# On Morbidity and Mortality in Norovirus Infection

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*“When you are up to your neck in shit, all you can do is sing”*

- Samuel Beckett

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## ABSTRACT

Norovirus causes epidemic gastroenteritis. The extent of excess mortality related to norovirus infections is not established and factors that influence the duration of viral shedding have not been determined. The aims of this thesis were (i) to describe the mortality among hospitalised patients with norovirus enteritis (NVE), (ii) to identify factors that indicate an increased mortality risk and a prolonged duration of viral shedding, and (iii) to examine if rectal swab samples can be used for the diagnosis of norovirus infection.

In **paper I**, we retrospectively studied 598 adult hospitalised patients with gastroenteritis and a stool sample positive for norovirus. For ages >80 years, 30-day mortality was higher among patients with community-onset NVE, compared to patients with hospital-onset NVE and to matched controls. In **paper II**, 82 patients with community-onset NVE were included. The adjusted odds ratio for death within 30 days was 2.5 for one mmol/L increase in the venous lactate measured on arrival to the emergency department. **Paper III** presents a prospective study of 28 patients admitted with NVE. Rectal swab samples were obtained weekly during follow-up. Slow clearance of norovirus was associated with low serum levels of the chemokine CCL5 and high viral load. In **paper IV**, PCR was performed on paired rectal swab and stool samples, obtained simultaneously from 69 patients with suspected viral gastroenteritis. In 38 sample pairs virus was detected in both samples. One pair was stool+/swab− and one pair was stool−/swab+.

In conclusion, norovirus infection may be associated with increased short-term mortality. Venous lactate can be used to identify patients with high mortality risk and a low level of CCL5 is associated with a long duration of viral shedding. Rectal swab samples can be used to diagnose norovirus infections.

**Keywords**: norovirus, mortality, lactate, viral shedding, CCL5, rectal swab
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# SAMMANFATTNING PÅ SVENSKA

Norovirus är det virus som orsakar vinterkräksjuka. Viruset är mycket smittsamt och sprids lätt från person till person. Under vinterhalvåret uppträder ofta epidemier med norovirus, särskilt på sjukhus och äldreboenden. Fler personer dör under vinterhalvåret än under sommaren och det är möjligt att norovirusepidemier bidrar till den ökade dödligheten under vintern. Dödsfall som direkt orsakas av norovirusinfektion förekommer men är ovanligt. Att vinterkräksjuka indirekt bidrar till dödsfall hos sköra patienter kan vara betydligt vanligare.

I delarbete I undersöktes dödligheten hos vuxna patienter på Sahlgrenska sjukhuset som haft vinterkräksjuka. I studien var dödligheten inom 30 dagar förhöjd hos äldre patienter (över 80 år) som fått vinterkräksjuka innan de kom till sjukhuset, både i jämförelse med patienter som blivit sjuka under vårdtiden och jämfört med patienter som inte haft kräksjuka alls. I delarbete II undersöktes olika faktorer som tidigt kan identifiera de patienter med norovirusinfektion som riskerar att avlida. Höga värden av mjölksyra (laktat) i blodet var kopplat till en ca 2-3 gånger högre risk. Laktat stiger vid många allvarliga tillstånd och patienter som har förhöjda värden behöver undersökas noggrant och behandlas på akutvårdsavdelning.

När symtomen försvunnit avtar smittsamheten snabbt. Dock fortsätter norovirus att utsöndras i avföringen under flera dagar och ibland veckor. I delarbete III undersökte vi vilka faktorer som har betydelse för hur långvarig virusutsöndringen blir. Det verkar som att patienter som utsöndrar virus en längre tid är något äldre, har mer uttalade symtom och utsöndrar stora mängder virus redan under den akuta sjukdomsperioden. De hade också låga blodhalter av en signalsubstans i immunsystemet, CCL5, som stimulerar och lockar till sig T-celler (lymfocyter). T-lymfocyter har en nyckelroll i försvaret mot virusinfektioner och det är möjligt att skillnader i hur dessa lymfocyter hanterar infektionen har betydelse för hur länge norovirus finns i avföringen.

En misstänkt norovirusinfektion bekräftas genom att norovirus kan påvisas i ett avföringsprov med hjälp av molekylär teknik (PCR). Avföringsprover kan vara svåra att ta, till exempel om patienten är ett spädbarn eller en äldre person med demenssjukdom som inte kan samarbeta. Pinnprov från ändtarmen kan däremot tas enkelt och utan dröjsmål. Därför gjorde vi i delarbete IV en jämförelse mellan pinnprov och avföringsprov från patienter med magsjuka. Vi fann att provtyperna är likvärdiga och att bägge kan användas för att diagnostisera norovirus med hjälp av PCR.

# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Gustavsson L, Andersson L-M, Lindh M, Westin J
Excess mortality following community-onset norovirus enteritis in the elderly
*Journal of Hospital Infection* 2011; 79: 27-31.

II. Gustavsson L, Andersson L-M, Brink M, Lindh M, Westin J
Venous lactate levels can be used to identify patients with poor outcome following community-onset norovirus enteritis.
*Scandinavian Journal of Infectious Diseases* 2012; 44: 782-787.

III. Gustavsson L, Skovbjerg S, Lindh M, Westin J, Andersson L-M
Low serum levels of CCL5 are associated with longer duration of viral shedding in norovirus genogroup II infection
*In manuscript*

IV. Gustavsson L, Westin J, Andersson L-M, Lindh M
Rectal swabs can be used for diagnosis of viral gastroenteritis with a multiple real-time PCR assay
*Journal of Clinical Virology* 2011; 51: 275-278.

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| BLAST | Basic local alignment search tool |
| CCL5 | Chemokine (C-C-motif) ligand 5. “RANTES” |
| CDC | United States Centers for Disease Control and Prevention |
| CI | Confidence interval |
| Ct | Cycle threshold |
| CTL | Cytotoxic T lymphocyte |
| CXCL | Chemokine (C-X-C-motif) ligand |
| EIA | Enzyme immunoassay |
| GI | Norovirus genogroup I |
| GI.1 | Norovirus genogroup I, genotype 1. “Norwalk virus” |
| GII | Norovirus genogroup II |
| GII.4 | Norovirus genogroup II, genotype 4 |
| HBGA | Histo-blood group antigen |
| IEM | Immune electron microscopy |
| IFN | Interferon |
| IL | Interleukin |
| MHC | Major histocompatibility complex |
| MIF | Macrophage migration inhibitory factor |
| MNV | Murine norovirus |

| | |
|---|---|
| NCBI | National center for biotechnology information |
| NLV | Norwalk-like virus |
| NoV | Norovirus |
| NVE | Norovirus enteritis |
| ORF | Open reading frame |
| qPCR | Real-time PCR |
| RAG | Recombination activating gene |
| RANTES | Regulated on activation, normal T cell expressed and secreted |
| SLV | Sapporo-like virus |
| STAT | Signal transducer and activator of transcription |
| ULN | Upper limit of normal |
| vB-lactate | Venous blood lactate |
| VF1 | Virulence factor 1 |
| ViGGo | The viral gastroenteritis in Gothenburg study |
| VLP | Virus-like particle |
| VP1 | Viral capsid protein 1. “Major capsid protein” |
| VP2 | Viral capsid protein 2. “Minor capsid protein” |

# 1 INTRODUCTION

*"- It is an inferno!*

*JM knows what she is talking about. As the head of a geriatric ward at the hospital of Kungälv, she is familiar with the depredations of the virus [...] - But early spring was the worst, she says, flipping the pages in a yearbook where the first three months is a constant chain of outbreaks, cohort care, ward closures, sick staff, extra staff etc." [1]*

This quote, from a Swedish newspaper story, illustrates the perception of norovirus in hospitals. The outbreaks cause major disturbances in patient care and seem to go on endlessly. The whole ward, or parts of it, has to shut down for long periods of time. Available hospital beds, which were few to begin with, become even more rare. And then the doctors and nurses catch it. The experience, true as it may be, has led to a widespread fear of gastroenteritis.

*"A man with cerebral haemorrhage suffered permanent brain damage when the ambulance did not take him to the hospital immediately. Since the man was vomiting, the ambulance nurse suspected winter vomiting disease, and did not want to bring him to the ambulance. After a couple of hours, when his condition had deteriorated, he was taken to the hospital." [2]*

*"One woman, 67 years old, was admitted in January because of a wound in one hand. As she also had vomiting and diarrhoea she was isolated awaiting further treatment. Not until 8 hours later, when the hand had become discoloured, was sepsis suspected and antibiotic treatment started. Twenty-four hours later, the woman died." [3]*

This thesis may describe morbidity and mortality in norovirus infection. For most of us, though, norovirus phobia is the real danger. The only thing *we* have to fear is fear itself.

## 1.1 The ascent of norovirus

In 1929 a paediatrician in St Louis, John Zahorsky, described "winter vomiting disease" [4]. This was an illness that was characterised by the sudden onset of vomiting and diarrhoea and occurred in outbreaks that peaked during the colder months. The cause of the disease remained unknown for over 40 years, until Kapikian and co-workers in 1972 could show that it was caused by "a 27-nm particle" [5]. In a truly impressive effort

(including over 20 months of examining stool samples with the electron microscope) they demonstrated small virus particles in diarrhoeal stool from healthy volunteers. Gastroenteritis was induced in the volunteers by oral administration of bacteria-free faecal filtrates, which were prepared from a 1968 school outbreak of "winter vomiting disease" in Norwalk, Ohio. As a result, the new virus was called "Norwalk virus".

Following this original discovery, other "small, round structured viruses" causing gastroenteritis, such as the Hawaii and Snow Mountain viruses, were described [6, 7]. These small, round gastroenteritis viruses were subsequently classified as "Norwalk-like viruses" (NLV) [8]. The NLVs were estimated to cause less than 10% of gastroenteritis cases [9]. NLVs could not be cultured and studied with traditional virological methods, and they remained obscure for several years, until new molecular methods became available. In 1990 the full genome was described and cloned [10], which led to the development of PCR assays (in 1992). With access to genetic information it was recognised that the various NLVs were different genotypes of a single genus, eventually termed *norovirus* [11]. When PCR assays were applied in clinical studies of gastroenteritis patients, it became clear that this virus was responsible for a majority of the cases where previously no aetiologic agent had been found [12]. The importance of norovirus could now be fully acknowledged. In recent estimates, norovirus causes between 12% and 40% of all gastroenteritis cases, in all parts of the world [12-14]. For epidemic gastroenteritis, norovirus is by far the most common cause, responsible for up to 90% of gastroenteritis outbreaks [15]. Each year, around 400 000 people get sick from norovirus in Sweden [16].

