glycans (Boren *et al.* 1993, Ilver *et al.* 1998). However, the association does not seem to be as strong as expected (Azevedo *et al.* 2008), probably because of the many other binding specificities identified for the bacterium (Angstrom *et al.* 1998, Gold *et al.* 1993, Kamisago *et al.* 1996, Mahdavi *et al.* 2002, Miller-Podraza *et al.* 2009, Teneberg *et al.* 2002). The possibilities for an association between secretor status and predisposition to infection and lung disease in cystic fibrosis patients have also been discussed. However in a recent study including 808 patients homozygous for the ΔF508 mutation in the cystic fibrosis gene, no polymorphisms in the *ABO*, *FUT2* or *FUT3* genes were found to correlate with severity of disease or *P. aeruginosa* infection (Taylor-Cousar *et al.* 2009).

Secretor status has also been associated with non infectious diseases. In a recent genome-wide study to identify loci that influence plasma vitamin B12 levels, a strong association to *FUT2* was identified. Non-secretors showed higher B12 levels than secretors (Hazra *et al.* 2008).

1.5. Norovirus-glycan interactions

Clinical studies during the late 1970s and early 80s were confusing concerning the linkage between antibodies against the Norwalk virus and susceptibility to illness (Baron *et al.* 1984, Dolin *et al.* 1972, Parrino *et al.* 1977). Especially puzzling was that some individuals who didn't have antibodies against norovirus were constantly resistant to challenge (Johnson *et al.* 1990). In addition, a study published in 1982 indicated a familial clustering of resistance to norovirus, suggesting a genetic factor to be involved (Koopman *et al.* 1982).

The successful production of norovirus VLPs facilitated binding studies to search for potential receptors (Jiang *et al.* 1992). A breakthrough came in 2000 when RHDV was demonstrated to bind to the histo-blood group antigens H type 2 and A type 2 (Ruvoen-Clouet *et al.* 2000).

1.5.1. Norwalk virus challenge studies and VLP binding studies

Two years after the demonstration that RHDV recognized α1,2-fucosylated glycans (Ruvoen-Clouet *et al.* 2000) researchers from the same group showed that the Norwalk virus VLP also recognized the H antigen (Marionneau *et al.* 2002). In contrast to RHDV, the Norwalk virus primarily recognized H antigens based on the type 1 and type 3 chains. This study also showed that Norwalk VLPs recognized epithelial cells of the gastroduodenal junction from secretor-positive, but not from secretor-negative individuals. A challenge study demonstrating the resistance of non-secretors to infection with the Norwalk virus was published in 2003 (Lindesmith *et al.* 2003). A few years later the authors of an earlier challenge study were able to include secretor status in their analysis, by genotyping for secretor status using the serum samples available (Hutson *et al.* 2005). Together these two challenge studies included 97 secretors and 30 non-secretors. Seventy-six of the secretors, but none of the non-secretors, showed symptoms of infection, excreted detectable amounts of viral RNA in feces or showed an increase in anti-Norwalk virus antibody titers. Furthermore, even though not statistically significant, secretor-positive blood group B individuals were underrepresented among the infected in both studies. In addition, *in vitro* studies showed weaker binding for Norwalk virus VLPs to saliva from secretor-positive B than from A and O individuals (Harrington *et al.* 2002a, Lindesmith *et al.* 2003, Marionneau *et al.* 2005). Similarly, another study showed that Norwalk VLPs hemagglutinated blood group O, A an AB erythrocytes, but only few blood group B erythrocyte samples (Hutson *et al.* 2003).

1.5.2. Binding studies of other norovirus VLPs

The glycan repertoire of the mucins in saliva resembles the one on the epithelial cells in the gastrointestinal tract (Ravn & Dabelsteen 2000). A number of studies have used ELISAs with immobilized saliva samples to study norovirus binding (Marionneau *et al.* 2002, Tan & Jiang 2005a). VLPs from a large number of norovirus strains have been assayed against saliva samples from individuals of different ABO(H) and Lewis histo-

blood groups (Table 3). Binding information for specific glycans have been achieved in a similar assay with immobilized neoglycoproteins or polyacrylamide (PAA) glycoconjugates.

By combining the saliva ELISA and the neoglycoprotein/PAA ELISA, different binding patterns have been identified for different strains of noroviruses (Harrington *et al.* 2002a, Huang *et al.* 2003, Huang *et al.* 2005, Shirato *et al.* 2008). A general conclusion from these studies is that most strains from the clinically common genoclusters only bind to saliva samples from secretor-positive and not from secretor-negative individuals. One group consisting of the VA387 and Grimsby VLPs, both from the dominating GII.4 genocluster, showed binding to secretor-positive individuals of all ABO groups whereas another group did not recognize saliva from blood group O individuals (Table 3). In the latter group the Paris Island (GII.3) and MOH (GII.5) VLPs were localized since they only recognized saliva from secretor-positive blood group A and B individuals. The Mexico (GII.3) strain recognized saliva from secretor-positive blood group A individuals only, but was included in this group as it recognized BSA conjugated with the B trisaccharide. Interestingly, some strains showed a secretor gene independent binding pattern. Of these, one group (best exemplified with the VA207 strain (GII.9)) seems to recognize α1,4-linked fucoses and consequently binds to saliva from all Lewis positive

Table 3 The saliva binding pattern of a number of VLPs, as determined by ELISA with saliva samples from non-secretors and secretors of blood group O, A and B. The binding strength have been scored as no binding (-), intermediate binding (+) and strong binding (++). The data is adapted from Harrington et al. (2002a), Huang et al. (2005), Lindesmith et al. (2008) and Marrioneau et al. (2005).

Strain	Genocluster	Non-secretor	Secretor		
			O	A	B
VA387	GII.4	-	+	++	++
Grimsby	GII.4	-	+	++	++
1987	GII.4	-	++	++	++
1997	GII.4	-	++	++	++
2002	GII.4	-	++	++	++
NV	GI.1	-	++	++	+
MOH	GII.5	-	-	++	+
Paris island	GII.3	-	-	+	+
Mexico	GII.3	-	-	++	-
C59	GI.2	-	-	+	-
BUDS	GII.2	-	-	++	-
SMV	GII.2	-	-	-	+
VA207	GII.9	+	+	+	-
OIF	GII.13*	++	+	-	-
2002a	GII.4	+	++	++	++
DSV	GI.3	-	-	-	-
VA115	GI.3	-	-	-	-
Hawaii	GII.1	-	-	-	-
2004	GII.4	-	-	-	-
2005	GII.4	-	-	-	-

* 81 % amino acid identity to Fayetteville, GII.13

individuals. In addition, some strains (e.g. DSV (Desert shield virus, GI.3) and Hawaii (GII.1)) did not recognize any saliva samples or neoglycoconjugates assayed. When comparing the amino acid sequence of the major capsid protein, strains with a similar binding pattern tended to cluster in the phylogenetic tree (Huang *et al.* 2005). However, no clear correlation could be established suggesting that a small number of amino acid mutations may alter the binding pattern. In support of this, a recent study has suggested that VLPs from different epidemic groups of GII.4 strains show differences in binding pattern (Table 3) (Lindesmith *et al.* 2008). Notably, one VLP constructed from a strain isolated in 2002 (2002a), bound to saliva from both secretors and non-secretors. However, these different GII.4 binding patterns have been questioned in a study from another research group (Tan *et al.* 2008c).

The results from the saliva and neoglycoconjugates ELISAs have been validated by experiments showing that the VLP-binding may be blocked by anti-blood group specific antibodies or glycosidases (Huang *et al.* 2003, Huang *et al.* 2005). In addition, the interaction between norovirus VLPs and glycans has been studied in a few other assays. A reversed ELISA, where VLPs were immobilized and interacting biotinylated polyacrylamide conjugates assayed for binding, was reported (Harrington *et al.* 2002a). In recent studies surface plasmon resonance has been used for label-free detection of VLPs binding to immobilized monovalent glycans (Shirato *et al.* 2008) and multivalent glycoconjugates (Choi *et al.* 2008).

In addition to VLP binding studies, RT-PCR has been used to detect norovirus captured from clinical stool specimens using magnetic beads conjugated with glycans (Harrington *et al.* 2004). Native viruses have also been assayed in the traditional saliva-ELISA (Thorven *et al.* 2005). Even though no systematic comparison has been performed between the binding characteristics of VLPs and native viruses, the studied native viruses showed similar binding patterns compared to the related VLPs. The relevance of the use of VLPs is furthermore strengthened by the match between VLP binding and infectivity of the Norwalk virus (Harrington *et al.* 2002a, Hutson *et al.* 2005, Lindesmith *et al.* 2003, Marionneau *et al.* 2005). As described in the next section GI.3 viruses show similar patterns in infectivity and saliva binding (Nordgren 2009, Shirato *et al.* 2008).

A limitation to most of the neoglycoconjugates used in the described binding studies is that the glycans are short, composed merely of the minimal antigen epitopes. Thus, the impact of different core structures and modifications closer to the reducing end has been poorly addressed.

In addition to the binding to ABO(H) and Lewis histo-blood group structures a specific binding of norovirus VLPs have been identified to heparan sulfate (Tamura *et al.* 2004). In this study three GII VLPs were shown to bind to a number of cell types in a heparan sulfate dependent manner, whereas two GI VLPs bound only weakly via heparan sulfate. Cell culture binding of the GII VLPs could be blocked by preincubating the VLPs with the glycosaminoglycans heparan sulfate, chondroitin sulfate and dermatan sulfate, but not with hyaluronic acid which lacks sulfate groups. Heparan sulfate was suggested to be the cell surface molecule responsible for the binding since pre-treatment of the cells with heparinase, but not with chondrotinase, reduced the binding.

The principal novel finding that GII VLPs also recognize SLe^x and structural analogues, as described in Paper II, will be discussed in section 4.2.

