

Original article

# Development and maturation of norovirus antibodies in childhood

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## Abstract

The burden of norovirus (NoV) gastroenteritis is substantial in young children. Maternal antibodies are thought to protect a child from NoV infection in early infancy but subsequent development of NoV-specific protective immunity in children is still largely unexplored.

We have determined NoV-specific antibody seroconversion to GII.4 virus-like particles as an indicator of NoV infection in two children prospectively followed from birth to eight years of age. Blocking activity and affinity maturation of maternal and serum IgG antibodies were evaluated.

Our results show that multiple infections occur in children up to eight years of age. The titer, blocking activity and avidity of maternal antibodies determined susceptibility of an infant to NoV infection. NoV GII.4-specific antibodies with high blocking potential and avidity were developed at two to three years of age and were retained throughout the follow-up. Subsequent NoV infections may have contributed to the duration of protective NoV-specific immune responses that lasted for several years.

This study adds to current understanding of the duration of passive protection by maternal antibodies and the duration and quality of acquired immunity following primary and subsequent NoV infections in infants and young children, who are the main target group for NoV vaccine development.

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## 1. Introduction

Infants and children under five years of age are most susceptible to norovirus (NoV) acute gastroenteritis (AGE) [1–3], and are therefore one of the potential target groups for NoV vaccination. Young children have higher and longer viral

shedding compared to adults [4] and may be considered as a pool for transmission of NoV to other vulnerable populations, e.g. senior citizens. NoV infects all age groups and in adults the NoV seropositivity is near 100% as a result of lifetime NoV exposure history [5].

Most NoVs infecting humans belong to genogroups GI and GII, which are genetically so distant that no intergenogroup cross-protective immune responses are generated [6]. However, after natural infection [7–11] and also vaccination [12] variable degree of intragenogroup cross-reactivity has been observed. NoV GII.4 genotype is responsible for

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approximately 55–80% of all NoV infections worldwide [13–15]. New epidemic antigenically distinct GII.4 variants emerge every two to three years [16–18] but considerable antibody cross-reactivity between GII.4 variants has been observed even in young children [19].

Correlates of protective immunity to NoV are not well established. A common finding has been that protection is not long lasting [5]. Early homologous challenge studies by Parrino et al. [20] and Johnson et al. [21] have shown that duration of protective immunity varies from two months to two years. There are a limited number of studies on NoV-specific immune responses in children. In contrast to those of adults, IgG responses to NoV during the first year of life are relatively weak and short-lived and the antibodies are of a low avidity [22,23]. Saito et al. [24] found that most infections in less than six-month-old infants are asymptomatic, probably due to maternally acquired antibodies and, possibly, breastfeeding. Altogether, the build-up of protective immunity after primary and secondary NoV infections is not well characterized.

NoV virus-like particles (VLPs) are commonly used for studying immune responses against NoV. The expression of the NoV capsid VP1 protein results in formation of VLPs that are morphologically and antigenically similar to native virions [25], thereby being also promising vaccine candidates [26,27]. Blocking assay has been used as a surrogate measure for NoV neutralization, determining the ability of serum antibodies to block binding of NoV VLP to its putative histo-blood group antigen (HBGA) receptors/attachment factors [12,28,29]. We have previously used NoV GII.4 VLPs to determine NoV seroprevalence in Finnish children [10]. Also, more recently an endpoint titer >51,200 and/or 90% blocking titer (BT<sub>90</sub>) >100 was suggested as an indicator of protection from NoV infection in these children [10]. The recent challenge studies in adults have further affirmed correlation between the blocking antibodies and protection [12,30]. Antibody avidity is low in primary and recent NoV infections and increases with time [23], but the relevance of the Ab avidity in NoV protective immunity is not currently known.

In this study we determined seroconversion to NoV GII.4 VLPs as an indicator of NoV infection in two children prospectively followed from birth up to the age of eight years and examined the duration, blocking ability, and avidity of the antibodies.