Public awareness of norovirus was raised in 2002-2003, when Europe and Sweden saw large, nationwide epidemics of a new variant of norovirus genotype II.4 [17]. Much media attention was focused on outbreaks in hospitals across the country. In an interview in a local newspaper in October 2002, an infection control physician made a direct translation into Swedish of Zahorsky's "winter vomiting disease". The term, "vinterkräksjukan", quickly caught on and was accepted as a new word in Swedish from 2002 [18].

### 1.1.1 Calicivirus

Other enteric viruses, also discovered in the 1970s, with a different appearance on electron microscopy, were designated caliciviruses [19]. The name was derived from characteristic cup-shaped depressions (calyces) in the virion. The prototype for the human caliciviruses was the Sapporo virus, described in Japanese children with gastroenteritis in 1979 [20]. When the

entire genomes were analysed in the 1990s, the close genomic relatedness between the caliciviruses and the Norwalk-like viruses became apparent [21]. The human caliciviruses were re-branded Sapporo-like viruses, and together the Norwalk-like and Sapporo-like viruses were placed as different genera in the family *Caliciviridae* [8]. These awkward designations have since been revised to *norovirus* and *sapovirus*, and the term calicivirus refers to both viruses. Although the illness caused by the two viruses is similar, they have distinct epidemiological and virological properties, and are seldom referred to collectively in the scientific literature.

## 1.2 The disease

The typical norovirus illness begins with vomiting, sometimes forceful and with a sudden onset, followed by abdominal cramps and watery diarrhoea. Associated symptoms can be low-grade fever, headache, myalgia and chills (explaining the other common nick-name, "stomach flu"). The symptoms recede relatively quickly and usually disappear within 2 to 3 days [22]. In older ages, however, the symptoms are less specific. Vomiting is often absent and diarrhoea dominates, and the duration of disease sometimes stretches to one week or longer [23]. Table 1 shows the frequencies of symptoms in different age groups in a study of 1544 cases of norovirus gastroenteritis in Catalonia, Spain [24].

*Table 1. Distribution of symptoms in patients with norovirus gastroenteritis (reprinted with permission from Arias et al, Clin Microbiol Infect 2010 [24]. Copyright 2010, European Society of Clinical Microbiology and Infectious Diseases)*

| | Age group | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | All cases[a] | | 1–4 years | | 5–11 years | | 12–17 years | | 18–64 years | | ≥65 years | |
| Symptoms | Cases | % | Cases | % | Cases | % | Cases | % | Cases | % | Cases | % |
| Diarrhoea | 1150 | 78.4 | 44 | 77.2 | 108 | 49.8 | 57 | 53.8 | 504 | 85.6 | 437 | 87.9 |
| Vomiting | 940 | 65.1 | 44 | 74.6 | 180 | 82.2 | 91 | 84.3 | 369 | 64.2 | 256 | 52.9 |
| Abdominal pain | 922 | 67.2 | 54 | 91.5 | 180 | 86.1 | 73 | 83.0 | 416 | 74.4 | 199 | 43.5 |
| Nausea | 712 | 51.9 | 17 | 30.4 | 110 | 54.7 | 66 | 75.0 | 362 | 64.0 | 157 | 34.1 |
| Fever | 448 | 31.7 | 19 | 32.2 | 71 | 33.3 | 42 | 40.0 | 230 | 41.5 | 86 | 17.8 |
| Headache | 417 | 31.3 | 11 | 20.4 | 81 | 40.1 | 49 | 57.0 | 221 | 41.5 | 55 | 12.0 |
| Myalgia | 303 | 24.1 | 10 | 18.5 | 13 | 6.8 | 17 | 22.1 | 203 | 39.4 | 60 | 14.2 |
| Chills | 181 | 15.7 | 3 | 7.7 | 17 | 12.8 | 24 | 30.0 | 107 | 22.2 | 30 | 7.1 |
| General malaise | 117 | 7.8 | 10 | 16.7 | 45 | 20.1 | 5 | 4.5 | 44 | 7.2 | 13 | 2.6 |

[a]In 40/1544 cases, age was unknown.

The incubation time is 12-60 hours, but occasionally longer [25]. Following resolution of symptoms a period of self-quarantine is recommended, to

reduce the risk of transmission [26]. How long this period should be is not clearly defined, but 48 hours is recommended in Sweden [27].

Norovirus disease has also been studied experimentally, where gastroenteritis is induced in healthy volunteers by oral administration of suspensions of norovirus. Here, the concept of norovirus infection is more complicated. The clinical incubation time is 1-2 days but detection of virus in stool can be delayed up to 5 days following inoculation. The peak faecal concentration of virus appears around 2 days after symptoms have disappeared, when stool is again solid. Viral shedding is often prolonged, and norovirus can be detected in stool for up to 8 weeks [28] (and for up to 2 weeks in mouthwash samples [29]). About one-third of those infected do not develop gastroenteritis symptoms, but participants with asymptomatic infection shed similar amounts of virus for equally long times [30]. This finding is supported by observations from clinical and population-based studies, where norovirus is frequently detected in persons without recent gastroenteritis symptoms [12, 31].

Knowledge about the pathophysiology of norovirus enteritis is limited, but villus blunting and reduction of villus surface area has been reported to occur, in the duodenum and jejunum [32]. The duodenal epithelium is invaded by CD8+ cytotoxic T cells and the rate of apoptosis increases sharply. Diarrhoea appears to be caused by a combination of reduced sealing tight junction protein expression and increased active anion secretion to the lumen [33]. The mechanism behind the onset of vomiting has not been described in detail. Stimulation of brain stem structures by vagal afferents, similar to the proposed mechanism in rotavirus infection, is one possibility [34].

There is no effective treatment available against norovirus infection. Supportive therapy with oral rehydration or intravenous fluids, and temporarily discontinuing selected drugs, such as ACE-inhibitors, warfarin and metformin, may be appropriate. The efficacy of anti-emetics for symptom relief has not been studied systematically.

## 1.3 Epidemiology

Norovirus infection is common. The incidence was estimated to 3800/100,000 person-years in a recent study from the Netherlands [16], and to 4700/100,000 in the 2012 UK infectious intestinal disease study [35]. In an often-cited report from the US Centers for Disease Control and Prevention (CDC) the annual number of norovirus cases in the United States is approximately 19-21 million [36]. For Sweden these figures translate to

between 370,000 and 650,000 cases of norovirus gastroenteritis annually. In addition, norovirus activity is reported to increase up to 50% in years when new strains emerge [37]. This would mean that almost 1 million people, or 10% of the population, are affected by winter vomiting disease during such years. Since the illness is usually brief and self-limiting, only a minority of cases require medical attention. Even so, around 9000 Swedes visit a doctor, and 1200 patients become hospitalised, because of norovirus gastroenteritis in a normal year (based on the Dutch data). As with many other infectious diseases, the risk of serious norovirus enteritis, that requires medical attention, is highest among small children and in the elderly (Figure 1) [38].

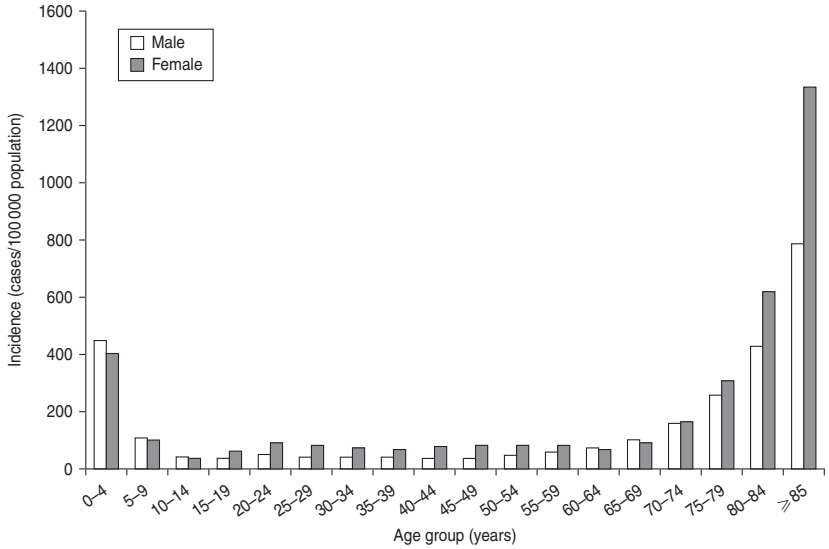


*Figure 1. Incidence of reported norovirus cases in different age groups, Germany 2001-2009 (reprinted with permission from Bernard et al, Epidemiol and Infect 2014 [38]. Copyright 2014, Cambridge University Press)*

The incidence of norovirus infection is not constant over the year. In temperate climates it displays a distinct seasonal variation that is nearly identical from year to year (Figure 2). In the northern hemisphere, the number of cases is low during summer and fall, begins to rise in late November, and increases sharply in December. The incidence rises more slowly, but steadily, through January and peaks in late February or early March, before declining to virtually disappear in May. In tropical or sub-tropical climates there is less seasonal variation, although a peak can be noted during the rainy season [39]. The recurring seasonal epidemics are dominated by one specific type of norovirus, the genogroup II, genotype 4 (GII.4) virus, which eclipses the other genotypes almost completely, and can cause 90-95%

of cases [40]. During non-epidemic periods, the relative contribution of other norovirus lineages, such as the original Norwalk virus (GI.1), is greater, but GII.4 is still the most common norovirus [37].

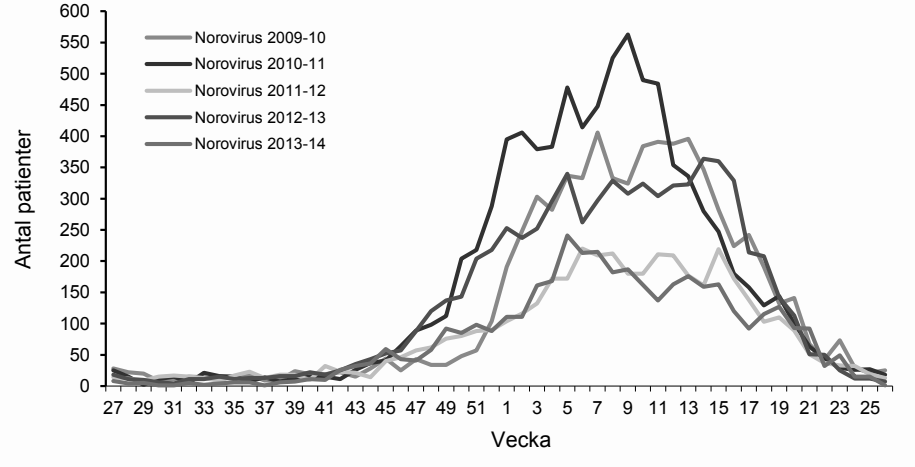


*Figure 2. Reported cases of norovirus per week (reprinted with permission from the Calicivirus final report 2014, The Public Health Agency of Sweden, 2014)*

Norovirus GII.4 is a rapidly evolving virus. Since 2002 new strains of GII.4 have emerged with regular intervals of two to three years. The new strain replaces the previously dominant variant [38, 41] and causes a large seasonal epidemic with more norovirus-associated morbidity [42]. The burden of norovirus on the healthcare system is therefore variable, as high-incidence years are commonly followed by one or more years with a rather low incidence. The association between emergence of new GII.4 strains and widespread norovirus epidemics is not straightforward, however, which is illustrated in Figure 3. In Sweden, the GII.4 2012/Sydney strain was projected to replace the previous (GII.4 2010) during 2012, but for some reason the older strain remained dominant in most healthcare settings. Even though the community epidemic appeared to be large, the number of nosocomial cases did not increase as expected [43].