1.5.3. Outbreak studies

The influence of the secretor status on susceptibility to norovirus infection in authentic norovirus outbreaks was investigated firstly in Sweden (Thorven *et al.* 2005). In the study, 38 symptomatic individuals from three outbreaks caused by GII.4 strains at a hospital were genotyped for secretor status. The same analysis was performed on 15 symptomatic individuals from three community outbreaks caused by GI.6 and GII.6 strains. Thus, in total, 53 symptomatic and 62 asymptomatic individuals were genotyped for polymorphisms of *FUT2* at nt 385, 428 and 571. As expected, mutations were identified only at nt 428. Strikingly, no non-secretors were identified among the symptomatic individuals. Fischer's exact test was used to show that the distribution of secretor status was significantly different between the symptomatic and asymptomatic individuals ($p<0.00001$) as well as between the symptomatic individuals and 104 Swedish blood donors ($p<0.0002$). As the secretor status was determined by genotyping, the influence of homozygosity and heterozygosity could be determined. No difference was identified between heterozygous and homozygous secretors. Subsequently, the resistance of non-secretors to GII.4 strains in authentic outbreaks has been confirmed by a similar study in Denmark ($p<0.003$, symptomatic vs asymptomatic) (Kindberg *et al.* 2007). Similar results were also obtained in a study of a GII.4 outbreak in China ($p<0.003$, symptomatic vs asymptomatic), where secretor status was determined by phenotyping of saliva samples in an ELISA with monoclonal antibodies. (Tan *et al.* 2008b). In the latter study, the weak-secretors were grouped with the secretors. In addition, a number of studies of noroviruses from other genogroups have reported that only secretors were identified among the symptomatically infected patients, even though the materials were not large enough to prove any statistical correlation (Bucardo *et al.* 2009, Tan *et al.* 2008b).

Reports of symptomatic infections in non-secretors do exist. In one study, 4 out of 24 symptomatically infected individuals were found to be non-secretors (Rockx *et al.* 2005b). The GI.3 virus, causing the outbreak investigated, could be detected in stool samples from all four of the infected non-secretors. The secretor status was determined by measuring the inhibition potential of saliva samples in a microtiterplate hemagglutination assay with blood group O erythrocytes and an anti-H lectin. Similar results were achieved in a recent study of a GI.3 outbreak in Sweden. Using both pheno- and genotyping, 7 out of the 33 symptomatic individuals in the outbreak were shown to be non-secretors (Nordgren 2009). In addition, even though not statistically significant an unexpectedly low proportion of the symptomatic individuals in these studies were blood group B secretors, indicating a similar protection of these individuals as observed for the Norwalk virus. Interestingly, the disease profile identified for these GI.3 strains agrees with the saliva and neoglycoconjugate binding pattern of a GI.3 VLP (Shirato *et al.* 2008). In addition, infection of one non-secretor (geno- and phenotyped), has been demonstrated in a challenge study with the Snow Mountain virus (GII.2) (Lindesmith *et al.* 2005).

A few additional outbreak studies have addressed the impact of ABO-blood group and Lewis status as well as secretor status on susceptibility to norovirus infection (Bucardo *et al.* 2009, Tan *et al.* 2008b). The already described Chinese outbreak study demonstrated that blood group A individuals were overrepresented among the infected individuals compared to the asymptomatic individuals ($p<0.02$) (Tan *et al.* 2008b). Blood group O individuals, in contrast, showed the opposite distribution ($p<0.03$). No binding studies

were performed with VLPs representing this outbreak virus or the outbreak virus itself. However, it is interesting to note that many GII.4 VLPs recognize saliva samples from blood group O individuals less well than those from blood group A, B and AB individuals (Table 3).

1.5.4. Protective effects of human milk

Human milk is known to contain histo-blood group antigens in the form of free glycans, GSLs and glycoproteins (Le Pendu 2004). *In vitro* studies have demonstrated that milk from secretor mothers, in contrast to non-secretor mothers, inhibits binding of norovirus VLPs to α1,2-fucosylated glycans (Jiang *et al.* 2004, Le Pendu 2004). Phenotyping of milk samples have suggested that the blood group A and B antigens are not expressed in milk (Jiang *et al.* 2004). In analogy with this, no blocking activities were observed for the MOH (GII.5) VLP recognizing only blood group A and B antigens (Jiang *et al.* 2004). Clinical evidence of breast-feeding protection of infants has been obtained in an infant-mother cohort in Mexico (Morrow *et al.* 2004). Calicivirus infections occurred less often in infants fed with milk containing higher levels of α1,2-linked fucoses.

1.5.5. The viral carbohydrate binding sites

One study of Norwalk virus VLP binding to CaCo-2 cells described a monoclonal antibody that could inhibit the virus from binding to the cells (White *et al.* 1996). The antibody binding epitope was mapped to the P2 domain of the capsid protein, suggesting this domain to be responsible for the cell binding. In addition, the demonstration that P particles and P dimers, but not S particles, maintained the binding specificities of the corresponding VLPs strongly suggested the binding site to be located in the P domain (Tan *et al.* 2004a, Tan & Jiang 2005b). A conserved RGD motif, surrounded by strain specific amino acids, was identified in the P2 domain by multiple sequence alignment analysis (Tan *et al.* 2003). Site-directed mutagenesis suggested the identified motif to be involved in the binding pocket. In another study, evolutionary trace was used to locate the binding pocket to another position (Chakravarty *et al.* 2005). Alignment analysis followed by a superimposition on the Norwalk virus capsid protein structure suggested the two sites to be distinct, but located close to each other (Chakravarty *et al.* 2005).

The location of the actual binding pocket was eventually revealed when the X-ray crystal structure of the P dimer of the GII.4 strain VA387 in complex with the blood group B trisaccharide was solved (Fig. 11) (Cao *et al.* 2007). In fact, the amino acids proposed by the evolutionary trace studies (Chakravarty *et al.* 2005) were surrounding the binding pocket. The crystal structure showed that the α1,2-linked fucose was tightly bound by interactions with 7 amino acids, all highly conserved in GII strains. The terminal αGal residue was also bound whereas the βGal residue seemed to act more like a scaffold. Subsequently, the amino acids involved in the recognition of the glycans have been confirmed by site-directed mutagenesis (Tan *et al.* 2008c). The structure of co-crystals, identifying the binding site of the Norwalk virus has also been solved (Bu *et al.* 2008, Choi *et al.* 2008). Interestingly, this site has a location distinct from the binding site in the VA387 structure. One of the Norwalk structures revealed the basis for the binding to blood group A- and H-, but not B-terminated glycans (Choi *et al.* 2008). The structure showed that the αFuc residue of the H type 1 structure and the αGalNAc residue of the A structure were engaged in similar hydrophobic interactions with a tryptophan (Trp-375).

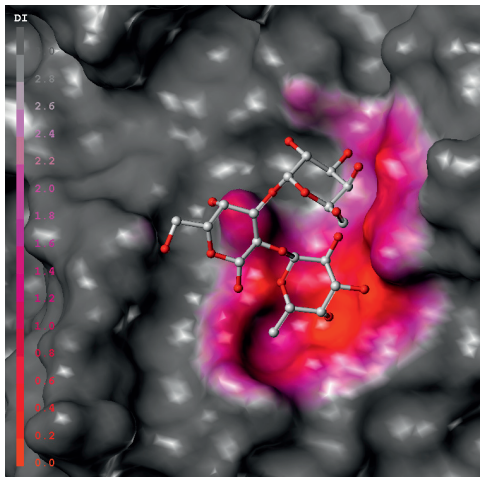


Figure 11
The blood group B trisaccharide in the binding pocket of the VA387 (GII.4) strain. The coordinates were obtained from PDB id 2obt Cao et al. (2007) and the image was generated using Sybyl (Tripos Inc., St Louis). The surface of the protein is color coded according to the distance between the glycan and the surface residues with a cut-off of 3Å. By courtesy of C. Koppisetty, Biognos AB.

This interaction may not take place for the αGal residue of blood group B structures, as this has a hydroxyl group instead of an N-acetamido group. Thus, the H type 1 pentasaccharide and the A trisaccharide were bound in the same binding site, but in different modes. Furthermore, the αFuc seemed to be minimally involved in the binding of the blood group A trisaccharide.

1.6. Antiviral therapy

1.6.1. Attachment inhibitors

Viruses constitute a diverse group of infectious agents and so far no common Achilles' heel has been identified to exploit for therapy against viruses. Thus, no drugs comparable to the bacterial broad spectrum antibiotics have been developed for viruses (Coen & Richman 2007).

Inhibition of viral attachment is an appealing antiviral strategy as it prevents all subsequent steps in the infection process and keeps the virus accessible for the immune system. Furthermore, the possibility for pathogens to develop resistance to a drug targeting the receptor binding site is expected to be low. This is because most amino acids interacting with the receptor must be conserved to keep the interaction (Coen & Richman 2007).

Anti-adhesion drugs mimicking glycans are not only of relevance for virus therapy, but also against glycan binding bacteria and bacterial toxins (Bovin *et al.* 2004, Collins & Paulson 2004, Imberty *et al.* 2008, Mammen *et al.* 1998). Furthermore, much effort has been invested in the development of selectin antagonists based on SLe^x-analogues, with applications for inflammatory diseases and cancer metastasis (Magnani 2004). Promising *in vivo* results for glycan based anti-adhesins have been obtained with dendrimers containing the globo trisaccharide (Galα4Galβ4Glc) in studies of mice challenged with Shiga toxin or enterohemorrhagic *E. coli* (Mulvey *et al.* 2003, Nishikawa *et al.* 2002). Other promising examples are the various sulfated polysaccharides that are in clinical trials as therapy against herpes simplex virus and as anti HIV-1 drugs (Ghosh *et al.* 2009).

Most attachment inhibitors are multivalent. The multivalency does not only enhance the binding strength, i.e. the competitive inhibition, but also provides the ability to block interactions through steric hindrance (Mammen *et al.* 1998). An additional mechanism for inhibition may be agglutination of the pathogens. Design of effective multivalent attachment inhibitors requires consideration of both enthalpy and entropy (Mammen *et al.* 1998). To make it simple: a flexible scaffold molecule has a large entropic cost upon binding whereas a rigid molecule has a large enthalpic cost, unless the geometric fit is perfect. In this aspect, bacteria and enveloped viruses, with mobile lectins may require different approaches than bacterial toxins and non-enveloped viruses with more rigid structures. The increasing structural information on carbohydrate-protein interactions provides opportunities for a structural design of inhibitors (Imberty & Varrot 2008, von Itzstein 2008). An alternative approach to synthetic constructs, is to genetically engineer probiotic bacteria to express receptor glycans for pathogens on their surface (Paton *et al.* 2006).