## 2. Materials and methods

### 2.1. Study samples

Two healthy children (Subject 1 and 2) taking part in the Type I Diabetes Prediction and Prevention (DIPP) study [31] were prospectively followed for NoV GII.4-specific antibodies from birth to eight years of age. The DIPP study protocol was approved by the ethics committee of the Pirkanmaa Hospital District (Permit number: 97193M) and a written informed consent was obtained from the parents. Health records were collected during the follow-up visits

and symptoms related to acute gastroenteritis episodes were recorded. Subject 1 had been breastfed exclusively for first five months and partially until the age of 25 months. Breastfeeding of the Subject 2 was exclusive for first two months, partial for one month. All procedures performed were conducted according to the principles expressed in the Declaration of Helsinki. Cord blood samples were taken at birth in year 2000 and thereafter blood samples were taken in sodium citrate tubes at the age of 3, 6, 12, 17 and 24 months and thereafter once per year up to eight years of age. Plasma fraction was stored at  $-70^{\circ}\text{C}$  until analyzed. Non-diarrheal stool samples were collected monthly at the age of 3–9 months and every two months until the age of 1 year, 9 months. Viral RNA was extracted from the stool suspensions and stored at  $-70^{\circ}\text{C}$  until reverse transcription-polymerase chain reaction (RT-PCR) and open reading frame 1 polymerase (region A) sequencing were used for NoV genotyping according to previously described methods [32].

### 2.2. Norovirus VLP production

GII.4-1999 capsid VP1 sequence originated from a patient sample collected in 1999 (GenBank reference strain accession no. AF080551) [23,33]. NoV GII.4-1999 VLPs used as antigens in analytical methods were produced by a baculovirus expression system (Invitrogen) in *Spodoptera frugiperda* (Sf9) insect cell cultures. VLPs were purified twice with discontinuous sucrose gradient ultracentrifugation as previously described [33]. The total protein concentration was quantified with Pierce<sup>®</sup> BCA Protein Assay (Thermo Scientific, Rockford). Protein purity, integrity, and morphology were determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and transmission electron microscopy (EM) as described earlier [26,33].

### 2.3. Serum IgG ELISA

NoV GII.4-1999-specific IgG antibody levels were analyzed by ELISA as earlier described [10]. Serum specimens were diluted two-fold starting at 1:100 and plated on GII.4-1999 VLP coated (0.5  $\mu\text{g}/\text{ml}$  in phosphate-buffered saline, PBS) 96-well half-area microtiter plates (Corning Inc., Corning, NY) blocked with 5% skimmed milk in PBS. Serum dilutions were incubated on plates for 1 h at  $37^{\circ}\text{C}$ . Bound GII.4-1999-specific antibodies were detected with goat anti-human IgG-HRP (Invitrogen, CA, USA) followed by o-phenylenediamine (OPD) substrate (Sigma–Aldrich, MO, USA) and  $\text{H}_2\text{O}_2$ . Optical density (OD) was measured at  $\lambda$  490 nm using the Victor2 1420 Multilabel Counter (Wallac, Perkin Elmer) plate reader. Background signal from the blank wells (wells without serum) was subtracted from all of the OD readings on the plate. Each plate contained NoV negative and positive control serum sample as an assay control. The cut-off value was determined as the mean OD reading of the negative control serum wells at a dilution  $1:200 + 3 \times$  standard error

and at least 0.100 OD. Endpoint titer was expressed as a reciprocal of the final serum dilution giving an OD above the cut-off value. Seroconversion was defined as at least four-fold increase in the titer of successive sera.

#### 2.4. Avidity assay

Avidity of serum IgG antibodies was determined in ELISA by urea elution steps according to a previously published method [23,34]. Sera were tested at 1:100 dilution on GII.4-1999 VLP (1.0 µg/ml) coated and blocked microtiter plates. After incubation for 1 h at 37 °C, wells were exposed twice for 5 min to 8 M urea in PBS-Tween solution or to phosphate buffer. After washing, the wells were incubated with peroxidase-labeled anti-human IgG and bound antibodies were detected as described above. Avidity index (%) was calculated using the equation:  $[\text{OD with urea}/\text{OD without urea}] \times 100\%$ . An avidity index >50% was considered as high avidity [7,23].