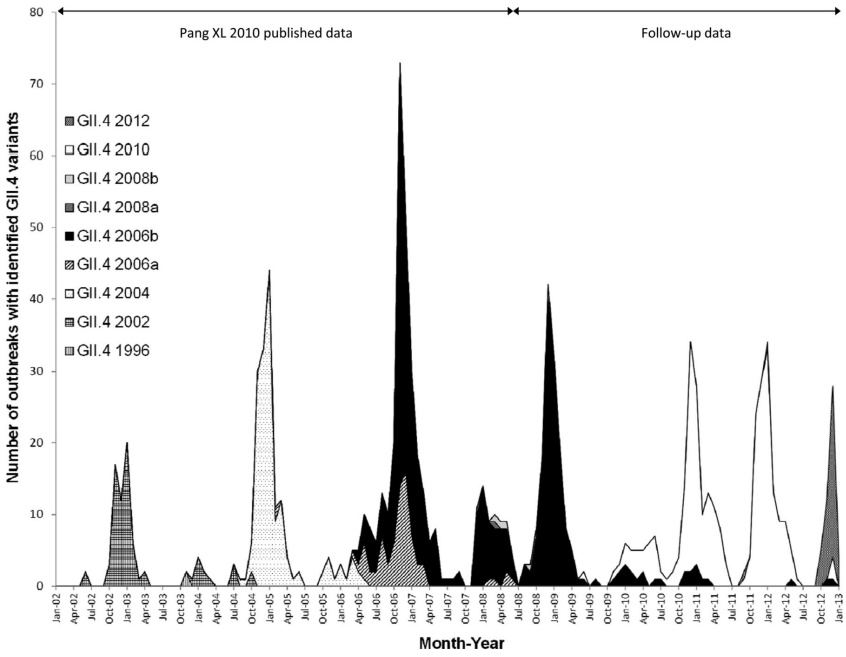


*Figure 3. Norovirus GII.4 variants in outbreaks in Alberta, Canada. Note that in 2002-2008 there is a bi-annual pattern with large epidemics following the emergence of new strains. From 2008/2009 this pattern disappears, as the seasonal epidemic was large without a GII.4 shift, followed by a limited epidemic in 2009/2010 despite the emergence of the new GII.4 2010 variant. The next two annual epidemics were similar even though they were dominated by the same strain (reprinted with permission from Hasing et al, J Clin Microbiol 2013 [44]. Copyright 2013, American Society of Microbiology).*

## 1.4 The virus

Norovirus is a small (≈38 nm), non-enveloped RNA virus that belongs to the *Caliciviridae* family. The norovirus genus is divided into five genogroups, of which three (GI, GII and GIV) infect humans. Each genogroup contains several genotypes. An overview of the noroviruses is shown in Figure 4.

The human norovirus genome is a single positive RNA strand, approximately 7,500 bases long, that contains three open reading frames (ORFs). ORF 1 encodes the non-structural viral proteins and enzymes, while ORF 2 and 3 encode the two capsid proteins, VP1 and VP2 (Figure 5) [21]. VP1 is the major structural protein, and contains the protruding P domain.

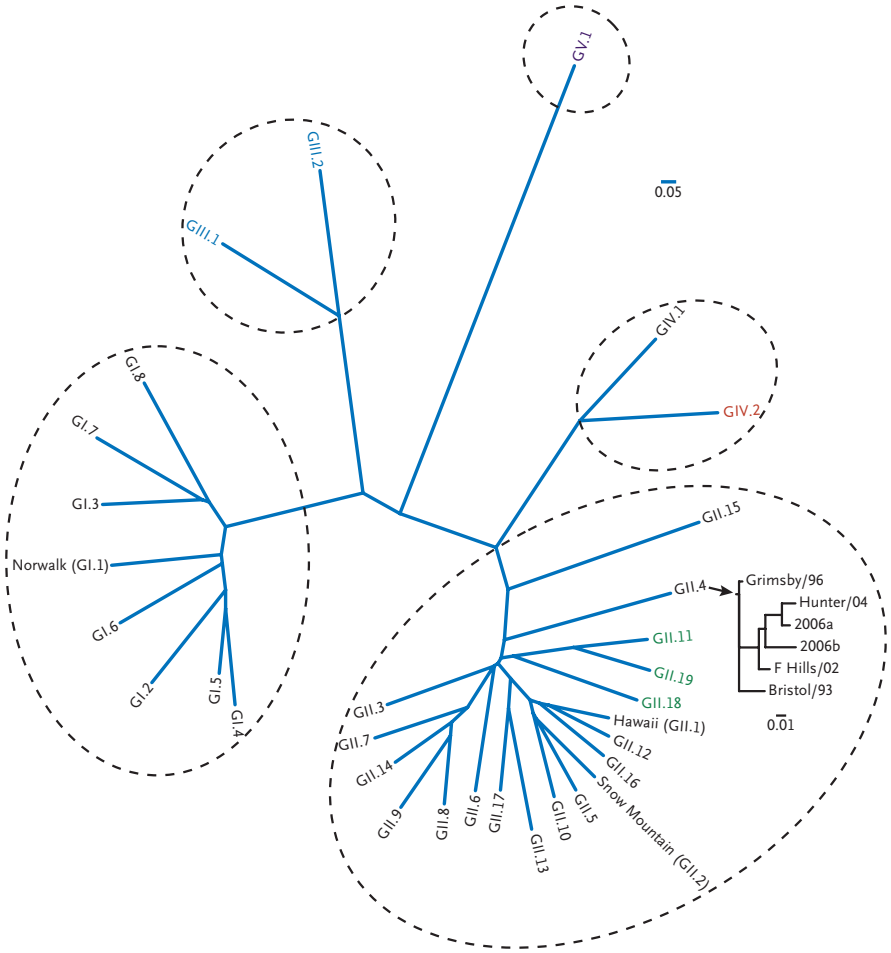


*Figure 4. Overview of the norovirus genus. Genotypes that infect humans are black, and can be found in genogroups I, II and IV. Some of the original "small, round structured viruses" are named. For the genogroup II, genotype 4 (GII.4) virus, subtype strains are shown in a separate section. These successively emerging strains are named after year of appearance. Genogroup V is the murine norovirus (reprinted with permission from Glass et al, NEJM 2009 [45]. Copyright 2009, Massachusetts Medical Society).*

This P domain is further subdivided into the P1 and P2 subdomains, of which the latter is the most surface-exposed (Figure 6) [46]. Ninety dimers of VP1 assemble to form the virus capsid. The protruding P domains describe an icosahedral structure over the cup-shaped depressions (calyces) that give the *Caliciviridae* family its name [47]. Translation of ORF 1 results in a poly-protein that contains the non-structural viral proteins. The virus-encoded protease then cleaves the protein into six functioning units: the protease; three

enzymes involved in the replication process (N-terminal protein, nucleoside triphosphatase, and p20); VPg, which attaches to the 5´end of the genome; and the viral RNA-dependent RNA polymerase [48].

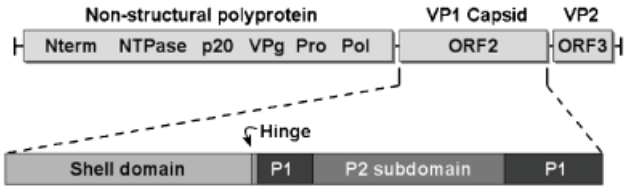


*Figure 5. The norovirus genome, which is an approximately 7,500 bases long positive RNA strand with three open reading frames (ORF). ORF1 encodes the non-structural proteins (Nterm, N-terminal protein (p48); NTPase, nucleoside triphosphatase; VPg, viral protein genome-linked; Pro, viral proteinase; Pol, RNA-dependent RNA polymerase), ORF2 encodes the major capsid protein VP1, and ORF3 a minor capsid protein, VP2, with largely unknown functions. VP1 consists of the shell domain and the protruding P1 and P2 subdomains (reprinted with permission from Donaldson et al, Immun Rev 2008 [49]. Copyright 2008, Blackwell Munksgaard).*

The details of host-virus interactions are difficult to study, due to the lack of an *in vitro* cell culture model. When the major capsid protein is expressed in recombinant systems it self-assembles into virus-like particles (VLPs). These VLPs can then be used to study aspects of virus-host interactions [50]. Thus, it has been shown that the P2 subdomain of VP1 recognises and binds to human histo-blood group antigens (HBGAs) [51]. The HBGAs are genetically determined carbohydrate structures present on the surface of enterocytes and other human cells (including erythrocytes, where the HBGAs determine a person's blood group). Virus strains that are not able to bind to a specific HBGA are not able to infect patients who express that same HBGA. The classic example is the so-called "non-secretors", who are naturally resistant to infection with the original Norwalk virus (norovirus GI.1) [52]. Non-secretors have a type of HBGA to which GI.1 VLPs (and most GII.4 VLPs) do not bind [53, 54]. The importance of HBGAs is further illustrated by the finding that the best correlate of protective immunity is not virus-specific antibodies, but rather HBGA-blocking antibody titers [55]. The HBGAs are now regarded as viral receptors and key host-susceptibility factors [45].