An example of a monovalent attachment inhibitor is Pleconaril (De Palma *et al.* 2008). This drug is in clinical trials for treatment of the common cold, caused by rhinovirus infection. The receptor (ICAM-1) binding site is located in a deep surface repression on the viral capsid and was early hypothesized to be inaccessible for antibodies (Rossmann 1989). Pleconaril binds into a hydrophobic pocket located underneath the floor of the receptor binding site. In the absence of the drug, this pocket is occupied by a lipid, suggested to be sphingosine (Ding *et al.* 2002). The binding of Pleconaril alters the conformation of the receptor binding site and hampers the binding to the receptor. As a second mechanism, the drug increases the rigidity of the virion, which prevents the uncoating process.

An alternative to blocking the receptor binding pocket on the pathogen is to target the receptor on the host cell. This has been a fruitful approach for HIV. One drug directed against the HIV co-receptor CCR5 has been approved and others are in clinical trials (Fox 2007). In analogy with the effect of these drugs, individuals who are homozygous for the Δ32 deletion in CCR5 are protected from HIV-1 infection (Dean *et al.* 1996). However, these individuals seem to have an increased susceptibility for infection with West Nile virus (Lim *et al.* 2006) and tick-borne encephalitis (Kindberg *et al.* 2008), which may have implications for usage of these drugs. Based on the successful treatment of patients with GSL storage diseases using glycosylation inhibitors, the use of these substances as a therapy against infections caused by GSL binding pathogens has been proposed (Svensson *et al.* 2006).

1.6.2. Other antiviral drugs

Among the most successful antiviral drugs are the protease and reverse transcriptase inhibitors directed against HIV, the interferon therapy for hepatitis B and C, Amantadine against influenza virus A and the neuraminidase inhibitors against influenza virus (Levine & Enquist 2007, von Itzstein 2007). The majority of the more than forty antiviral drugs approved are directed against viral enzymes (Coen & Richman 2007).

1.6.3. Vaccines

Vaccination is an efficient method to prevent viral infections. The complete elimination of natural smallpox by vaccination is among the most remarkable medical achievements of

the last century (Levine & Enquist 2007). The present Swedish national vaccination program includes vaccination against polio, measles, mumps and rubella virus. In total, vaccines have been developed for about 15 viral pathogens infecting humans (Graham & Crowe Jr 2007). Most vaccines generate long time immunity, whereas for the influenza virus new vaccine has to be developed for every season, because of the extensive antigenic drift. To be effective, a vaccine must generate neutralizing antibodies towards the virus. Since most such antibodies are directed against the receptor binding site on the virus, knowledge about the virus-receptor interaction is of relevance for vaccine development.

1.6.4. Anti-norovirus therapy

Only very few and recent studies have presented attachment inhibitors for norovirus. One study presented inhibitors based on α1.2-fucosylated glycans conjugated to hydrogels (Zhang *et al.* 2006). Another study identified 14 molecules showing 50% inhibition at <15μM in a VLP-saliva ELISA through a screening of a library of 5000 drug-like small molecules (Feng & Jiang 2007).

For several reasons we consider norovirus a promising target for anti-adhesion therapy. Firstly, the potential of abolishing the interaction between norovirus and α1,2-fucosylated glycans has been demonstrated in challenge and outbreak studies of different norovirus strains. In addition, the protection from gastroenteritis of breast-fed infants with secretor mothers is a direct indication of the potential of inhibiting the virus-glycan interaction (Morrow *et al.* 2004). Secondly, vaccine development for norovirus is challenging because of the complex immunity and the large antigenic diversity between different strains (Estes *et al.* 2000, LoBue *et al.* 2009, Tanaka *et al.* 2006). The recent demonstration of epochal evolution of norovirus GII.4 strains suggest that new vaccines have to be developed as new epidemic variants emerge similarly to the influenza vaccine (Allen *et al.* 2008, Lindesmith *et al.* 2008, Siebenga *et al.* 2007). Thirdly, norovirus infection is well suited for prophylactic therapy, given the large outbreaks caused in closed settings such as hospitals and cruise ships. This is of importance as the largest effect of anti-adhesion therapy is expected to appear when administered prior to exposition to the pathogen, in analogy with the neuraminidase inhibitors for influenza virus (Moscona 2005). Finally, the administration of a norovirus inhibitor may be simple, as it is to function in the gastrointestinal tract as a decoy mechanism. Thus, the drug does not have to be optimized for uptake or distribution, which lowers the risk for side effects in the rest of the body.

2. Aim

The aim of this thesis is to characterize the interactions between human noroviruses and host cell surface glycans. Knowledge about the carbohydrate binding specificities and the kinetics of different strains of norovirus may be used not only to predict susceptibility to infection, but also for development of anti-adhesion therapy. The long term goal of this study is to design a drug inhibiting the attachment of norovirus to host cell glycans.

The specific aims are:

- To investigate whether norovirus specific IgG antibody prevalence and titer correlate with secretor, Lewis or ABO histo-blood group status in Swedish blood donors.
- To characterize the glycan binding characteristics of human norovirus VLPs using a wide array of structurally and antigenically well defined glycoconjugates:
    - Saliva samples from Swedish blood donors, well characterized according to secretor, Lewis and ABO histo-blood group status
    - Neoglycoconjugates, with oligosaccharides synthetically linked to albumin or polyacrylamide
    - Glycosphingolipids, as naturally occurring membrane bound glycoconjugates
        - Chromatographed on thin layer-plates
        - Incorporated in laterally fluid supported lipid bilayers

3. Methodological considerations

3.1. Virus-like particles as a model for norovirus

One of the major obstacles in research on norovirus has been, and still is, the lack of cell culture methods for propagation of the virus. Consequently, most of the knowledge about the virus stem from epidemiological studies, challenge studies and studies using VLPs. When this thesis project started the murine norovirus had just been discovered (Karst *et al.* 2003) even though the propagation of the virus in mouse dendritic cells and macrophages was still to be revealed (Wobus *et al.* 2004). However, the symptoms of the norovirus infection in mice differ from the human norovirus infection (section 1.1.9). In addition, the glycome of the small intestine differs between mice and humans, as GSLs of the ganglioseries (and not of the lactoseries) are the most prominent in epithelial cells of mouse small intestine (Hansson *et al.* 1982, Iwamori & Domino 2004). When taking these aspects together, we considered VLPs to be the most favourable model to study human norovirus-host glycan interactions. The VLPs used are described in Table 4 and were produced in insect Sf9 cells using the baculovirus system (Jiang *et al.* 1992). VLPs may also be produced in mammalian cells using the venezuelan equine encephalitis virus system (Baric *et al.* 2002). This system, however, requires a high level of safety measures during laboratory work. Since the VLPs used in this thesis have been produced in different labs, some minor differences between the production protocols exist. Notably, the Houston VLP was produced using a recombinant baculovirus which contained the open reading frames coding for both the major and minor capsid proteins. In contrast, the baculoviruses used for the production of the other VLPs contained the open reading frame coding for the major capsid protein only. The Norwalk virus is the prototype norovirus strain and belongs to GI.1. The Chron1 strain belongs to GII.3 and was isolated from the first viral isolate from the chronically infected patient described in section 1.1.2 and 1.1.10. The Dijon and Houston strains both belong to GII.4, the genocluster responsible for more than half of all outbreaks of norovirus (section 1.1.5). The Dijon strain was isolated from an outbreak in France during the winter 1995-1996 (Nicollier-Jamot *et al.* 2003). The Houston strain was isolated from a stool sample from a 2-month old boy with gastroenteritis who was seen at the Texas Children's hospital in Houston in 2002 (Paper I).

3.2. Typing for ABO(H) and Lewis blood group status

The ABO(H) and Lewis blood group status of an individual may be determined either by pheno- or genotyping. In phenotyping, antibodies or lectins are used to detect the presence of the specific antigens, characterizing the various blood groups. In genotyping various

Table 4 The VLPs studied in this thesis.

Name	cluster	Genbank no	Used in	Original reference
Norwalk (NV)	GI.1	AAB50466	Paper II, III & IV	Jiang *et al.* 1992
Chron1	GII.3	AAP03029	Paper II	Paper II
Dijon	GII.4	AAL79839	Paper II, IV	Nicollier-Jamot *et al.* 2003
Houston (HOV)	GII.4	ABY27560	Paper I	Paper I

SNPs, known to characterize the activities of the enzymes synthesizing the antigens, are analyzed.

The main problem with phenotyping is the cross-reactivity of the antibodies (Manimala *et al.* 2007). Accurate phenotyping thus often requires the use of several different monoclonal antibodies. Clinically, phenotyping for the ABO(H) Lewis blood group antigens is mainly performed on erythrocytes (section 1.2.3). In the norovirus field, phenotyping has commonly been performed on saliva samples, which are used also for VLP-binding studies.

The major disadvantage with genotyping is that usually only the known SNPs are characterized. Uncommon inactivating mutations may thus be missed, leading to false positive results. *FUT2* genotyping in the Caucasian population is straight forward, since the G428A mutation is so dominating. In our lab about 300 Caucasians have been genotyped without ever finding the A385T or C571T mutations.

3.2.1. Saliva and plasma samples

The plasma and saliva samples used in Paper I and II are uniquely well characterized according to the secretor, Lewis and ABO blood group status of the donors. The material was collected from healthy donors arriving at the Sahlgrenska University hospital for plasmapheresis. From 105 individuals, EDTA-anticoagulated plasma was obtained and separated into plasma, buffy coat and erythrocytes. All of the 105 donors were phenotyped for ABO blood groups by hemagglutination and genotyped for inactivating *FUT2* G428A and *FUT3* T59G, T202C, C314T, G508A, T1067A point mutations. Fixed erythrocytes from 93 of the donors were in addition analyzed for Lewis blood groups by fluorescence activated flow cytometry (FACS) and the results were compared to the hemagglutination scores (Larson *et al.* 1999). Saliva samples were obtained from 81 of the 105 donors. These samples were collected as mixtures of parotid, sublingual, and submandibular secretions and stored at -20 °C. In Paper II, the material was further characterized by phenotyping the saliva samples for Le^a, Le^b, A, B and SLe^x antigens using ELISA.

The ABO(H) Lewis histo-blood group distribution of the material is presented in Table 1 in Paper I. The histo-blood group distribution of the individuals donating saliva samples is presented in Figure 2 in Paper II.