#### 2.5. Blocking of HBGA binding

The ability of antibodies to block GII.4-1999 VLP binding to synthetic HBGA was tested using H type 1 HBGA carbohydrates that have been shown to be biologically relevant for NoV attachment in infection [28] as well as to bind to GII.4 NoV VLPs [35]. The blocking assay was carried out as previously described [35]. Briefly, the pre-coated and pre-blocked NeutrAvidin plates (Pierce, Rockford, IL) were coated with H type 1 (Glycotech, Gaithersburg, MD) HBGAs for 1 h at room temperature. Serially two-fold diluted serum samples starting at 1:50 dilution were first preincubated with NoV GII.4-1999 VLPs (0.4 µg/ml) for 1 h at 37 °C, before incubating the samples on HBGA-coated NeutrAvidin plates for 2 h at +4 °C. Bound VLPs were detected by GII.4-1999-specific mouse antisera and anti-mouse IgG-HRP (Sigma–Aldrich, Saint Louis, MO), followed by OPD substrate and H<sub>2</sub>O<sub>2</sub>. Mean OD reading of the blank wells was subtracted from the OD values of the sample wells. A maximum binding signal was the mean OD of the wells with VLP alone, lacking serum. The blocking index was defined as follows:  $100 - [\text{OD for wells with serum}/\text{OD for wells without serum}] \times 100$ . A blocking titer (BT) value of 50 or 90 was determined as the reciprocal of the final serum dilution that blocked at least 50% or 90% of VLPs binding to the HBGA. For statistical analyses an arbitrary BT of 25 (half of the starting reciprocal serum titer 50) was assigned to all sera which lacked blocking.

#### 2.6. Statistical analysis

The Spearman rank correlation coefficient was used to examine the differences between antibody titers, blocking titers BT<sub>90</sub> and avidity index. All hypothesis testing was two-tailed. Statistical analyses were performed using IBM SPSS Statistics (SPSS, Chicago, IL) version 22.0.  $P < 0.05$  was considered statistically significant.

### 3. Results

The integrity and morphology of NoV GII.4 VLPs used for the analytical methods were verified by western blot (Fig. 1A) and EM (Fig. 1B). EM images identified NoV VP1 capsid proteins self-assembled into the VLPs of ~38 nm in size.

The Subject 1 had moderate levels (endpoint titer 3200) of GII.4-1999-specific cord blood antibodies that waned in the following three months (Fig. 2 and Table 1). Seroconversion to NoV GII.4 VLPs was observed at the age of six months and again at the three and five years of age, indicating at least three NoV infections by this age. No NoVs were detected in the stool samples of the Subject 1 collected during the first two years of life. Only one episode of AGE between the age of 17–24 months had been recorded for Subject 1, which did not coincide with the stool or serum samples collected.

Despite the seroconversion, the primary NoV exposure before the age of six months did not increase the blocking (BT<sub>90</sub> < 50) or avidity index (19%) of GII.4-1999-specific antibodies (Table 1). Blocking antibodies with BT<sub>90</sub> value of 100 and high avidity (95% avidity index) were observed after the presumably second NoV infection at the age of three years. The ability to block the binding of VLPs to HBGA ligand declined within one year (BT<sub>90</sub> < 50), but remained still above the initial level until the age of seven years (BT<sub>50</sub> 50). Similarly, the avidity index declined within one year to 67%, but remained >50% for all the subsequent time points. A two-fold increase in endpoint titer was detected again at the age of eight years but no change in blocking or avidity index was observed.

In comparison to the Subject 1, the second child (Subject 2) had considerably higher levels of GII.4-1999-specific cord blood antibodies (endpoint titer 51,200) with BT<sub>90</sub> 100 and avidity index 95%, which dropped by the age of six months (Fig. 3 and Table 2). The primary NoV infection was acquired between 1.5 and 2 years of age (Table 2), approximately a year later than in the Subject 1 (Table 1). In addition, this infection induced highly functional (BT<sub>90</sub> 100 and avidity index 99%) GII.4-1999-specific antibodies. NoV RT-PCR and sequencing of the stool samples detected NoV GII.2 genotype infection at the age of 1 year and 9 months, prior to the first seroconversion to NoV GII.4 VLPs at the age of two years (Table 2 and Fig. 3). Correspondingly, an episode of AGE had been recorded between the age of 17–24 months. Despite the high levels of presumably protective antibodies at the age of two years, another NoV infection already appeared during the next year as indicated by the four-fold increase in BT<sub>90</sub> at the age of three. Judging by the seroconversion, the Subject 2 acquired two additional NoV infections at the ages of five and eight years (Table 2). Additionally, the Subject 2 had AGE symptoms at the age of 4.5 years, corresponding to seroconversion at the age of 5 years.