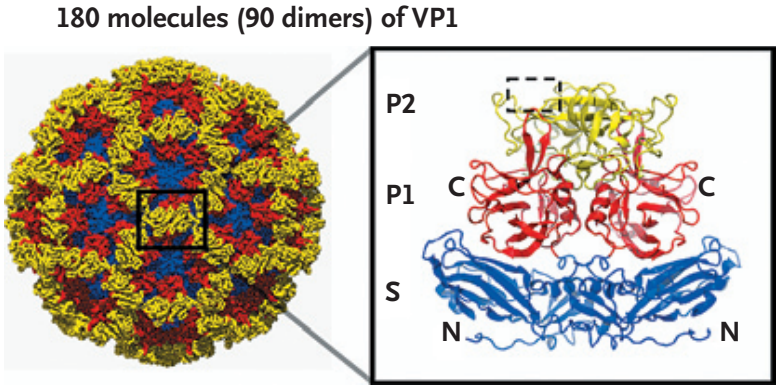


*Figure 6. The capsid structure of norovirus. Note the surface structure with depressions (blue) typical of caliciviruses. The capsid is formed by 90 dimers of viral capsid protein 1 (VP1), shown on the right. The shell is formed by the S domain (blue), with the P domain protruding from the surface. The exposed P2 subdomain (yellow) contains the histo-blood group antigen-binding region (dashed box). Antibodies that block this region are a correlate of protective immunity. In GII.4 viruses, the P2 subdomain is highly variable (reproduced with permission from Glass et al, NEJM 2009 [45]. Copyright 2009, Massachusetts Medical Society).*

### 1.4.1 Escaping the immune system

Presence of GII.4 antibodies is almost universal by the age of five [56], but this fact does not hinder the virus from causing outbreaks with attack-rates exceeding 50% [57]. Early challenge studies found that when volunteers were re-challenged six months after an infection they were protected from illness, but if they were re-challenged after more than two years they became infected, and ill, once again [58, 59]. This led to the assumption that immunity following norovirus infection for some reason wanes quickly and does not provide long-term protection. The validity of observations from these early pilot studies for non-experimental settings has been questioned [60]. For instance, GII.4 viruses are highly prevalent and limited exposure to the virus, acting as a booster of immunity, is likely to occur frequently. In a more recent publication, where a mathematical model of transmission was constructed from available data on incidence, infectiousness, duration of shedding, etc., the duration of immunity was estimated to last for a period of between 4 and 9 years [61].

The coincidence of large epidemics with the emergence of new GII.4 strains in the 2000's suggested that protection against circulating strains was widespread but that antigenically novel strains could infect readily, *i.e.* that antigenic drift was occurring. A hypervariable region in the P2 subdomain of the capsid appears to be central to this process [62]. Amino acid replacements

in this surface-exposed, protruding structure are thought to cause the loss of blocking ability in existing antibodies, allowing the virus to escape the protective memory immune response. Such mutations are likely selected under the pressure of herd immunity [63]. In contrast, GI viruses display only minor variations in the P2 subdomain, with considerable cross-protection between strains and without evidence of antigenic drift [60]. Another possible source of new norovirus strains are immunocompromised patients with chronic norovirus infections [64]. Here, the likelihood for the appearance of fit escape mutants is increased by long-term replication under lax immune control [65]. Onwards transmission of such mutants from chronic norovirus patients has been shown [66], and if the antigenic appearance is sufficiently different, they may form a new successful strain.

The same variable region of the P2 domain is also responsible for the HBGA-binding described above [67]. This means that immune-driven changes in this part of the capsid can also affect the binding capacity of the virus to different HBGAs [62]. If strains with new HBGA affinities appear, they can infect individuals that have previously been off-limits to the virus and are naïve (immunologically speaking). Thus, the virus might enter new non-immune populations and thrive.

## 1.5 Methods to study norovirus

The story of norovirus is also a story of the development of new diagnostic methods.

### 1.5.1 Electron microscopy

Norovirus was first detected using immune electron microscopy (IEM). Stool samples were incubated with convalescent serum from an ill volunteer followed by ultracentrifugation, staining and other preparations for electron microscopy [5]. In a positive sample aggregates of virus particles could then be visualised. IEM is a technique that requires highly skilled microscopists and expensive equipment, but even in the best of circumstances has a very low sensitivity, ranging from 20-30% [68]. This is too low to be useful for sporadic cases of gastroenteritis. In practice, its use was limited to outbreak investigations. Yet for over 20 years it remained the most reliable method to directly diagnose norovirus infections.

### 1.5.2 Antigen tests

Norovirus (NoV) can be detected with immunoassays. Norovirus-specific antibodies, derived from humans or animals, are used to capture antigen from

stool samples. Labeled NoV antibodies are then added to measure the amount of captured antigen. Present-day enzyme immunoassay (EIA) antigen tests use monoclonal NoV-specific antibodies for capture and detection. They have a very high specificity (>98%) with sensitivities between 50-70% [69], and can detect asymptomatic shedding [70]. Commercial test kits, including point-of-care tests, are available [71]. Due to the low sensitivity, antigen tests are unsuitable for diagnosing norovirus in a clinical setting. Still, EIAs can be useful for outbreak investigations, if access to PCR is limited [72].

### 1.5.3 Serology

Originally, serology was also performed with immune electron microscopy. Positive stool samples were added to patient serum and the mixture was examined for aggregates of virus [5]. In a development parallel to that of antigen tests, EIAs were created for the detection of norovirus-specific antibodies. The use of these tests was restricted to experimental studies and occasional outbreak investigations [73]. Today, norovirus antibodies are detected by EIAs that utilise virus-like particles (VLP) as the capture antigen [74]. Strain specific VLPs can be used to detect strain-specific antibody responses and allows detailed seroprevalence investigations [56]. In clinical virology, however, there is no place for serology in the diagnosis of norovirus infections.

### 1.5.4 Real-time PCR

The cloning of the Norwalk virus genome in 1990 was a major breakthrough. It paved the way for the development of PCR-based detection of norovirus in stool [75, 76]. Real-time PCR (qPCR), developed in the late 1990s, enabled reliable and rapid detection of viral RNA [77]. It also enabled an estimation of viral load, since the amount of virus present in a sample is inversely related to the cycle threshold value of the positive PCR reaction (Figure 7) [78]. These assays have since been continually improved, automated and updated. Modern qPCR assays have very high analytical sensitivity and specificity, and are the gold standard for the diagnosis of norovirus at present [79]. qPCR is also the standard method used in clinical virology practice, across high-income countries.

When qPCR is used in epidemiological studies, norovirus is detected in a proportion of asymptomatic patients [12, 80]. Also, in both clinical studies and volunteer experiments, norovirus RNA can be detected in stool samples obtained several weeks after the symptoms have disappeared [28, 81]. However, qPCR assays amplify and detect the RNA segment targeted by the primers and probes, disregarding the rest of the viral genome and whether

functional virions are present or not [82]. In order to distinguish between shedding of viral RNA remnants and genuine norovirus infection, an equally sensitive assay that detects only infectious virions, essentially replacing qPCR as gold standard, is required.

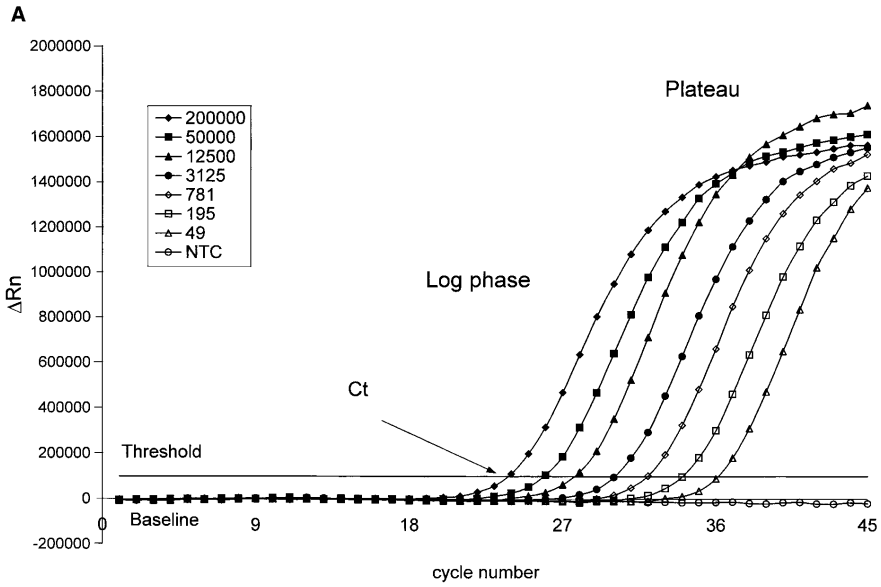


*Figure 7. Example output from real-time PCR on samples with different amounts of target sequence (box). Fluorescence (ΔRn) is plotted on the y-axis and cycle number on the x-axis. Amplified sequences (amplicons) are detected with fluorescent reporters or dyes, and the cycle threshold value denotes how many PCR cycles were run before the fluorescence threshold was reached. Samples with high amounts of the target sequence (norovirus RNA, for instance), that require few amplification cycles before the number of amplicons reach the detection threshold, have low cycle threshold values (and vice versa). NTC, no template control; Ct, cycle threshold (reprinted with permission from Giuletti et al, Methods 2001 [83]. Copyright 2001, Elsevier Science).*

Although cycle threshold (Ct) values are not necessarily lower (indicating higher viral load) in symptomatic than in asymptomatic patients [28, 31], attempts to find a Ct value cut-off that improves the predictive value of a qPCR-positive sample have been made. A cut-off at Ct 30 was suggested in one influential study [84]. The infectious dose of norovirus is low [85], however, and a safe level of faecal viral load, where transmission is unlikely, has not been presented. To the contrary, onward transmission from symptomatic patients with Ct >30 has been documented [86].

### 1.5.5 Sequencing

For the clinical diagnosis of norovirus infection, ideally all variants and strains of existing human genotypes, present and emerging, should be detected. Consequently, clinical qPCR assays are directed at highly conserved regions of the viral genome. In outbreak investigations or epidemiological and viral evolution studies, on the other hand, the focus is on variations in the infecting strains. Variable regions, typically ORF 2, where the P domain of the capsid is encoded, are studied with sequencing of the viral genome.

The most widespread sequencing technique currently is dye-termination sequencing. This approach uses coloured di-deoxynucleotides that terminate the elongation of DNA if incorporated by the polymerase. Thus, during polymerisation a mixture of DNA fragments with various length will be produced, all marked by one out of four colours. Subsequently this mixture is migrated through a capillary tube. The fragments will pass through the tube in order of size, starting with the shortest. By registering the colour changes as the fragments pass the sequence can be deduced. The sequence can then be submitted to a sequence database, to establish the degree of relatedness in comparison with known genotypes or outbreak strains. Alternatively, phylogenetic analysis is performed with the sample sequence and a selection of reference sequences.

Dye-terminating sequencing is limited by a restriction in the length of sequences that can be read. To overcome this, various other techniques are increasingly used for sequencing. With so-called next-generation sequencing techniques, whole-genome sequences can be determined, often directly from clinical samples [87]. Whole-genome sequencing has several applications in norovirus research, and has already been put to use for detailed chain-of-transmission analysis [66].

### 1.5.6 Animal models

There are several animal noroviruses, with the murine norovirus (MNV) placed as a single genotype within genogroup V [11]. MNV offers a small animal model of norovirus infections that is useful in immunology studies [88]. Genetically engineered knockout mice, who lack defined pathways in the innate or adaptive immune system (such as $STAT1^{-/-}$, $RAG1^{-/-}$, $IFN^{-/-}$ or MHC class $II^{-/-}$ mice), are infected. Symptoms, viral kinetics or pathology can then be assessed and compared, and conclusions can be drawn about the role of different immune mechanisms involved in MNV infection. Animal models are also important for vaccine development [89].