3.3. Glycosphingolipids

The GSLs used are listed in Paper III and were purified essentially as described (Karlsson 1987). The type 1 series GSLs originate from human meconium samples from single individuals or from meconium samples pooled according to ABO blood groups. The other reference GSLs used in this thesis originate from human erythrocytes pooled according to ABO blood groups or from the small intestines of dogs. The identity and purity of the reference GSLs were analyzed by ^{1}H-NMR spectroscopy (Paper III). Chemical shifts and coupling constants for the H1 anomeric protons and fucose H-5 and H-6 protons were compared to published values (Angstrom *et al.* 2004, Clausen *et al.* 1986, Clausen *et al.* 1985a). This analysis complemented previous characterizations by ^{1}H-NMR and mass spectrometry (Angstrom *et al.* 1982, McKibbin *et al.* 1982). Figure 12 shows the anomeric region of the ^{1}H-NMR spectra of the H type 1 and Le^a GSLs, used in Paper III and IV.

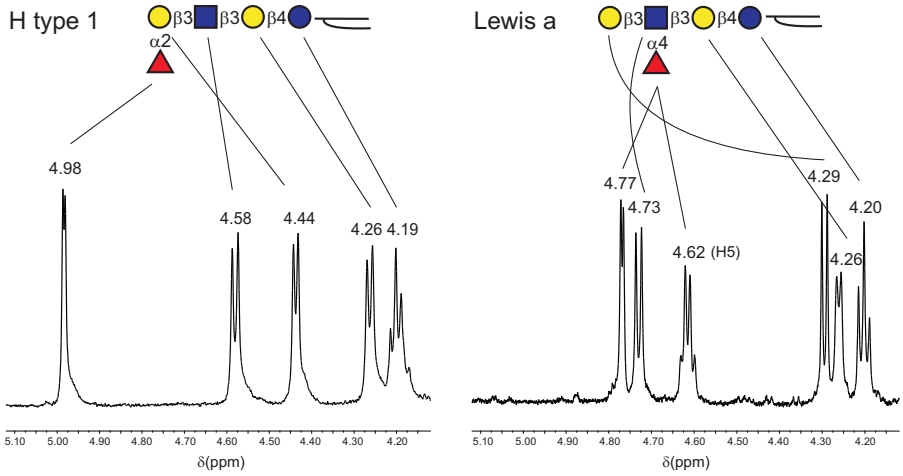


Figure 12
The anomeric region of the ^{1}H-NMR spectrum of the H type 1 and Lewis a GSLs in DMSO:D2O (98:2) at 303 K with corresponding shifts for each monosaccharide indicated. Both GSLs were judged to be more than 98% pure.

3.4. Antibody titers

An ELISA with immobilized VLPs was established to determine titers of norovirus specific IgG antibodies. The assay was used together with human serum samples in Paper I and is described there.

3.5. VLP binding studies

An ELISA was used to study VLP binding to saliva samples, serum albumin neoglycoconjugates and PAA neoglycoconjugates, whereas CBA and Quartz crystal microbalance with dissipation (QCM-D) monitoring was used to study VLP binding to GSLs. Antibodies were used for VLP detection in the ELISA. The CBA was established using radiolabeled VLPs, but was also used together with antibody detection. QCM-D is a label-free method not requiring use of antibodies.

3.5.1. ELISA

The most commonly used assay for characterization of norovirus VLP interactions with glycans is an ELISA with immobilized glycoconjugates (section 1.5.2). We used such an assay to study VLP binding to saliva samples and serum albumin conjugates. For the saliva samples we chose to coat saliva according to protein concentration, even though in most other studies coating has been according to certain dilutions. The reason for coating according to protein concentration was mainly to correct for differences in water content between the saliva samples. For detection of bound VLPs antisera obtained by immunizing rabbits or mice with purified VLPs were used. In addition also a human antiserum, of a blood group ABLeSe individual with a high titer of anti norovirus antibodies was used. An inhibition-ELISA was also established, where the VLPs were preincubated with inhibitors, before being added to wells coated with either saliva or neoglycoproteins.

An inverted ELISA was established for the PAA conjugates. Thus, VLPs were coated and interacting biotinylated PAA conjugates detected using peroxidase conjugated streptavidin. Because Norwalk VLPs have been reported to dissociate at pH above 8 (Ausar *et al.* 2006) the coating buffer for this assay was changed from carbonate buffer (pH 9.6) to PBS (pH 7.2). Notably, no antibodies were used in this assay. However, these experiments complemented results obtained with the standard ELISA.

The glycoconjugate ELISAs were mainly used in Paper II and are thoroughly described there. The neoglycoproteins and PAA conjugates used are listed with complete structures in Table I in the same paper.

3.5.2. Chromatogram binding assay

The chromatogram binding assay (CBA) is a classical method for studies of protein-GSL interactions. In this method GSLs are chromatographed on thin layer plates. Thereafter the plates are coated with plastic and overlayed with the glycan binding protein studied. The method was originally established for studies of interactions between cholera toxin and gangliosides (Magnani *et al.* 1980) and later for studies of other glycan binding proteins including viruses (Hansson *et al.* 1984). A CBA enables screening for binding structures in complex mixtures of GSLs. However, use of pure substances is preferred to accurately demonstrate binding activity for specific GSLs. The methodology has been extensively described (Karlsson & Stromberg 1987).

In Paper III a CBA was used together with ^{125}I-labeled Norwalk VLPs. The results were confirmed in Paper IV using antibody detection of non-labeled VLPs. The antibody detection was less sensitive than the radiography detection of ^{125}I-labeled VLPs and thus required higher concentrations of VLPs to be used. The CBA is described in detail in Paper III.

Radiolabeling of VLPs using ATE

In Paper III a method for radiolabeling of VLPs via an activated tin-containing ester (ATE, *N*-succinimidyl-3-tributylstannyl benzoate) was established (Fig. 13) (Garg *et al.* 1993). The alkylstannyl group of this compound is a good leaving group in the electrophilic substitution reaction with iodine resulting in the formation of a ^{125}I-containing succinimidyl ester. In the second step, this ester is conjugated to accessible lysine residues on the VLP. This two-step procedure admits quenching of the oxidizing agent before the addition of the protein, which eliminates the risk of oxidative damage to the protein. The ATE method thus provides a milder treatment of the VLPs than the commonly used Iodo-Gen method (Fraker & Speck 1978). The major advantages with the

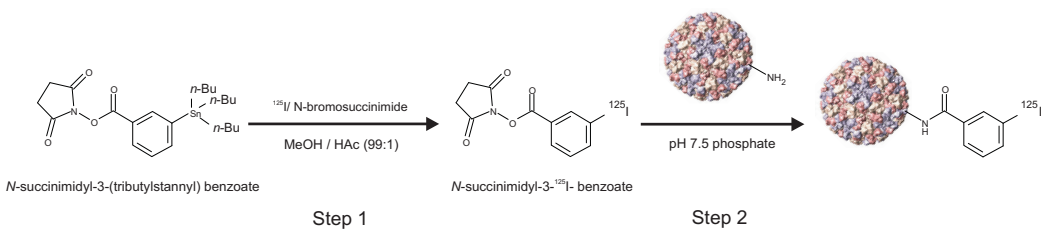


Figure 13
The chemical principles for iodination of VLPs using N-succinimidyl-3-tributylstannyl benzoate (ATE).

ATE method compared to the similar Bolton-Hunter method (Bolton & Hunter 1973) are that the ATE method is a one-pot procedure and that the benzene extraction step used in the Bolton-Hunter methodology is not required.

The incorporation of ^{125}I in the ATE labelling agent was controlled with Radio-HPLC before addition of the VLPs. A solid phase radio-binding assay was established and used in comparison with ELISA of non-labeled VLPs to verify that the labeling did not affect the VLP binding specificity to saliva samples.

3.5.3. Quartz crystal microbalance with dissipation (QCM-D) monitoring

The chromatogram binding assay is an ideal tool when screening GSLs for protein interactions. However, when further characterizing GSL-protein interactions, supported lipid bilayers provide a milieu more resembling the dynamic environment in the cell membrane.

Quartz crystal microbalance with dissipation (QCM-D) monitoring is an efficient method to study protein binding to GSLs incorporated in laterally fluid supported lipid bilayers (Fig. 14). QCM-D is an extension of QCM (Rodahl *et al.* 1995). The basis of both methods is the piezo electric properties of quartz crystals (Andreas Janshoff 2000). Due to this property an external electric field induces mechanical stress in quartz crystals. By cutting the crystals in a specific angle, a deformation in shear-mode may be obtained. If an alternating electric field is applied across the crystal, it is driven into mechanical oscillation and at certain frequencies resonance occurs. Importantly, the resonance frequency is dependent on the mass of the crystal. Adsorption of an object to the crystal therefore induces a change in the resonance frequency. This change is as a first approximation proportional to the mass coupled to the crystal. The resonance frequency, *f,* may be obtained by studying the decay of a free oscillation induced by a pulse of alternating current. By also recording the life time of the oscillation, information on changes in energy dissipation or the dampening, *D,* of the oscillating system may be obtained. The dampening gives information about the rigidity of the adsorbed object and is the basis for the QCM-D extension (Hook *et al.* 1998). Measurements of the *D* parameter can for instance be used to distinguish adsorbed lipid vesicles, inducing high dampening, from planar bilayers, inducing little dampening (Keller & Kasemo 1998). The combined *f* and *D* measurements make QCM-D well suited for studies of supported lipid bilayers as the formation of the bilayer from lipid vesicles may be continuously followed over time (Hook *et al.* 2008).

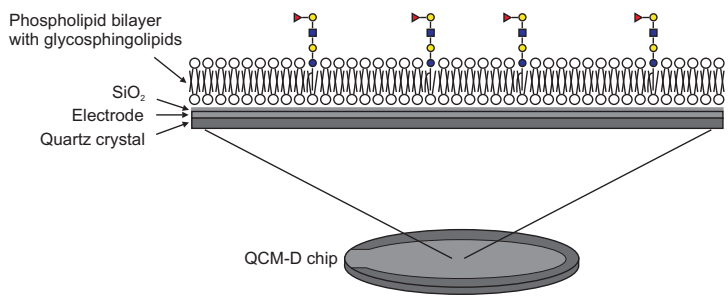


Figure 14
Schematic representation of GSLs in a supported lipid bilayer formed on a QCM-D chip.

QCM-D provides label-free monitoring of the kinetics of binding to surfaces. An estimation of the coupled mass in terms of the number of interacting molecules is however complicated by the influence of water coupled to the adsorbed molecules. In that perspective, new instruments combining QCM-D with surface plasmon resonance or reflectometry are promising (Reimhult *et al.* 2004, Wang *et al.* 2008).