Altogether, both subjects acquired at least three to four NoV infections (Tables 1 and 2). The Subject 1 who had low maternal antibody titer, lacking blocking activity and avidity, acquired first infection already by the age of six months, whereas the Subject 2, with a high titer and functionality of maternal antibodies acquired first infection at least one year

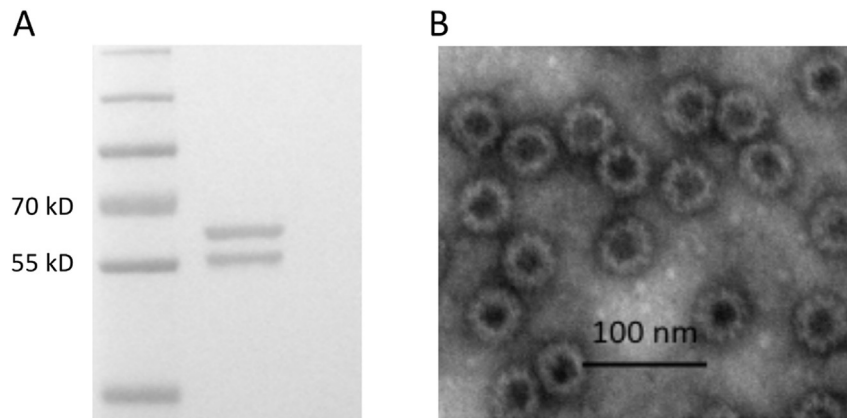


Fig. 1. The integrity and morphology of NoV GII.4 VLPs as characterized by (A) western blot and (B) the electron micrograph examined by FEI Tecnai F12 electron microscope (Philips 487 Electron Optics, Holland). The protein weight marker is shown on the left lane and the purified NoV VLP on the right lane, using a human convalescent serum against NoV GII.4 for detection.

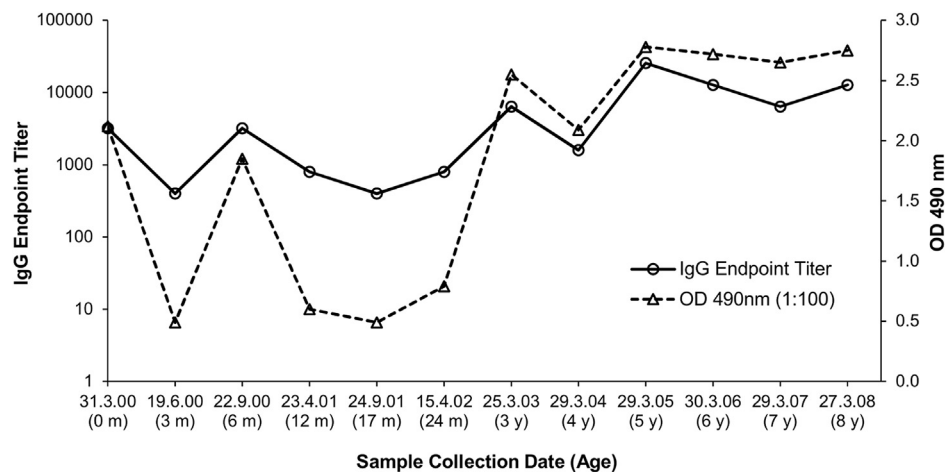


Fig. 2. Norovirus GII.4-specific serum IgG responses during the first eight years of life in Subject 1. Serum specimens of the child were collected between March, 2000–March, 2008. Sera were analyzed at 1:100 dilution (OD 490 nm) and serially diluted to determine the endpoint titer (OD  $\geq$  0.1) for each sample.

later. Antibodies with high blocking activity and avidity were developed only at (Subject 2) or after (Subject 1) the two years of age. Although the avidity remained high up to eight years for both subjects, only the Subject 2 retained high level of blocking antibodies. However, these GII.4-specific antibodies did not protect the Subject 2 from a new infection prior to the age of eight years with presumably NoV genotype highly divergent to GII.4. Altogether, a positive correlation was detected between BT<sub>90</sub> and IgG endpoint titers ( $r = 0.808$ ,  $P < 0.01$ ) and avidity index ( $r = 0.775$ ,  $P < 0.01$ ) of serum antibodies in both subjects.

#### 4. Discussion

In the present study we followed the development of NoV GII.4-specific humoral immune responses in two children from birth up to eight years of age. Seroconversion was detected in at least three instances, suggesting a minimum of three NoV GII infections in both children in eight years. NoV GII.4 VLPs were chosen to measure antibody responses to GII

NoVs as it is the most common genotype circulating for >20 years and causing sporadic acute gastroenteritis in children worldwide [15,18,36]. Also, most infections in children during the years 2000–2013 in Finland were caused by the GII.4 viruses [14,37].