### 1.5.7 Virus-like particles (VLP)

In the absence of a cell culture system for human norovirus, the most important model for studies of capsid structure and host-virus interactions is the virus-like particle. The gene for the major capsid protein, VP1, is inserted into a virus that readily infects cell culture and produces large quantities of viral proteins. The two most common systems are the Baculovirus system and the Venezuelan equine encephalitis virus replicon particles (VEE-VRP) system [50, 90]. When VP1 is expressed in this way, it forms dimers which then self-assemble into a 90-dimer sphere with a very virus-like appearance on electron microscopy [47]. These virus-like particles are used to determine the molecular structure of the capsid or visualise the interaction between the P2 domain and carbohydrate structures [46, 51]. They are used to determine key antibody and T-cell epitopes [91], and serve as highly specific capture antigens in serology EIAs and in histo-blood group antigen-blocking assays [55]. Last, but not least, VLPs are immunogenic and atoxic when administered to humans, and provide a promising concept for a norovirus vaccine [92].

## 1.6 Infection control aspects

The importance of norovirus in clinical medicine is chiefly related to the wintertime hospital outbreaks of gastroenteritis. Norovirus GII.4, in particular, seems well adapted to the health care setting [93]. Outbreaks can become large and linger on for weeks [94], disturbing the normal operations of medical and geriatric wards, and they are costly [95, 96]. For these reasons, prevention of nosocomial spread of norovirus is a priority for infection control units across Europe and elsewhere. This work is hampered by a relative lack of evidence-based preventive measures. A review made on behalf of the Swedish Institute for Infectious Disease Control found that almost no evidence of high quality has been published [27]. In the absence of proven effective protocols, we rely on expert opinion and consensus guidelines that are based on data from observational studies and clinical experience.

The best available evidence is that close physical contact facilitates spread [97]. The basis of all infection control measures is therefore to keep infectious patients separated from susceptible patients. New admissions with suspected viral gastroenteritis should be placed in single rooms [97]. To avoid unnecessary isolation of patients, and minimise the risk for misinterpretation of gastroenteritis symptoms, a qualified medical assessment is warranted [27]. In the British guidelines, it is explicitly stated: "*rapid*

*clinical assessment of the patient by a doctor with full competence to decide on the destination of the patient. Preliminary assessment by more junior doctors should be avoided*" [98]. When gastroenteritis symptoms develop in patients after admission to multi-occupancy rooms, however, isolation of the suspected case does not necessarily protect fellow patients [86]. Instead, most guidelines stress that incubated patients should be isolated for the duration of the incubation period [27]. There is also support for the division of the affected ward into separate cohorts of infected/incubated and unaffected patients [99]. The infected cohort should be closed to new admissions, but the practice of closing entire wards for new admissions does not appear to be more effective in limiting the duration of outbreaks [99, 100]. Nosocomial spread of norovirus occurs mainly around symptomatic cases and screening for norovirus and isolating patients with asymptomatic shedding is not recommended [101]. Still, guidelines prescribe an extended period of quarantine following resolution of symptoms, to ensure that symptoms do not reappear, and because of the clinical experience that this precaution prevents onward transmission. A symptom-free interval of 48 hours is usually required before infection control measures can be terminated [27, 98].

Norovirus is stable on most surfaces, and it is ubiquitous in the hospital environment during outbreaks [102, 103]. Alcohol-based disinfectants are ineffective [104]. In outbreak situations and after tending to patients with suspected or confirmed viral gastroenteritis, standard hand disinfection should be complemented with thorough hand washing with soap and water [98]. All contact surfaces surrounding a norovirus patient should be thoroughly disinfected, or discarded, to prevent point-source transmission [105, 106]. For surface disinfection, available data primarily support the use of sodium hypoclorite, but potassium peroxymonosulfate (Virkon), in a 1% solution, also appears effective in experimental studies and from clinical experience [27].

# 2 AIM

The overall aim of this thesis was to assess factors influencing disease severity and complications from norovirus infection.

Put in more specific terms the aims were:

- To describe all-cause mortality following norovirus gastro-enteritis in hospitalised patients, in relation to similar patients without gastroenteritis.
- To examine the association between venous lactate levels and mortality, in patients with norovirus gastroenteritis.
- To investigate factors that may affect the duration of viral shedding in norovirus infections, with special reference to cytokine profile.
- To analyse if rectal swab samples can be used for diagnosing norovirus infection.

# 3 PATIENTS AND METHODS

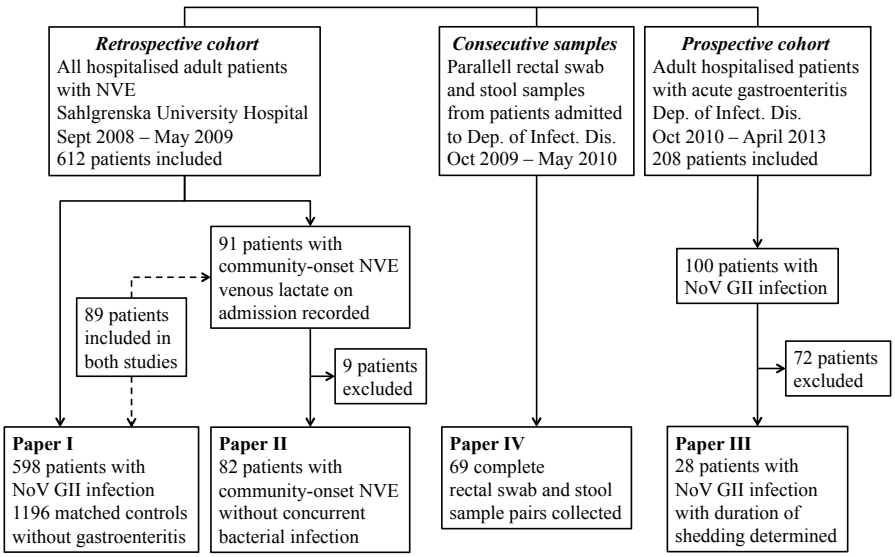


*Figure 8. Overview of the study participants in this thesis. NVE, norovirus enteritis; NoV GII, norovirus genogroup II.*

## 3.1 Patients

The participants in the first three studies included in this thesis were recruited from two different cohorts of patients with norovirus gastroenteritis. Paper IV was based on a series of consecutive clinical samples.

### 3.1.1 Retrospective cohort (paper I & II)

All hospitalised adult patients (>18 years) treated at Sahlgrenska University Hospital between September 2008 and May 2009, who had provided a stool sample for virological analysis where norovirus (NoV) was detected, were included in the cohort. We reviewed medical records to ensure that patients had gastroenteritis symptoms at the time of sampling. Community-onset norovirus enteritis (NVE) was defined as vomiting and/or diarrhoea with reported onset before arrival to the hospital. Consequently, hospital-onset NVE was defined as the reported onset of gastroenteritis symptoms after admittance to the hospital. The following variables were registered: type of ward (medical, surgical etc.), date of admittance and discharge, reported date of symptom onset, concurrent relevant medical conditions, vital signs and

routine blood chemistry tests on admission or at symptom debut, diagnosis at discharge, and date of death. Survival time was defined as the number of days from positive sample to date of death, up to 90 days. All patients were treated according to current clinical practice. The retrospective cohort comprised 612 patients.

In paper I, patients with NoV genogroup II infection were included (n=598). A control group was created, consisting of 1196 randomly selected hospitalised patients without gastroenteritits (two for each included study patient) matched for age, sex, type of ward and month of the year. Ward types were divided into geriatric, surgical, infectious disease, pulmonary and non-pulmonary medical wards, and other types. The control subjects were selected from a list of all patients hospitalised at Sahlgrenska University Hospital (excluding those with gastroenteritis diagnoses on discharge) arranged chronologically by date of birth. For each study participant, the first two patients with matching age, sex, ward type and month were included in the reference cohort. For the controls, survival time was defined as number of days from date of admittance to date of death.

In paper II, patients with community-onset NoV gastroenteritis (genogroup I and II) and with venous lactate recorded on arrival to the emergency department, were included (n=91). From 2009 and onwards, venous lactate was measured routinely in all patients presenting to the emergency department (unless their condition was designated "not life-threatening and not in need of immediate care" by the hospital's triage protocol). Since elevated lactate is common in several other medical emergencies, patients were excluded if one of the discharge diagnoses was serious bacterial infection, *Clostridium difficile* infection, shock, appendicitis, diverticulitis, cholecystitis or intestinal ischemia. The remaining 82 patients were included in the analysis.

### 3.1.2 Prospective cohort (paper III)

The prospective study of *Vi*ral *G*astroenteritis in *Go*thenburg (*ViGGo*) was conducted at the Department of Infectious Diseases, from September through April in three successive years (2010-2013). Adult patients who were admitted with suspected gastroenteritis were screened for inclusion. The inclusion criteria were: Age >18 years; vomiting ≥2 times/24 h and/or ≥3 loose stools/24 h; and symptom duration ≤5 days. Patients with bacterial gastroenteritis and patients who failed to provide informed consent were excluded. Demographic and clinical data were recorded for each participant. We used the Vesikari score to assess gastroenteritis disease severity [107].

This score was originally developed for grading rotavirus infections in children. It gives a numerical value, based on duration and intensity of the gastroenteritis symptoms and on the degree of dehydration, which enables quantitative comparisons between groups. Similarly, co-morbidity was summarised with the Charlson co-morbidity score [108]. Here, concurrent medical conditions are weighted and added to give a numerical score predicting the background 1-year mortality rate. Rectal swab samples were secured on enrolment and if patients were positive for norovirus, we asked them to provide follow up samples on day 7, 14, 21 and 28 of the study period. Serum and whole blood samples were also obtained on enrolment. During the study period, 957 patients were screened for participation. Two hundred eight patients could be included in the cohort, of whom 100 had NoV GII infection.

In paper III, we included 28 patients without immune suppression from this cohort, who had consented to provide follow-up samples after discharge and whose duration of viral shedding could be determined.

### 3.1.3 Consecutive samples (paper IV)

Between October 2009 and April 2010 parallel rectal swab samples and stool samples were collected at the Virological Laboratory, Department of Clinical Microbiology, Sahlgrenska University Hospital. The samples were obtained consecutively from hospitalised patients with gastroenteritis admitted to the Department of Infectious Diseases. We compared the 69 complete sample pairs in paper IV.

In the prospective cohort there were 69 patients who had provided a clinical stool sample within 24 hours from enrolment, when the study swab sample was collected. Consequently, we had access to 69 additional representative sample pairs that could be included in the analysis, for the purpose of this thesis.