QCM-D together with supported lipid bilayers was used in Paper IV. In these studies, a Q-Sense E4 instrument (Q-Sense AB, Västra Frölunda, Sweden) with 4 parallel 40 µl sample chambers was used. The working temperature for this instrument may be adjusted between 18°C and 45 °C. Our experiments were performed at 22 °C.

Normally, QCM-D experiments are performed in flow mode, keeping the bulk concentration of the analyte constant. However, because of limited amounts of VLPs our experiments were performed in batch mode. The concentration of VLPs used was in the same order of magnitude as in the CBA experiments with antibody detection (Paper IV). Each QCM-D experiment with bilayers containing 10 wt% of GSLs required 5 µg of GSLs per chamber. This is similar to the amount of pure GSLs used per lane in the CBA (2-5 µg) (Paper III and IV).

4. Results and discussion

When this project was initiated the first challenge study clearly demonstrating resistance of non-secretors to infections with the Norwalk virus had just been published (Lindesmith *et al.* 2003). Additionally, binding studies had suggested that a large number of norovirus strains recognize secretor gene dependent glycans (Harrington *et al.* 2002a, Huang *et al.* 2003). The next step was to investigate whether secretor status was associated with susceptibility to natural norovirus infections. We addressed this question in two studies. In the first one, symptomatic and asymptomatic individuals in authentic norovirus outbreaks were genotyped for secretor status (Thorven *et al.* 2005). This study revealed that among 53 symptomatically infected individuals not a single one was a non-secretor. The distribution of secretor status in this group was significantly different from the 20% non-secretors among 104 Swedish blood donors ($p<0.0002$) and the 29% non-secretors among the 62 asymptomatic individuals ($p<0.00001$) (Fisher's exact test). In the second study, we investigated concentrations of norovirus specific IgG antibodies in plasma from Swedish blood donors (Paper I). Our hypothesis was that if non-secretors are protected from norovirus infection, they should have lower titers of antibodies to the virus than secretors.

4.1. Antibody titers to norovirus GII correlate with secretor status (Paper I)

Titers of norovirus specific IgG antibodies were assayed in plasma samples from 105 Swedish blood donors. These donors constitute a uniquely well-characterized material considering ABO(H) histo-blood group glycans. They have been genotyped for *FUT2* and *FUT3* status and phenotyped for ABO and Lewis blood groups on erythrocytes (Larson *et al.* 1999). Norovirus specific IgG antibody titers were determined in an ELISA using immobilized VLPs from the Houston strain as antigen. This strain belongs to genocluster GII.4, which has been the dominating genocluster in outbreaks during recent years (section 1.1.5).

The result showed that the non-secretors had lower antibody titers than secretors ($p<0.0001$, Mann-Whitney) and were also more often antibody negative ($p<0.05$, Fisher's exact test). However, some non-secretors had high antibody titers (>1600), suggesting that secretor independent strains may exist. No significant difference in antibody prevalence or titers was identified between homozygous (SeSe) and heterozygous secretors (Sese). In addition, no significant differences were found between *FUT3* genotypes or different ABO phenotypes, neither in the whole material nor among the secretor-positive individuals. To exclude the possibility that secretor status was associated with susceptibility to viral infections in general, 63 randomly selected plasma samples were assayed for IgG antibody titers also towards rotavirus. No correlation of IgG titers with *FUT2*, *FUT3* or ABO blood groups was found in this case.

The identified association between antibody titers and secretor status does not only reflect GII.4 strains, but also other strains giving rise to antibodies cross-reacting with the Houston VLP. In contrast to outbreak studies, where susceptibility to one strain at the time is investigated, this study thus investigated susceptibility to a number of strains simultaneously. However, to determine which and how many strains is not trivial, since immunity to norovirus is complex (section 1.1.10). We concluded that non-secretors are

significantly less prone to be infected with GII noroviruses than secretors. This conclusion supports the hypothesis that non-secretors are relatively, but not absolutely, resistant to norovirus infection. In addition, our study suggests that secretor and non-secretor dependent virus strains have common antigenic epitopes.

Recently, we identified one non-secretor ($se^{428}se^{428}$) among 116 symptomatically infected individuals in a GII.4 norovirus outbreak (Carlsson *et al.* 2009a). RT-PCR was used to demonstrate the presence of norovirus in the stools of the symptomatically infected non-secretor, who was also phenotyped on saliva as Le^{a+b-}. In addition, ELISA binding studies demonstrated that the outbreak virus recognized saliva from one non-secretor individual. The study suggests that non-secretors can indeed get symptomatically infected by GII.4 viruses. In addition, one GII.4 VLP (2002a) has been demonstrated to bind to saliva samples from both secretors and non-secretors (Lindesmith *et al.* 2008).

4.2. Human noroviruses recognize SLe^x (Paper II)

To map the glycan binding characteristics of norovirus VLPs, an ELISA using immobilized neoglycoproteins or saliva samples was established. The first VLP to be investigated in the assay was Chron1. This VLP was constructed from the first viral isolate from the chronically infected patient described in section 1.1.2 and 1.1.10. Surprisingly, the Chron1 VLP showed a strong interaction with SLe^x-HSA. Initially, we thought that this novel binding specificity was related to the chronic infection caused by the virus, as SLe^x is a well known marker for inflamed tissue (section 1.2.3). However, subsequent experiments with the Dijon VLP, belonging to the common GII.4 cluster, demonstrated that also this VLP recognized SLe^x-HSA. In contrast, the Norwalk strain (GI.1) did not recognize this sialylated conjugate. The characteristics of the identified sialic acid dependent binding specificity were reported in Paper II. This paper also describes the binding properties of the Chron1 and Dijon VLPs to secretor gene dependent neoglycoproteins and to saliva samples from individuals representing different ABO(H) histo-blood groups.

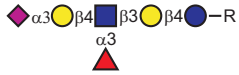
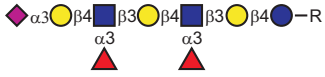
A number of 81 individuals, who donated plasma samples used in Paper I, also donated saliva samples. In order to further extend the ABO(H) and Lewis histo-blood group characterization of the donors, the saliva samples were phenotyped for A, B, Le^a, Le^b and SLe^x antigens, before being assayed for VLP binding. As the saliva binding characteristics of the Norwalk strain had already been well described (Marionneau *et al.* 2005), it was not included in the analysis. The result showed that both the Chron1 and the Dijon VLP recognized saliva samples from secretor-positive individuals only. In addition, both VLPs bound stronger to saliva from secretor-positive A, B and AB individuals compared to O individuals (Chron1, $p<0.0001$; Dijon $p<0.001$, Mann-Whitney). The Chron1 strain, furthermore, bound stronger to saliva from B than from A_2 secretors ($p<0.01$). This VLP also showed a larger interindividual variance in binding than the Dijon VLP. Both the total variance and the variance within each blood group were larger for the Chron1 strain.

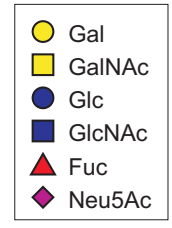
The saliva binding pattern determined for the Dijon strain is in agreement with most GII.4 strains studied (Huang *et al.* 2005, Shirato *et al.* 2008) even though, for some GII.4 strains, other binding patterns have been reported (Lindesmith *et al.* 2008). The Chron1 strain has a somewhat broader saliva binding pattern than other GII.3 strains, as it recognizes saliva from some secretor-positive blood group O individuals. Of the other

GII.3 strains described, the Paris Island and r336 strains recognize saliva from secretor-positive blood group A and B, but not O individuals whereas the Mexico strain recognizes saliva from A, but not O or B individuals (Huang *et al.* 2005, Shirato *et al.* 2008) (Table 3). The recognition of blood group O structures by the Chron1 strain was further supported by the fact that the chronically infected patient was a secretor and Lewis positive blood group O individual.

The binding to specific glycans was studied using neoglycoproteins, either immobilized directly in the ELISA or used as inhibitors in the saliva ELISA. The sialic acid dependent binding specificity identified for the Chron1 and the Dijon strain in these experiments is summarized in Table 5. Three sialylated structures, SLe^x, $SdiLe^x$ and S Type 2, on neoglycoproteins were shown to bind to the two VLPs. The binding to SLe^x-HSA was strictly sialic acid dependent as Le^x-BSA did not bind. However, non-sialylated type 2-HSA showed weak binding to the Dijon VLP. The binding of both VLPs to S Type 2-HSA demonstrated that the Fucα residue of SLe^x is not absolutely required for binding. A terminal sialic acid in itself was however not enough for binding since SLe^a-HSA did not bind. The lack of binding to SLe^a-HSA suggests that the binding is specific since the only difference between SLe^x and SLe^a is a switch in positioning between the Fucα residue and the Neu5Acα2,3Galβ disaccharide to GlcNAc. The binding of $SdiLe^x$-HSA illustrated that modifications at the reducing end of the SLe^x pentasaccharide may be tolerated. As suggested by the saliva binding experiments, the Chron1 and Dijon VLPs, in addition to the sialylated structures, recognized secretor gene dependent glycans. The α1,2-Fuc-dependent binding specificity was reflected by binding to the Le^b conjugate for the Chron1 strain and to the H type 1, Le^b, Le^y, and B trisaccharide conjugates for the Dijon strain. In contrast, the Norwalk strain did not recognize the neoglycoproteins with

Table 5 Binding activity for various neoglycoproteins towards the Chron1 and Dijon VLPs. The data has been compiled from the direct binding and inhibition experiments presented in Paper II.

Structure	Name	Binding
	Type 2	(-)*
	S Type 2	+
	SLe^x	+
	Le^x	-
	$SdiLe^x$	+
	SLe^a	-



* *The Dijon strain showed weak binding to the type 2 precursor*

sialylated glycans. The Norwalk VLP bound strongly to the H type 1 and Le^b conjugates, and weakly also to the Le^y and H type 2 conjugates.