In here, the routine stool specimens collected prior to two years of age were used for determining possible NoV infection. For the Subject 1 NoV infections were not detected in the stool samples collected at 4 and 5 months, although the seroconversion happened at the six months of age. It is known that NoV shedding can be highly variable, an average of 8–60 days [38] and therefore the infections may be readily missed if the samples are not collected weekly. However, GII.2 NoV infection of the Subject 2 was detected in a stool specimen collected prior to blood sample taken at the age of 24 months, when the first seroconversion was detected. The above result shows that although a child may be infected with a diverse NoV GII genotype it seroconverts to the GII.4 VLPs, as we have previously shown [10,11]. NoV-specific cross-reactive antibody epitopes are present in the N-terminal region of the

Table 1

NoV GII.4-1999 specific antibody responses in Subject 1. Shown are GII.4-specific endpoint titers, GII.4-specific blocking titers (BT) 50 and 90 and avidity indexes, (%).

Age (months/years)	Presumed infection	GII.4 endpoint titer	Blocking antibodies		Avidity index (%) <sup>c</sup>
			BT <sub>50</sub> <sup>a</sup>	BT <sub>90</sub> <sup>b</sup>	
0 m		3200	<50	<50	43
3 m		400	<50	<50	38
6 m	x	3200 <sup>d</sup>	<50	<50	19
12 m		800	<50	<50	25
17 m		400	<50	<50	33
24 m		800	<50	<50	38
3 y	x	6400 <sup>d</sup>	200	100	<b>95</b>
4 y		1600	50	<50	<b>67</b>
5 y	x	25,600 <sup>d</sup>	100	<50	<b>98</b>
6 y		12,800	50	<50	<b>75</b>
7 y		6400	50	<50	<b>82</b>
8 y		12,800	<50	<50	<b>69</b>

<sup>a</sup> BT<sub>50</sub>, 50% blocking titer.

<sup>b</sup> BT<sub>90</sub>, 90% blocking titer.

<sup>c</sup> The avidity indexes >50% are shown in bold.

<sup>d</sup> >4-fold increase in antibody titer.

Table 2

NoV GII.4-1999 specific antibody responses in Subject 2. Shown are GII.4-specific endpoint titers, GII.4-specific blocking titers 50 and 90 and avidity indexes, (%).

Age (months/years)	Presumed infection	GII.4 endpoint titer	Blocking antibodies		Avidity index (%) <sup>c</sup>
			BT <sub>50</sub> <sup>a</sup>	BT <sub>90</sub> <sup>b</sup>	
0 m		51,200	200	100	<b>95</b>
3 m		6400	<50	<50	<b>91</b>
6 m		1600	<50	<50	46
17 m		400	<50	<50	42
24 m	x	102,400 <sup>d</sup>	400	100	<b>99</b>
3 y	x	204,800	800	400	<b>99</b>
4 y		51,200	200	100	<b>96</b>
5 y	x	204,800 <sup>d</sup>	1600	800	<b>98</b>
5.5 y		51,200	200	100	<b>100</b>
6 y		25,600	100	100	<b>97</b>
8 y	x	102,400 <sup>d</sup>	200	100	<b>97</b>

<sup>a</sup> BT<sub>50</sub>, 50% blocking titer.

<sup>b</sup> BT<sub>90</sub>, 90% blocking titer.

<sup>c</sup> The avidity indexes >50% are shown in bold.

<sup>d</sup> >4-fold increase in antibody titer.