## 3.2 Methods

### 3.2.1 Rectal swab samples

The rectal swab samples were collected with a regular flocked swab in a standardised manner. A specially trained nurse collected all rectal swab samples in paper IV, and all samples obtained on enrolment in paper III. The follow-up samples (in paper III) were collected by the participants themselves at home, according to instructions provided by the study nurse.

Following sampling the swab was placed in a dry sterile tube for transportation. Stool samples were obtained according to clinical routine. In the laboratory, stool samples were homogenised in 4.5 mL buffered NaCl. For swab samples 1.5 mL NaCl was added and the tube was vortexed briefly.

### 3.2.2 Real-Time PCR

PCR was used to detect NoV and diagnose NoV infections. Real-time PCR (qPCR) is a sensitive, reliable and fast technique. It is the standard method to diagnose viral gastroenteritis, in Sweden and elsewhere. The qPCR system that is used at the Department of Clinical Microbiology is a multiplex system, set up to detect six clinically relevant enteric viruses; adenovirus, astrovirus, NoV genogroup I and II (GI and GII), rotavirus and sapovirus. In addition, *Clostridium difficile* toxin B is included in the panel. Details on the primers and probes, and on the composition of the PCR mixes, are presented in the Appendix (see page 56).

The RNA polymerase-major capsid protein junction, which is a conserved region across genotypes, was the target region for both NoV GI and GII. The assay for NoV GII was combined with the rotavirus assay, whereas NoV GI was analysed in a separate mix. The lower limit of detection can be determined by serial dilution of plasmids containing inserts of the target region. For these assays the limit of detection was estimated to 10-30 copies for each PCR reaction.

The extraction of nucleic acids is automated. We used two types of extraction robots for the analyses in this thesis: the NucliSENS easyMAG robot (Biomerieux, Marcy l'Etoile, France) and the Magnapure LC robot (Roche Molecular Systems, Mannheim, Germany). Amplification and detection was then performed in an ABI7300/7500 qPCR instrument (Applied Biosystems, Foster City, CA, USA).

### 3.2.3 Genotyping

To distinguish between the genotypes in genogroup II we used nucleotide sequences. cDNA was produced by reverse transcriptase, and a segment of the viral genome was then amplified with nested PCR (primers are presented in the Appendix). An approximately 1000 base long fragment, including the RNA polymerase and major capsid protein genes, was amplified in the first step. In the following step, a 350 base fragment, covering the polymerase-capsid junction and first part of the major capsid protein gene, was generated from the PCR product. This amplicon was subjected to dye-terminating

sequencing, and the genotype was identified by submission to the NCBI BLAST database.

### 3.2.4 Lactate measurements

Venous lactate was measured with Radiometer analysing robots (Radiometer ABL800, Radiometer medical, Bronshøj, Denmark). This is a common type of analysing robot used for blood gas and electrolyte measurements in intensive care units and emergency departments. It uses an amperometric ion-selective electrode to determine the lactate concentration in heparinised whole blood.

### 3.2.5 Cytokine detection

We measured cytokines in serum with the ”Bio-rad Pro Cytokine, Chemokine and Growth factor assays” (Bio-Rad laboratories, Hercules, CA, USA). This assay is based on xMAP technology (Luminex, Austin, TX, USA), which uses microscopic colour-marked beads coated with specific reagents. A large number of different beads can be distinguished in a single well, due to the diversity of the colour marking. This technique permits the detection and quantification of multiple molecules simultaneously. We used the premixed Bio-Plex human cytokine 27-plex and 21-plex assays in paper III. Here, the beads are coated with specific immunoassays for 48 different cytokines and growth factors. The concentrations are interpolated from standard curves produced from wells with known concentrations of the analytes. For the cytokine analysis, serum samples from 20 healthy blood donors were used as controls.

### 3.2.6 Statistical methods

Several statistical tests were used in this thesis. In papers I-III, we made univariate comparisons of continuous variables with the Mann-Whitney U test or one-way analysis of variance (ANOVA). Univariate comparisons of proportions and risk ratios were made with $\chi^2$-test, Fisher’s exact test or $\chi^2$-test for trend. We used one-step logistic regression for multivariate analyses. In paper I, we also used Kaplan-Meier survival analysis with the log-rank test. For the purpose of this thesis, we performed additional multivariate modelling on data from paper I and II, with Cox proportional-hazards regression. We made the calculations with PASW Statistics 18 or IBM SPSS Statistics 20 software (IBM corp., Armonk, NY, USA).

## 3.3 Outcome measures

In papers I and II, the primary outcome was 30-day mortality. We also presented data on 90-day mortality and length of stay.

In paper III, we measured the duration of viral shedding and we defined two possible outcomes: rapid clearance, defined as a negative swab sample PCR at day 7 or 14 of the study period; and slow clearance, defined as a positive swab sample PCR on day 21 or 28 of the study period.

The primary outcome in paper IV was sensitivity of rectal swab samples for the diagnosis of viral gastroenteritis. We defined gold standard as the detection of norovirus in one or both sample types in paired stool and swab specimens.

## 3.4 Ethical considerations

The Regional Ethical Review Board in Gothenburg approved the creation of the patient cohorts and the studies in paper I, II and III. All participants in the prospective cohort (paper III) gave their written informed consent. The samples in paper IV had been collected for clinical purposes. They were de-identified prior to our analysis and no personal information was available.

# 4 RESULTS AND DISCUSSION

## 4.1 Mortality following norovirus infection (paper I & II)

### 4.1.1 Community-onset and hospital-onset norovirus gastroenteritis

In temperate climates, the general mortality is higher during winter. Annual winter epidemics of norovirus genogroup II (NoV GII) may contribute to this seasonal variation in mortality. In paper I, we investigated the extent of all-cause mortality following NoV GII gastroenteritis in a retrospective cohort study of hospitalised patients. The cohort consisted of 598 adult patients with gastroenteritis symptoms and a stool sample positive for NoV GII by qPCR. Overall 30-day mortality was 7.6%. There were no deaths among patients less than 60 years old ($n$=60) and we excluded these patients from further analysis. Here in the thesis frame, crude mortality risks are presented as unadjusted risk ratios (Table 2, not included in the published paper). As expected, the 30-day mortality risk increased with increasing age and the presence of co-morbidity. Interestingly, there was also a strong trend towards increased mortality in patients with community-onset norovirus disease.

*Table 2. Unadjusted risk ratios for death within 30 days, in 538 hospitalised patients aged >60 years with norovirus gastroenteritis*

| | Risk ratio | 95% CI | *p*-value |
|---|---|---|---|
| Age (+10 years) | 1.8[a] | 1.3-2.6 | 0.002 |
| One or more co-morbid conditions | 2.0 | 1.03-3.8 | 0.04 |
| Male sex | 1.2 | 0.7-2.1 | 0.6 |
| Community onset of symptoms | 1.7 | 1.0-3.2 | 0.07 |

[a]corrected odds ratio [109]

In paper I, we adjusted for confounding by stratification and could show that community-onset of symptoms was associated with increased mortality in the older age group, see figure 9. When patients were stratified by age, we did not detect any differences in co-morbidity between patients with community-onset and hospital-onset disease (paper I, Table III). However, the number of

co-morbid conditions is a rough estimation of co-morbidity. It does not take the prognosis of various concurrent diseases into account.

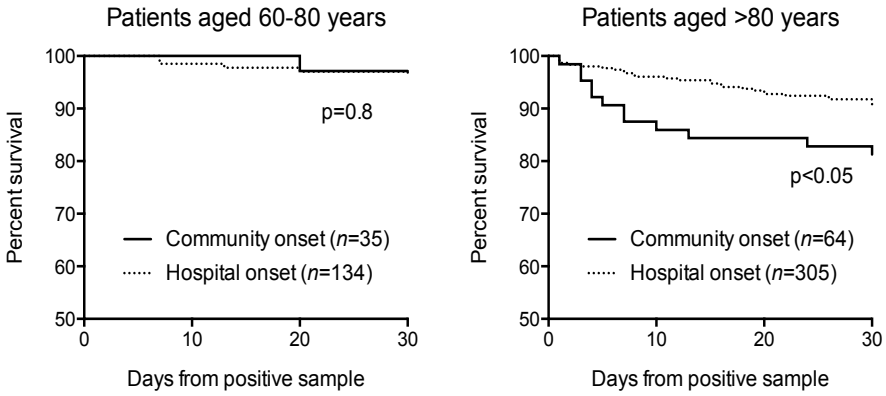


*Figure 9. 30-day survival curves for patients with community and hospital onset of gastroenteritis symptoms, in a retrospective cohort study of 538 hospitalised patients with norovirus genogroup II infection. In patients aged >80 years, 30-day mortality was 18.8% and 9.2%, respectively. Day 0 was the date of first norovirus-positive stool sample. The left panel was not included in the published version of paper I.*

A weighted index of co-morbidity would adjust better for baseline morbidity. In paper III, we used the Charlson co-morbidity score, a weighted co-morbidity index developed in the 1980s [108]. Although modern treatments have since improved the prognosis of most diseases, the *relative* mortality risks remain similar (with one notable exception – AIDS – that is assigned the highest possible score by Charlson *et al*. In the era of effective antiretroviral therapy, this no longer holds true).

*Table 3. Adjusted hazard ratios for death in the first 30 days following norovirus gastroenteritis, in 538 hospitalised patients aged >60 years.*

| | Hazard ratio | 95% CI | p-value |
|---|---|---|---|
| Age (+10 years) | 2.1 | 1.4-3.3 | 0.0004 |
| Charlson score (+1 point) | 1.3 | 1.1-1.5 | 0.001 |
| Community onset of symptoms | 1.9 | 1.0-3.7 | 0.05 |

With Charlson scores calculated for the patients from paper I, we were able to do multivariate analysis with Cox proportional-hazards regression,

adjusting for both confounders in the same model. In this model, community-onset disease was an independent risk factor for death within 30 days (Table 3, not included in the published version of the paper).

### 4.1.2 Norovirus patients versus matched controls

An alternative method to control for confounding was to create a reference cohort composed of matched control subjects. In paper I, we used a group of patients without gastroenteritis that was matched for age, month of the year and type of ward. We reasoned that patients hospitalised in the same type of ward are likely to have similar physical status and co-morbidities. It was also essential to match for month of hospitalisation, since the original hypothesis came from seasonal variations in mortality. Overall, there were no significant differences in mortality between patients with norovirus gastroenteritis and controls (8.6% *vs.* 8.0% for ages >60 years; 10.8% *vs.* 9,3% for ages >80 years). The survival of patients with community-onset norovirus gastroenteritis, compared to their matched controls, is presented in Figure 10.