The identified sialic acid dependent binding specificity is distinguished from the secretor gene dependent binding, as *FUT2* is not involved in the biosynthesis of SLe^x, S type 2 or $SdiLe^x$. Nevertheless, the saliva samples were assayed for SLe^x using the CSLEX-1 antibody to rule out the possibility that the expression of this antigen was dependent on secretor status. This experiment showed that SLe^x could be detected in saliva samples from both secretor and non-secretors in analogy with a previous study (Thomsson *et al.* 2005). It could be concluded that saliva samples from at least some non-secretors have detectable levels of SLe^x but still do not bind to the Chron1 or Dijon VLPs. To verify that these VLPs do not recognize the SLe^x epitopes found in saliva, the three saliva samples with the highest reactivity with the CSLEX-1 antibody were assayed at a 10-fold increased coating concentration. Even at this high concentration, no binding of either of the VLPs was observed. Furthermore, the saliva sample with the highest reactivity with the CSLEX-1 antibody was titrated together with the SLe^x-HSA conjugate and separately assayed with the CSLEX-1 antibody and the Chron1 VLP. This experiment indicated that the amount of SLe^x in the saliva sample would be sufficient for VLP binding, suggesting a difference between the VLP and the antibody in their specificity for the SLe^x epitope.

The SLe^x structure on the neoglycoprotein is the full length hexasaccharide found on GSLs, even though the innermost Glc residue is ring-opened to facilitate the conjugation to the protein. In contrast, the most abundant SLe^x epitope in saliva mucins is the core 2-based structure. This structure has the same terminal SLe^x tetrasacchride as the GSL structure, but differs from the GSL epitope in the rest of the structure. The presentation of the SLe^x epitope varies between different core structures, which is illustrated by the large number of antibodies directed towards specific SLe^x structures available (Clark *et al.* 1998). Studies of the core fucose substitution of N-glycans have demonstrated that carbohydrate substitutions distant from the binding epitope may alter the presentation and lectin binding properties of the epitope (section 1.3.5). A complementary explanation for the difference in VLP binding observed between SLe^x-HSA and SLe^x-expressing non-secretor saliva could be that the multivalency of the conjugates, but not of the saliva mucins, are optimal for VLP binding (section 1.3.4).

It is interesting to note that many other viruses recognize sialylated carbohydrate structures (Olofsson & Bergstrom 2005). Recently, Taube *et al*. demonstrated that murine noroviruses use the ganglioside GD1a (see Fig 10 for structure) as receptor (Taube *et al.* 2009). Previously, α2,6-linked sialic acid on an N-linked glycan has been shown to be part of the receptor for feline calicivirus (Stuart & Brown 2007). Thus, binding to sialylated glycans seems to be a common theme for several caliciviruses. As a speculation, SLe^x or structural analogues could function as co-receptors for some human norovirus strains. If this is the case it could have implications for the identification of the target cells in the intestine as well as functional cells for *in vitro* cultivation of the virus. For numerous viruses, the distribution of receptors and co-receptors is crucial for the tissue tropism (section 1.3.2). Interestingly, the herpes-simplex virus type-1 and varicella-zoster virus were recently demonstrated to induce SLe^x expression on target cells through upregulation of host fucosyltransferases (Nystrom *et al.* 2007, Nystrom *et al.* 2009).

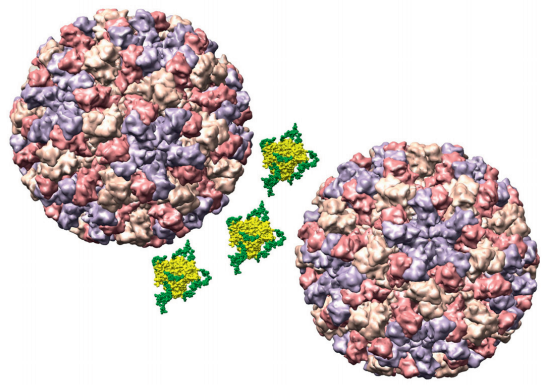


Figure 15
Schematic presentation of SLe^x-HSA conjugates and norovirus VLPs to visualize size ratios and possible mechanisms of interactions. The HSA molecules are colored yellow with the attached SLe^x oligosaccharides in green. The monomers in the VLPs are visualized by coloring the three monomers in the crystallographic unit cell in red, blue and beige respectively. The coordinates for the structures of the Norwalk VLP and HSA were adopted from Prasad et al. (1999) and Sugio et al. (1999). SLe^x pentasaccharides were modeled using Sweet2 (Bohne et al. 1999) and attached to randomly selected lysine residues on the HSA surface via a ring-opened Glc residue conjugated to an aminophenylethyl spacer. The picture was constructed using the Chimera package.

An obvious parallel to the dual binding specificities of norovirus is the adhesion characteristics of *H. pylori*. Just like norovirus, this bacteria is an intestinal pathogen recognizing both α1,2-fucosylated glycans and sialylated Lewis antigens. The expression of SLe^x in the gastric epithelium is induced during chronic *H. pylori* infections, suggesting the bacterium to induce glycans for attachment (Mahdavi *et al.* 2002). Recently a mechanism for the induction involving upregulation of the expression of β3GlcNAcTV (converting Galβ4GlcβCer to GlcNAcβ3Galβ4GlcβCer) was suggested for certain *H. pylori* strains (Marcos *et al.* 2008). In addition, the interaction between *H. pylori* and SLe^x has been shown to be essential for the bacterial stimulation of neutrophils (Unemo *et al.* 2005). This stimulation is an important virulence mechanism for the bacterium as it results in production of reactive oxygen species causing oxidative damage of the gastric epithelium. An opposite effect on neutrophils involving SLe^x has been suggested for the Staphylococcal superantigen-like protein 5 (SSL5). First, SSL5 was demonstrated to prevent neutrophil rolling by binding to SLe^x on PSGL-1 (Bestebroer *et al.* 2007). Thereafter, SSL5 was also shown to bind to G-protein coupled receptors on the neutrophils in a SLe^x dependent manner. This interaction blocked the binding of chemokines to the receptors and thus inhibited neutrophil activation by chemotactic factors (Bestebroer *et al.* 2009).

Irrespective of the biological function, the recognition of SLe^x by noroviruses may be exploited for anti-adhesion therapy. Our inhibition experiments demonstrated that at 10 μg/mL SLe^x-HSA was able to completely inhibit the binding of both the Chron1 and the Dijon VLP to saliva. The saliva sample used was from an ABLeSe individual, thus

theoretically presenting a maximum of potential receptor glycans. In these experiments, inhibition of neither the Chron1 nor the Dijon VLP was seen with the H type 1- or the Le^b-HSA conjugate, even though these conjugates inhibited the binding of the Norwalk VLP. Thus, the Le^b conjugate bound to both Chron1 and Dijon VLP if it was immobilized in the ELISA, but did not show any inhibition potential as a free conjugate in solution. This discrepancy may indicate that the accessible number of Le^b epitopes on one single HSA conjugate is not large enough to form a binding of a sufficient valency. Interestingly, the SLe^x conjugate has a lower valency than the Le^b conjugate (13 vs. 29 glycans/protein). The multivalency of the SLe^x conjugate makes it difficult to determine whether the sialylated structures accommodate the same binding site as the α1,2-fucosylated glycans. Figure 15 shows a schematic visualization of the interaction between VLPs and SLe^x-HSA conjugates. The picture shows that the size of SLe^x-HSA is larger than the capsid protein monomer and that the conjugate might therefore block a second binding site by steric hindrance without actually binding to it. The picture also shows that SLe^x-HSA is large enough to simultaneously interact with several monomers on the same VLP to allow a multivalent interaction. In addition the size ratios of the VLPs and the HSA conjugates suggest VLP aggregation to be a potential mechanism of inhibition.

4.3. Human noroviruses recognize glycosphingolipids (Paper III and IV)

GSLs have been shown to function as true receptors for a number of viruses and bacterial toxins (section 1.3.1). To investigate whether norovirus recognizes ABO(H) histo-blood group active GSLs, Norwalk VLPs were studied using a thin layer chromatogram binding assay (CBA) (Paper III). For detection, the VLPs were ^{125}I-labeled using an activated tin-containing ester (ATE). A solid phase radio-binding assay was used to verify that the labeling did not affect the VLP binding specificity to saliva samples. In addition, the binding results for the H type 1 and the Le^a GSL were validated in Paper IV, using antibody detection of non-labeled VLPs in the CBA.

4.3.1. The Norwalk virus recognizes glycosphingolipids terminated with A, H and difucosylated Lewis, but not B histo-blood group epitopes (Paper III)

Using the CBA, we could determine the binding pattern of the Norwalk VLP to many of the neutral GSLs of the lactotetra (type 1) and neolactotetra (type 2) series. For the type 1 chain structures, the VLP showed a secretor gene dependent binding pattern and did not recognize the precursor (lactotetra) or Le^a. The VLP bound to the α1,2-fucosylated structures H type 1, Le^b, A type 1 and ALe^b, but not to B type 1 or BLe^b. An analogous binding pattern was seen for the type 2 structures with binding to H type 2, Le^y, A type 2 and ALe^y, but not to Le^x. The determined GSL binding pattern is in agreement with the pattern expected from saliva binding studies and challenge studies with the Norwalk virus (section 1.5.1). The determined pattern is essentially in agreement also with binding data for specific glycans obtained in ELISA studies using PAA and HSA neoglycoconjugates (Harrington *et al.* 2002a, Huang *et al.* 2005, Hutson *et al.* 2003). Norwalk VLP binding to blood group A and B structures which are type 1 and type 2 specific, as well as difucosylated, has not been assayed before.

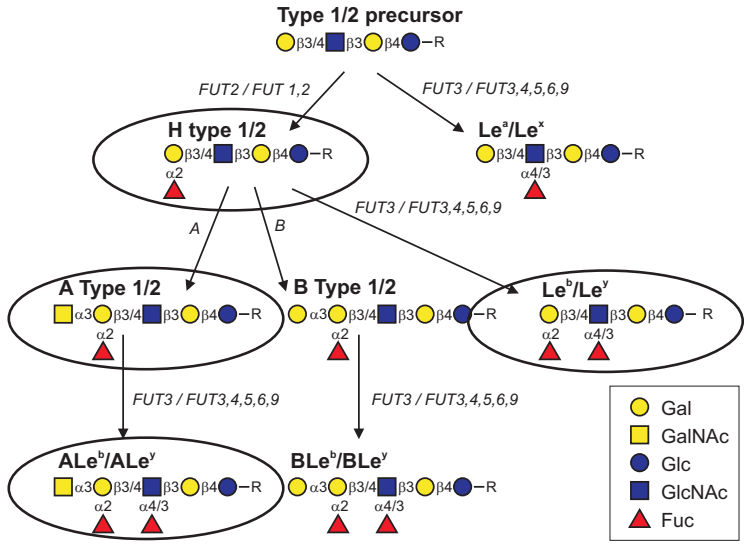


Figure 16
The biosynthetic scheme of the type 1 and type 2 ABO(H) histo-blood group GSLs with the structures demonstrated to bind to the Norwalk VLP encircled.