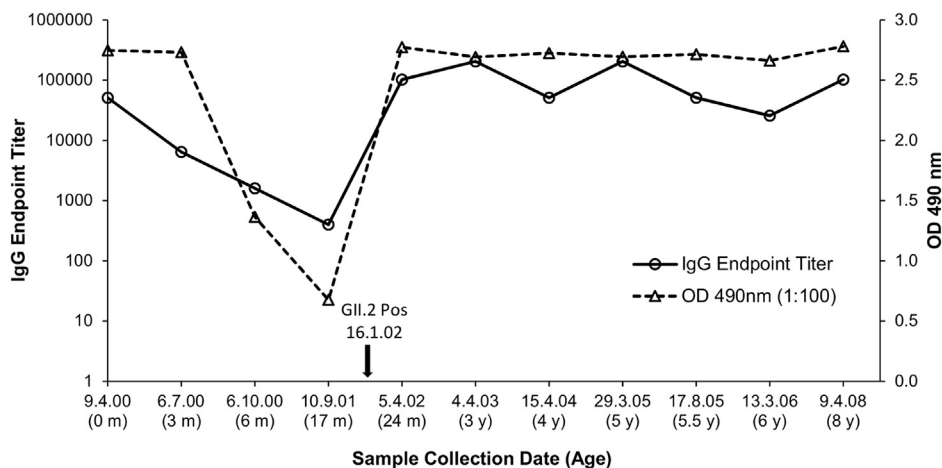


Fig. 3. Norovirus GII.4-specific serum IgG responses in Subject 2 from birth to eight years of age. Longitudinal serum samples were collected starting from cord blood on April 9th, 2000. Serum specimens were analyzed at 1:100 dilution (OD 490 nm) and serially diluted to determine the endpoint titer (OD  $\geq$  0.1) for each sample.

NoV capsid VP1 [39] therefore, it is possible to detect sero-conversion using VLPs heterologous to the infecting genotype.

The titer, avidity and blocking ability of maternal antibodies seemed to influence susceptibility of an infant to NoV infection. The Subject 1 had a low level and low avidity of maternally acquired antibodies and had the first NoV infection early (<6 months of age), while the Subject 2 with high maternal antibody level of high avidity was protected more than 1.5 years. Interestingly, the Subject 2 was breastfed for only three months in contrast to the Subject 1 who was breastfed for 25 months indicating no protective role of breastfeeding against NoV infection. Both children developed high titer functional antibodies only at the age of two years or later, probably because of the immaturity of the immune system in an infant [22]. Avidity indexes of sequential serum

samples in the present study confirm the earlier findings that the avidity is low in primary infections and increases over time, possibly as a result of serial heterotypic infections [7,23].

Young children are able to generate homologous protective blocking antibody titer (BT<sub>90</sub> > 100) [10,19], which is associated with greater protection from NoV infection and illness [30], but are lacking heterologous blocking response and therefore are prone to new infections by another NoV strain [10]. We have recently reported that pre-existing blocking antibody titers in acute sera were low in <2-year-old children and only children above 12 months of age generated protective cross-blocking antibodies following the infection, with the endpoint titer >50,000 and BT<sub>90</sub> > 100 [19]. In this study, at the ages of four and six years of the Subject 2 the endpoint IgG titer of  $\leq$ 51,200 and BT<sub>90</sub> 100 did not protect from new NoV



infection (Table 2), confirming the previously determined protective endpoint titer  $>50,000$  and  $BT_{90} > 100$  [10].

Early adult experimental infection studies have shown that NoV immunity following infection lasts up to six months [21] but less than two years [20]. More recent adult NoV challenge studies showed duration of NoV blocking antibodies up to six months [28]. Our results in naturally infected children support these findings. We found maximum duration of protection up to 3 years at older age, which contradicts calculations by a mathematical model that estimated four to eight years' duration of protective immune response to NoV [40]. It is possible that some infections with GI or non-GII.4 NoV were missed in this study as only GII.4-1999 VLPs were used in the analyses, but what we detected is the minimum number of infections. We have earlier studied homologous and cross-reactive antibody responses in GII.4-2010 New Orleans infected children [10,19] and found that 100% of 6–18 month old children seroconverted to GII.4-2010 NO and GII.4-2012 Sydney [19] and 67% to GII.4-1999, but only 17% to more distant GII.12 NoV [10]. However, as the most common strains circulating in Finnish children at the time of the sample collection in the present study were closely related to GII.4-1999 (e.g. GII.4-US95/96, GII.4-2001, Farmington-2002 and 2006) [14,41], these infections were likely detected by the seroconversion to GII.4-1999 VLPs.

Previous studies in large cohorts [24,36] as well as our own work [11] have suggested that a child may encounter 1–3 NoV infections within the first year of life. The results in here confirm these findings and also show that at least three infections are encountered by a child by the age of five years. Vaccination of infants against NoV would address the most common cause of hospitalizations due to gastroenteritis in children in countries where universal rotavirus vaccination is implemented [42]. Our results suggest that vaccine should be delivered the earliest to the one-year-old since before that age the immune system is immature. Women of a childbearing age could be considered as a vaccine target population to protect young infants. Even though this study is based on two subjects only, the results in this study add to the understanding of immunogenicity and duration of protection in infants and young children.

### Conflict of interest

None of the authors have conflict of interest.

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