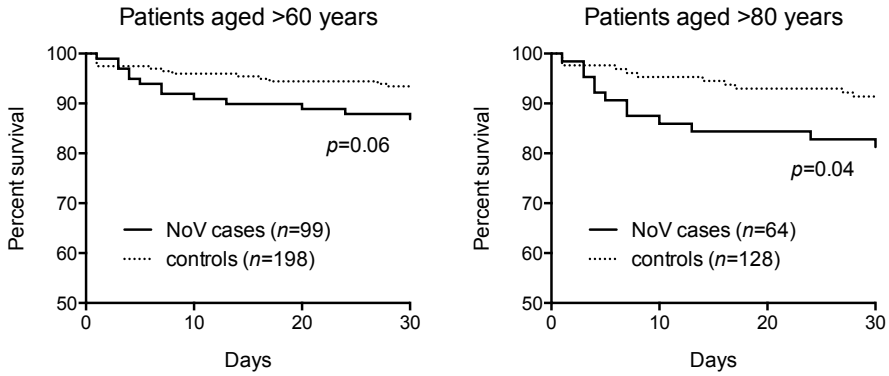


*Figure 10. Kaplan-Meier plots showing survival curves for patients with community-onset norovirus (NoV) gastroenteritis and matched control subjects without gastroenteritis. For all patients aged >60 years, 30-day mortality was 13.1% in NoV cases and 6.6% in controls (left panel, not included in the published version of paper I). For patients aged >80 years, 30-day mortality was 18.8% and 8.6%, respectively (right panel).*

Although not formally statistically significant, the diverging survival curves support the observation that community-onset norovirus infection was associated with increased short-term all-cause mortality. If the analysis was restricted to the older age groups, where the background mortality risk is higher, the difference was statistically significant. The focus of the published

paper was on elderly patients and results for the age group >80 years were presented.

### 4.1.3 Comments on 90-day mortality

We did not present detailed results for 90-day mortality in paper I. The effects of a norovirus gastroenteritis episode, usually lasting between 2 and 5 days, is not likely to extend for several months. The 90-day mortality rates were not significantly different between community-onset and hospital-onset disease (18.2% and 16.6%, respectively) or compared to controls (18.2% *vs.* 13.1%, p=0.25). If baseline mortality risks are similar, however, the increase in short-term mortality in an exposed group would still be apparent at 90 days. Otherwise, the proportion of deaths is lower in the exposed group for some time of the follow up period. This is illustrated in Figure 11 (not included in the published version of paper I). There are very few deaths in the community-onset group between day 30 and 90, while the mortality risk appears constant over time in the hospital-onset group and among controls. The most likely explanation is that this is a chance phenomenon caused by the low number of patients in the community-onset group. Alternatively, the finding suggests that among patients with community-onset norovirus gastroenteritis there was a subgroup of relatively healthy patients with low mortality risk.

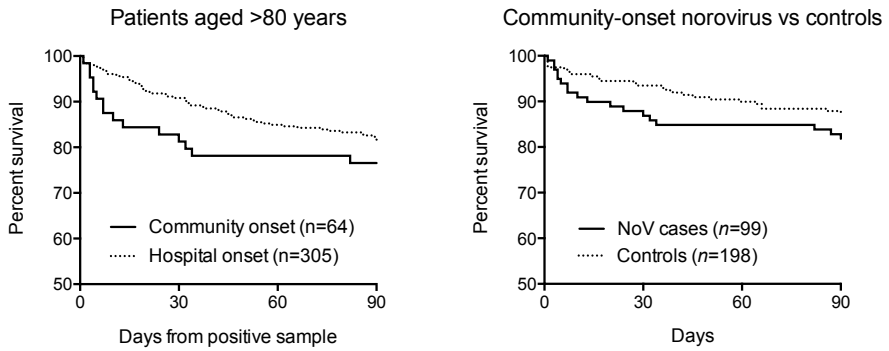


*Figure 11. 90-day survival curves for norovirus patients aged >80 years (left) and community-onset norovirus patients aged 60-101 years vs. matched controls (right). Differences were non-significant in both comparisons.*

The observation of low background mortality risk in a subset of patients with community-onset norovirus is supported by findings from the *ViGGo* study (the study cohort of paper III). That cohort included patients admitted with community-onset gastroenteritis, and the overall 90-day mortality was only 1.4% (4/208).

### 4.1.4 Lactate and norovirus gastroenteritis

In several medical emergencies, an increased lactate concentration is associated with mortality. We investigated if venous lactate measurements could be used to identify gastroenteritis patients who are at risk of a severe course of disease. In a retrospective cohort study of patients with community-onset norovirus gastroenteritis, presenting to the emergency department, we grouped the participants according to venous lactate on arrival. We used cut-off values derived from previous studies on emergency department lactate. After excluding patients with concurrent conditions known to give lactate elevation we were able to include 82 patients. The median age was 77 years (interquartile range 53-86) and 57% were women. One-third of the patients (34%) had two or more serious chronic conditions. Median venous lactate on arrival was 1.8 mmol/L and 55% of patients had a venous lactate above the upper limit of normal (ULN; 1.6 mmol/L). The distribution of lactate values and the 30-day mortality is presented in Figure 12.

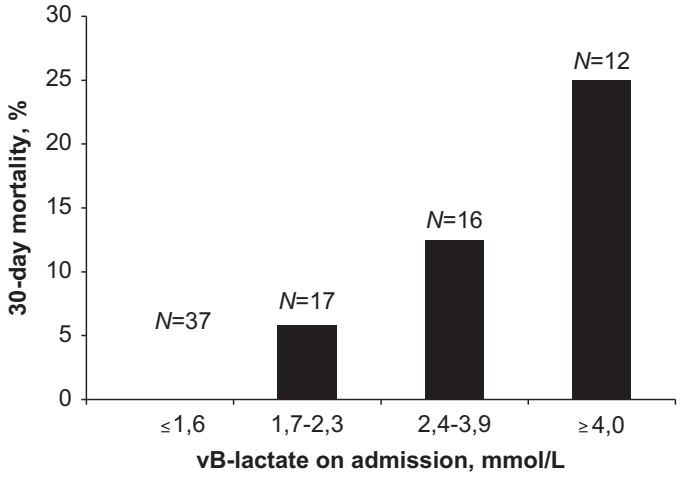


*Figure 12. Venous blood (vB-) lactate on arrival in 82 patients with community-onset norovirus gastroenteritis. The 30-day mortality rate in the four lactate intervals was 0%, 5.3%, 12.5% and 25 %, respectively; p<0.01.*

The most important differences between survivors and non-survivors were age, peripheral oxygen saturation and venous lactate (for the complete list of variables, please see paper II, Table III). Median venous lactate and age were significantly higher in patients who died within 30 days (4.5 *vs.* 1.7 mmol/L, p<0.01, and 88 *vs.* 66 years, p<0.01) and peripheral oxygen saturation was clearly lower (88% *vs.* 96%, p=0.25). To adjust for these factors, we included them in a multivariate logistic regression model. Venous lactate was

significantly associated with 30-day mortality in this model, independent of age and oxygen saturation. The adjusted odds ratio for death with an increase in venous lactate of 1 mmol/L was 2.5 (95%CI 1.003-6.3, $p<0.05$). For the purpose of this thesis, we also performed a multivariate Cox proportional-hazards regression analysis on the data. The result of this alternative model (not included in the published version of the paper) is presented in Table 4.

*Table 4. The independent association with the risk of death within 30 days for three emergency department variables, in patients with community-onset norovirus gastroenteritis (n=82).*

| | Hazard ratio | 95% CI | *p*-value |
|---|---|---|---|
| Age (+1 year) | 1.3 | 0.9-1.9 | 0.15 |
| Oxygen saturation (-1%) | 1.1 | 0.9-1.3 | 0.16 |
| Venous lactate (+1 mmol/L) | 1.9 | 1.2-2.9 | 0.003 |

In paper II, we found that a significant elevation of lactate (>2.4 mmol/L) was common in patients with community-onset norovirus gastroenteritis. We also found a relation between lactate and short-term mortality. This is in line with earlier investigations, where high lactate concentrations on presentation have been related to increased mortality in many medical emergencies, including fluid and electrolyte disturbances [110-112]. The adjusted odds ratio (and adjusted hazard ratio above) in our study was comparable to previously reported mortality risk ratios. In a much-cited paper from 2007, Howell *et al* showed that increased lactate predicted high mortality in patients with infection, regardless of systolic blood pressure [113]. This so-called occult hypoperfusion – high lactate with normal blood pressure – has since been established as an early warning sign in patients presenting to the emergency department with signs of bacterial infection. Untreated norovirus gastroenteritis, especially in combination with the ubiquitous anti-hypertensive diuretics and ACE-inhibitor/A2-receptor antagonist drugs or metformin, may also lead to dehydration, hypoperfusion and increased levels of lactate [114, 115]. There is also the possibility that other concurrent acute conditions, such as septicaemia [116] or an exacerbation of congestive heart failure [117], are present and need to be addressed. This multitude of possible causes of lactataemia makes lactate a poor differential diagnosis tool. On the other hand, in gastroenteritis as well as in other conditions, an elevated lactate gives an indication of the prognosis and severity of the condition.

The patients included in this study were elderly and age was, not surprisingly, another discriminating factor between survivors and non-survivors. Norovirus is primarily a threat to older patients, as seen in paper I and in other studies of norovirus-related mortality [118, 119]. We included peripheral oxygen saturation in our multivariate model, since respiratory distress is recognised as an early sign of systemic organ involvement in medical emergencies [120, 121]. Respiratory rate may be more sensitive in this respect, as hypoxia is a late and more sinister sign of respiratory failure. The median respiratory rate was indeed higher among the non-survivors, but the difference was small. Substituting oxygen saturation for respiratory rate did not improve the multivariate model (data not shown). This is likely due to the limited sample size, which restricted the analysis to variables with large effects on mortality. Because of the limited sample size and retrospective study design, other variables confounding the results may be overlooked. Moreover, we only included hospitalised patients. Since lactate values were available to the emergency physician, they may have influenced the decision to admit the patient, leading to a biased inclusion of patients with high lactate in this study.

In the *ViGGo* study (the study cohort of paper III), lactate values were available in 136 (65%) patients. Again, median lactate on admission was 1.8 mmol/L and it was elevated above the ULN in a majority of norovirus gastroenteritis cases (36/59, 61%), with a value above 2.4 mmol/L in 37% (22/59). Lactate was also elevated in the non-norovirus gastroenteritis cases, however, confirming that lactate values are neutral from a diagnostic perspective. Mortality in both groups was very low (90-day mortality was <2%).

In short, an elevated lactate is common in patients presenting with possible norovirus gastroenteritis. These patients should be carefully evaluated for other serious conditions that require specific treatment. The threshold for admitting elderly patients with gastroenteritis symptoms and an elevated lactate, for inpatient care and medical surveillance, should be low.