A difference between the Norwalk virus binding pattern to GSLs and to neoglycoconjugates was the weak binding of the Le^b GSL compared to the H type 1 GSL. Binding studies using PAA-conjugates have suggested these structures to be of similar binding strengths (Harrington *et al.* 2002a, Huang *et al.* 2005, Hutson *et al.* 2003). In our own inhibition studies with the Norwalk virus, Le^b-HSA was an even better inhibitor than H type 1-HSA (Paper II). However, in agreement with the weak binding of the Le^b GSL, the free Le^b tetrasaccharide has been reported to be unable to inhibit Norwalk VLP binding to tissue sections while near-complete inhibition was seen with H type 1 trisaccharide under the same conditions (Marionneau *et al.* 2002). The discrepancy in Le^b binding suggests differences in the presentation of glycans between different conjugates, highlighting the necessity to use different conjugates to completely map binding specificities. Differences in glycan presentation between different glycoconjugates have also been noticed by others in the norovirus field (Huang *et al.* 2009, Shirato *et al.* 2008).

In analogy with the binding to purified A- and H-, but not B-terminated GSLs, the Norwalk VLP recognized a large number of structures in mixtures of GSLs from blood group O, A_1 and A_2, but not from blood group B erythrocytes. In the B fraction, only one weak VLP binding band in the seven sugar region could be detected. This band most likely corresponds to an elongated H type 2 GSL. Using antibodies, the presence of H epitopes was demonstrated in the erythrocyte B fraction as well as in meconium GSLs from a secretor-positive B individual. Binding to such residual H epitopes, not converted to B epitopes, may explain the susceptibility of some secretor-positive B individuals to Norwalk virus infection. This explanation is also in agreement with the binding of Norwalk VLPs to saliva samples from some secretor-positive blood group B individuals only.

When comparing the binding to the A_1 and A_2 erythrocyte fractions an A_1 specific band was seen in the seven sugar region. This band most likely corresponds to the A type 4 (Globo A) GSL (GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4GlcβCer), typically found in A_1 but not in A_2 individuals (section 1.2.3).

A terminal GalNAc is not enough for binding of the Norwalk virus (Paper III)

The X-ray structures of the P dimer of the Norwalk virus capsid protein in complex with the A trisaccharide and the H type 1 pentasaccharide revealed that the αFuc residue was minimally involved in the binding of the A epitope (Choi *et al.* 2008). However, the lack of Norwalk VLP binding to the globoside (GalNAcβ3Galα4Galβ4GlcβCer) and the Forssman (GalNAcα3GalNAcβ3Galα4Galβ4GlcβCer) GSL in the chromatogram binding assay, suggests that a terminal GalNAc is not enough for binding.

4.3.2. The Norwalk and Dijon strains recognize supported lipid bilayers containing H type 1 glycosphingolipids (Paper IV)

In paper IV supported lipid bilayers were used to study the interaction between GSLs and norovirus VLPs in an environment resembling the plasma membrane. Quartz crystal microbalance with dissipation (QCM-D) monitoring is a suitable method for binding studies involving supported lipid bilayers. This is mainly because the parameters monitored provide unique signatures for a successful formation of a continuous fluid membrane. In addition, QCM-D provides label free monitoring of the binding kinetics.

The binding of Norwalk and Dijon VLPs to supported lipid bilayers with 10 wt% of either H type 1 or Le^a GSL was studied using QCM-D. In complete agreement with CBA experiments, both VLPs bound to bilayers containing H type 1, whereas no binding could be detected to bilayers containing Le^a. Thus, human noroviruses recognize GSLs both on thin layer chromatograms and in supported lipid bilayers. This suggests that GSLs can be considered as potential receptors. The idea is supported by the recent demonstration of gangliosides as receptors for murine norovirus (Taube *et al.* 2009).

Analysis of the VLP binding kinetics to the H type 1 bilayers revealed differences in total resonance frequency shifts (Δf) and equilibrium time between the two norovirus strains. These differences were probably caused by differences in concentrations of active VLPs. By plotting the monitored response in resonance frequency, f, versus the change in energy dissipation, D, the time and thus the VLP concentration was eliminated as explicit parameters. Such plots revealed a lower $\Delta f/\Delta D$ ratio for the Norwalk than the Dijon strain indicating that the Norwalk VLPs forms a more rigid structure together with the lipid bilayer.

To further compare the binding properties, binding of Norwalk and Dijon VLPs to bilayers containing different amounts of H type 1 GSLs was studied. In order to compensate for possible domain formations, Le^a was added to achieve a total GSL concentration of 10 wt% in all bilayers. However, identical results were obtained in experiments without addition of Le^a. An IgG1 antibody directed against H type 1 was included to confirm the availability of the GSL in the membrane. For both VLPs, the binding was dependent on the concentration of H type 1 in the bilayer. In support of multivalency, both strains showed a threshold concentration of H type 1 below which no binding was observed. Interestingly, the threshold concentration was one order of

magnitude larger for the Dijon compared to the Norwalk strain. The larger value observed for the Dijon strain suggests that this VLP must interact with a larger number of GSLs for detectable attachment to the lipid bilayer. No threshold concentration was observed for the antibody.

We propose a stepwise model for the interaction where the VLPs initially bind only a few GSLs but where the binding is subsequently strengthened by additional GSLs recruited by lateral diffusion in the bilayer (Fig. 17). At low GSL concentrations, the distance between the GSLs is too large to provide multivalent binding. The monovalent interaction at such densities is too weak to provide adhesion in a time scale long enough for additional GSLs to diffuse into the interaction area. As the concentration of GSLs increases, the distance between the GSLs decreases. Thus, a VLP approaching the bilayer gets in contact with a larger number of GSLs. Moreover, the time required for additional GSLs to diffuse into the interaction area decreases with increasing GSL concentration. At concentrations above the threshold value, VLPs arriving to the bilayer stay there long enough for additional GSLs to strengthen the interaction. These additional GSLs make the interaction essentially irreversible.

The difference in threshold concentration identified between the Norwalk and the Dijon strain suggests the latter to have a lower affinity for the H type 1 GSL. This difference is probably a reflection of the different binding sites used by GI.1 and GII.4 strains for binding to the H type 1 glycan (Cao *et al.* 2007, Choi *et al.* 2008).

For the Norwalk strain a maximal shift in resonance frequency was obtained at 1 wt% of H type 1. No such maximum was observed for the Dijon strain, up to 10 wt% of H type 1.

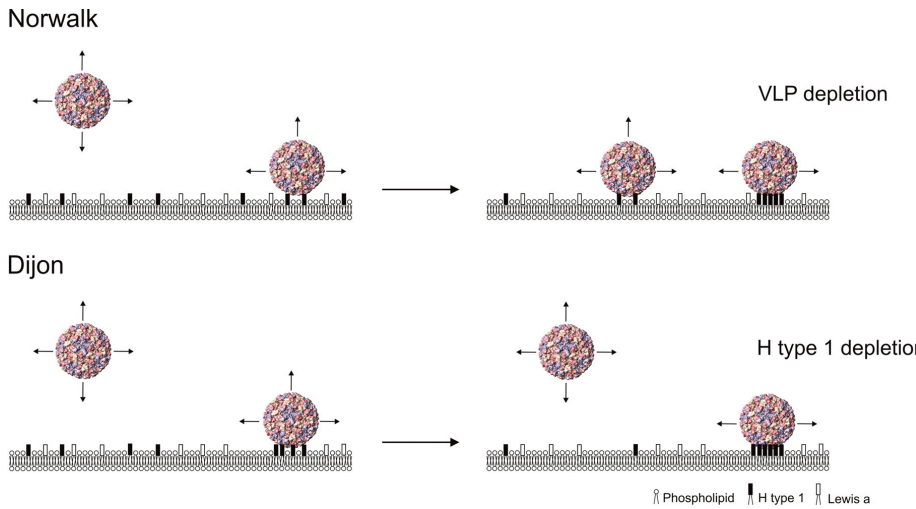


Figure 17
Schematic model illustrating the adhesion of Norwalk and Dijon VLPs to bilayers containing 5 wt% of H type 1 and 5 wt% of Le^a GSLs. Initially the VLPs bind only a few H type 1 GSLs, but the binding is subsequently strengthened by additional H type 1 GSLs moving into the interaction area. The number of GSLs needed to maintain at the bilayer is larger for the Dijon strain than for the Norwalk strain. At the H type 1 concentration shown, the binding was limited by VLP depletion for the Norwalk strain but by ligand depletion for the Dijon strain.

The resonance frequency shifts are measured when the binding process has reached equilibrium. As the experiments were performed in stationary reaction chambers, the equilibrium may be caused by depletion of either VLPs or GSLs. At low GSL concentrations, the GSLs are the limiting factor, whereas at high GSL concentrations the VLPs are limiting. The observation of a maximal shift for the Norwalk, but not the Dijon strain, is consistent with a lower concentration of active VLPs for the Norwalk strain. Thus, at 5 wt% of H type 1 in the bilayer, the binding process seems to be limited by VLP depletion for the Norwalk strain, but by ligand depletion for the Dijon strain (Fig. 17).

To analyze the binding kinetics, bi-exponential functions were fitted to the resonance frequency curves obtained for the bilayers with different concentrations of H type 1. These functions resolved the binding processes into a fast and a slow phase. An analysis of the rate constants for the fast phase revealed a sharp transition from non-binding to binding for both strains at their respective threshold concentrations. A maximal rate was reached for the Norwalk VLP at 1 wt% H type 1 and for the Dijon VLP at 5 wt% H type 1. Thus, even though no maximal shift in resonance frequency was identified for the Dijon strain a maximal binding rate was identified through this kinetic analysis.

The sharp transition from non-binding to binding at the threshold concentration, suggests the VLPs to be precise sensors of receptor density. Data in support of a biological relevance for such a threshold effect in relation to susceptibility to infection is provided by studies of the related lagovirus, RHDV. Young rabbits are protected from infection with this virus, which attaches through recognition of H type 2. The expression of the glycan is low in young rabbits, but increases with age. Interestingly, the increase in expression of H type 2 is paralleled by an increase in susceptibility to infection with RHDV (Ruvoen-Clouet *et al.* 2000).

The identification of a threshold concentration for human noroviruses suggests that individuals with a low expression of the potential glycan receptors may be protected also from high concentrations of the virus. In analogy, the difference in threshold concentration between the Norwalk and Dijon strains, could suggest that some individuals may be susceptible to infection with the Norwalk but not the Dijon strain. However, glycan binding viruses must not only get in contact with glycans on the target cell, but also avoid getting trapped on mucins and other decoy receptors. For viruses with receptor destroying enzymes, the balance between receptor binding and destruction is crucial. For instance, the requirement for a delicate balance between the hemagglutinin and neuraminidase activities of influenza A and B virus is well documented (Wagner *et al.* 2002). Many viruses lacking receptor destroying enzymes avoid getting stuck by possessing a weak affinity for their receptors (Harrison 2007). Such an inverse correlation between binding affinity and virulence have been described for murine polyomavirus and parvovirus, both recognizing sialic acid bearing glycans (Bauer *et al.* 1995, Nam *et al.* 2006). In analogy with the weak affinities observed for these viruses, the threshold concentrations identified for noroviruses may reflect a mechanism for the viruses to avoid decoy receptors. It has been hypothesized that the wide binding specificity typical for GII.4 norovirus strains is linked to their clinical dominance (Le Pendu *et al.* 2006, Shirato *et al.* 2008). Another speculation is that an optimal affinity for the receptor glycans may contribute to the high prevalence of this genocluster.

4.4. Conclusions

To summarize, our studies have extended the knowledge of the diversity of the histo-blood group glycans recognized by human noroviruses. We have strengthened the evidence for the existence of strains capable of infecting non-secretors, and at the same time shown that secretors are much more prone to be infected by GII strains (Paper I). Additionally, we have for the first time identified SLe^x and some of its structural analogues as potential co-receptors for GII strains (Paper II). The ability of SLe^x-HSA conjugates to inhibit binding of Chron1 (GII.3) and Dijon (GII.4) VLPs to saliva points to new strategies for anti-adhesion therapy. Additionally, we have shown that noroviruses do not only recognize secretor gene dependent glycans on glycoproteins, but also on GSLs. In Paper III we demonstrated that the Norwalk (GI.1) VLP recognizes blood group A and H, but not B, terminated GSLs of both the type 1 and type 2 series. In paper IV we validated the binding of GSLs in supported lipid bilayers using QCM-D. Thus, VLPs from the Norwalk and Dijon strains were shown to bind to bilayers containing H type 1, whereas no binding was observed to those with Le^a. In support of multivalency, both strains showed a threshold concentration of H type 1 below which no binding was observed. The threshold concentration was higher for the Dijon strain, suggesting a weaker affinity to the H type 1 GSL for that strain.

5. Ongoing and further studies

5.1. Docking of histo-blood group ABO active saccharides with the norovirus VA387 capsid protein

The crystal structures of the P dimers of the capsid protein of the VA387 norovirus strain in complex with the histo-blood group A and B trisaccharides provide structural information about the binding pocket of GII.4 strains (Cao *et al.* 2007). However, a complete understanding of the wide binding specificity typical for GII.4 strains requires structural information also for other histo-blood group glycans. In addition, a complete map of the amino acids involved in the recognition of the potential receptor glycans would facilitate structure-based inhibitor design. Since amino acids, interacting with an inhibitor may be mutated to confer resistance to the drug, an ideal inhibitor should have a ligand footprint not significantly exceeding the footprint of the natural ligands.

Together with P. G. Nyholm and co-workers, Biognos AB, Gothenburg (Koppisetty *et al.* 2009), we have computationally explored the fit of the VA387 capsid protein with a set of histo-blood group glycans containing an α1,2-linked fucose residue. Docking of α-fucose using Glide XP was in agreement with the crystal structure of the A and B trisaccharides indicating a distinct binding site for fucose. Because docking software has problems with large glycans we used a simple superimposition of the extended structures with the terminal α1,2-linked fucose in the binding site followed by molecular dynamics (MD) using Amber 8.0 (Case *et al.* 2005) with position restraints on the fucose. The trajectories of the MD simulations were analyzed and the relative binding energies were calculated with the Glide XP scoring function v5 (Schrödinger, LLC, NY). The result suggests that a wide variety of ABO and Lewis active type 1 chain structures may be well accommodated in a similar mode in the VA387 binding site. In contrast, A and H antigens bind to the Norwalk virus binding pocket in different orientations (Choi *et al.* 2008). Calculations of the binding energies of the complexes from the MD simulations suggested that in addition to the Fucα1,2, the GalNAcα/Galα1,3 of the A and B terminated structures made strong interactions with the VA387 protein. The difucosylated structures Le^b, ALe^b and BLe^b were well compatible with the binding site, but the Fucα1,4 modification had only a minor influence on the interaction energy. This observation is in agreement with saliva binding studies of the VA387 VLP suggesting the influence of Lewis status to be small (Huang *et al.* 2003, Huang *et al.* 2005). In addition, MD simulations of the H type 3 trisaccharide suggested that the type 3 chain is also compatible with the binding site. Notably, the O1 of the H type 3 GalNAcα pointed into the bulk medium. The accessibility of this oxygen may be the basis for the strong binding reported for neoglycoconjugates with the H type 3 trisaccharide (Huang *et al.* 2005). The structural fit of the α1,2-fucosylated glycans investigated was in agreement with mutational analysis of the VA387 binding site (Tan *et al.* 2008c).

5.2. Identification of the biological function of the SLe^x-related binding specificity

In search for a biological function of the SLe^x-related norovirus binding specificity histochemical binding studies using tissues with high expression of SLe^x will be initiated

together with Prof. Jacques Le Pendu and coworkers, INSERM, Nantes, France. These studies will include FACS analysis of VLP binding to SLe^x-expressing cultured cells.

To investigate whether norovirus recognizes sialylated GSLs, gangliosides are being screened for VLP binding using the CBA in collaboration with Prof. Susann Teneberg, University of Gothenburg.

5.3. Further characterization of VLP binding to glycosphingolipids in supported lipid bilayers using QCM-D

QCM-D will be used to further characterize the interaction between norovirus VLPs and GSLs in supported lipid bilayers. Specifically, the influence of the composition of the bilayer in terms of different phospholipids, sphingomyelin and cholesterol will be investigated at different temperatures. In addition, the QCM-D assay will be evaluated for studies of inhibitors of VLP attachment.

6. Populärvetenskaplig sammanfattning på svenska

Norovirus orsakar den s.k. vinterkräksjukan, som kännetecknas av kaskadkräkningar och diarré. Medvetenheten om norovirus har ökat under senare år, sannolikt beroende på en kombination av införandet av känsligare metoder för virusdiagnostik och uppkomsten av mer aggressiva virusstammar. Sedan 2002 har världsomspännande epidemier av norovirusinfektioner inträffat ungefär vartannat år. Idag anses norovirus vara den mikroorganism som orsakar flest utbrott av magtarm-infektion (kräkningar och diarré) i världen. Viruset orsakar varje år upp till 200 000 dödsfall bland barn i u-länder.

Inga vaccin eller läkemedel finns ännu mot norovirus. Upptäckten att norovirus sannolikt använder ABO-blodgrupper på cellytan för att ta sig in i cellen har lett till försök att hitta molekyler som blockerar interaktionen mellan viruset och cellytan. Sådana molekyler skulle kunna hindra viruset från att ta sig in cellen och därmed förhindra virusinfektionen.

Vi har upptäckt att norovirus från den vanligaste genogruppen (GII) inte bara binder till ABO-blodgrupper utan även till en annan grupp kolhydrater, som kännetecknas av att de ytterst bär monosackariden sialinsyra. Inhibitionsförsök visade att proteiner som på ytan exponerar dessa sialinsyrainnehållande kolhydrater var effektiva hämmare av norovirusbindning. Genom att vidare analysera virusets bindningsegenskaper till sialinsyrainnehållande kolhydrater hoppas vi kunna utveckla nya hämmare med ännu effektivare hämningsförmåga.

Intressant nog har det visats att individer, som tillhör den femtedel av den europeiska befolkningen som är s.k. sekretornegativa, är resistenta mot norovirusinfektioner. Sekretornegativa individer saknar det enzym som krävs för att uttrycka ABO-blodgrupper på tarmceller och i saliv. Sannolikt beror resistensen hos dessa individer på att viruset behöver ABO-blodgrupper för att ta sig in i tarmcellerna. Många virus använder flera molekyler för att ta sig in i målcellen. Möjligen är interaktionen med de sialinsyrainnehållande kolhydratstrukturerna, som vi upptäckt, också nödvändiga för norovirus intrång i celler.

Vår forskargrupp har tidigare visat att inga sekretornegativa individer fanns bland de symptomatiskt sjuka i ett antal svenska norovirusutbrott. Denna avhandling stärker kopplingen mellan sekretorstatus och mottaglighet för norovirusinfektion ytterligare genom att visa att sekretorpositiva individer har en statistiskt säkerställd högre koncentration antikroppar mot norovirus i blodet än sekretornegativa individer. Detta tyder på att sekretorpositiva individer haft betydligt fler norovirusinfektioner än sekretornegativa individer. Dock så hade vissa sekretornegativa individer betydande koncentrationer antikroppar, vilket tyder på att även sekretornegativa kan infekteras av vissa norovirusstammar.

I avhandlingen visas även för första gången att norovirus inte bara binder till ABO-blodgrupper på proteiner utan även på lipider, s.k. glykolipider. Glykolipider finns i stora mängder i cellmembranet och har för flera virus visats vara sanna receptorer, dvs de molekyler som aktivt hjälper viruset in i cellen. Med hjälp av nyutvecklade mätinstrument har vi studerat interaktionen mellan norovirus och glykolipider i syntetiska membraner,

som liknar cellmembranet. Dessa studier visar att viruset måste interagera med flera glykolipider samtidigt, s.k. multivalent bindning, för att fastna på lipidmembranet.

Sammanfattningsvis beskriver avhandlingen norovirus förmåga att binda till ett antal olika mänskliga kolhydrater. Kunskaper om virusets precisa bindingsegenskaper hoppas vi ska kunna användas till utveckling av läkemedel som förhindrar virusets intrång i cellen.

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