### 4.1.5 Aspects of the methodology

In this thesis, we did not aim to investigate deaths directly caused by norovirus but attempted to estimate the effect of norovirus enteritis (NVE) on all-cause mortality. Furthermore, we wanted to describe the effect on short-term mortality, including the period of convalescence. We hypothesised that NVE is overlooked in most death certificates when recent infection is related to the conditions directly causing mortality (by precipitating an exacerbation

in chronic heart failure, for instance). For these reasons, we omitted cause of death in the data registration. We focused on elderly patients, since they are more vulnerable and have much co-morbidity. The background morbidity and rate of death would have to be high if we were to detect an increased short-term mortality.

The foundation for papers I and II was a retrospective cohort study, and the major limitations of those papers are related to the study design. The first problem is that the data quality was not optimal. To account for this, all categories had to be very wide. Second, retrospective studies are prone to introduce selection bias. The participants were, strictly speaking, not NVE patients, but hospitalised patients with NVE. The latter group may be a very different group from the first. For the community-onset group there may have been other factors that necessitated the admission, in addition to the acute gastroenteritis. It is possible that this category represents a group of vulnerable patients, for whom hospitalisation was needed even for benign, self-limiting conditions. Patients in the hospital-onset group, on the other hand, were originally admitted and treated for other diseases. They might not have been admitted, or even been brought to the ED, if they had suffered community-onset NVE. This makes the cohort heterogeneous, with risk of confounding. We addressed these concerns by adjusting for obvious factors such as age and co-morbidity, but there might be other important differences that we were not able to control for.

For the comparison with an unexposed reference cohort in paper I, we attempted to create a control group that had a similar expected short-term mortality. Controls were matched for age, sex and time of the year (to account for the higher mortality during winter), and by ward type, since patients from the same type of ward are likely to have similar physical status. To match for co-morbidity in greater detail, such as by a weighted co-morbidity index score, would require thorough revision of the medical records of several thousand patients. This part of the study, where patients with NVE were compared to patients with other common acute conditions such as myocardial infarction or pneumonia, supported the conclusion that patients admitted with suspected NVE are frail and that careful evaluation and close attention is warranted.

### 4.1.6 The context of norovirus and mortality

When we started work on paper I, deaths specifically related to norovirus had been reported. In a case series published 2009 Roddie *et al* presented 12 patients, who had undergone allogeneic stem cell transplantation, who

suffered from severe gastroenteritis with a duration of several months, caused by norovirus genogroup II [122]. Two of the 12 patients eventually succumbed to the gastroenteritis. Rondy *et al* discovered 4 norovirus-related deaths among 144 elderly psychiatric patients during an outbreak in a long-term care institution in 2008 [123]. In addition, case reports of life-threating complications of norovirus gastroenteritis, such as bowel perforation and oesophageal rupture, had been published [124, 125].

Since the publication of paper I, several estimations of the mortality caused by norovirus, on a population level, have been published. These studies are based on outbreak data and information from death certificates. For the age group >65 years, it is estimated that norovirus directly causes 8-20 deaths/1,000,000 person-years [119, 126], or one death every 7$^{th}$ reported outbreak [127]. During epidemic seasons with emerging new strains, mortality increases by 50%. Assuming a norovirus incidence of 1/18-1/26 [35], this translates into mortality rates of 0.3-0.5/1,000 cases for normal years and up to 0.8/1,000 cases in epidemic years. Death certificates are not always accurate, however, and systematic under-reporting of conditions can occur. When *all-cause* mortality is related to norovirus infections, the mortality rate is estimated to 2-4/1,000 cases for old (>85 years) and vulnerable patients in long-term care institutions [93, 128]. A very comprehensive study from the U.S. found that season-adjusted, all-cause mortality in nursing homes increased 11% during norovirus outbreak periods (which in fact is close to the 16% increase we found for patients >80 years, although our sample size was too limited to detect such small differences at a 5% significance level) [129].

In view of these new results, it appears that the doubled mortality risk for community-onset norovirus infection, described in paper I, may be partly explained by selection bias. On the other hand, the new data support the main conclusion from this thesis; *i.e.* that norovirus may indeed contribute to excess mortality in the elderly.

## 4.2 The duration of viral shedding in norovirus infection (paper III)

The duration of faecal viral shedding in norovirus infections is variable. We investigated and described factors associated with brief and prolonged shedding of virus, in 28 patients admitted with NoV GII gastroenteritis. Twelve patients had cleared the virus within 14 days and constituted the "rapid clearance" group; 16 patients were still norovirus RNA-positive in

samples from day 21 or 28 of the study period, and they constituted the "slow clearance" group. The two groups are presented in Table 5. Due to the limited sample size, there were few significant differences between the two groups, although we noted several interesting trends (the apparent difference between men and women can be explained by age. The participating women were, on average, more than 20 years younger, and the sex difference disappeared when it was adjusted for age).

*Table 5. Characteristics of patients in the two study groups. Rapid clearance: faecal shedding of norovirus <14 days; slow clearance: shedding >21 days.*

| | Rapid clearance<br>*n*=12 | Slow clearance<br>*n*=16 | *p*-value |
|---|---|---|---|
| Age, years | 72 (34-86)[a] | 84 (71-89) | 0.21 |
| Women, *n* (%) | 10 (83) | 5 (31) | 0.006 |
| Charlson score (co-morbidity) | 0 (0-1) | 1 (0.25-1) | 0.11 |
| Vesikari score (symptom severity) | 10 (9-12) | 12 (10-13) | 0.12 |
| CRP, mg/L | 9 (3-22) | 26 (8-28) | 0.14 |
| Venous lactate, mmol/L | 1.6 (1.4-2.9) | 2.0 (1.6-2.8) | 0.51 |
| Viral load (Ct), day 0 | 27 (22-33) | 21 (19-25) | 0.03 |
| Viral load (Ct), day 7 | 34 (33-39)[b] | 27 (24-31) | 0.01 |

[a]median (interquartile range)
[b]*n*=5

Patients with long duration of shedding appeared to be older and have more co-morbidity, which was expected. But there were also trends towards higher CRP levels and more severe symptoms, indicating that their norovirus infection might be more intensive. The significantly lower cycle threshold (Ct) values in this group support this interpretation. Low Ct values mean greater amounts of virus, *i.e.* higher viral load. There are several possible explanations for high viral load; the infectious dose might be higher or the infecting NoV GII strain could be more virulent. In this observational study we could not control for the former, but genotyping revealed that most patients (87%) were infected with the genotype GII.4. The results did not change when we restricted the analysis to GII.4 patients and we concluded that major differences in the patients' norovirus strains could not explain the findings. Instead, we hypothesised that low viral loads and rapid clearance was related to host immune mechanisms. To investigate this further we

measured 42 inflammatory mediators in acute phase serum, obtained at enrolment (on average 3 days after onset of symptoms).

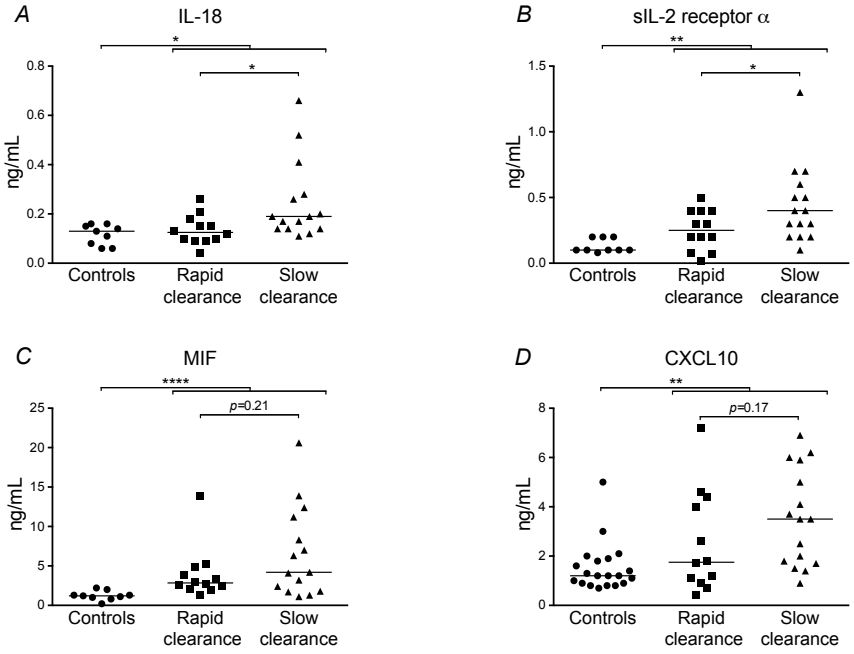


*Figure 13. Serum levels of four lymphocyte-related mediators in patients with rapid clearance (n=12) vs. slow clearance (n=16) of norovirus and healthy controls (n=9 in panel A-C, n=20 in panel D). The mediators are Interleukin-18 (A), soluble Interleukin-2 receptor α (B), macrophage migration inhibitory factor, MIF (C) and chemokine CXCL10 (previously IP-10, D). Horizontal lines denote median values. Stars denote significance level; ****, p<0.0001; ***, p<0.001; **, p<0.01; *, p<0.05. Outliers with very high values in the slow clearance group (n=1 in panel A-C) have been omitted for clarity.*

We discovered that patients with slow clearance of virus tended to have higher amounts of several T lymphocyte-related mediators, compared to the rapid clearance group (Figure 13). This was in line with the indications of more symptoms, higher CRP and higher viral load in the slow clearance group. However, the opposite was found for the chemokine, CCL5. This is an important T cell activating and –attracting cytokine (previously called RANTES, Regulated on Activation, Normal T cell Expressed and Secreted). The CCL5 levels were significantly lower in the slow clearance group, both compared to the rapid clearance patients and to healthy controls (Figure 14). This suggests that CCL5 could be involved in the immune mechanisms

responsible for the clearance of norovirus from the intestine. Alternatively, CCL5 secretion is suppressed by norovirus, resulting in lower CCL5 levels in patients with high viral load. Such a mechanism has been demonstrated for murine norovirus, which blocks interferon and CXCL10 expression through a viral protein called virulence factor 1 (VF1) [130].

In paper III, we speculate that the underlying mechanism is related to cytotoxic CD8+ T cell (CTL) activity. Norovirus-specific CTLs are critical for efficient clearance of norovirus in animal models [131, 132], and large numbers of CTLs are found in the duodenal mucosa of infected patients [33]. Since CCL5 levels are related to the amount and activity of CTLs [133-136], a possible explanation may be that the variations in CCL5 mirror differences in the activity of CTLs. In turn, such differences may explain the variations in duration of shedding.

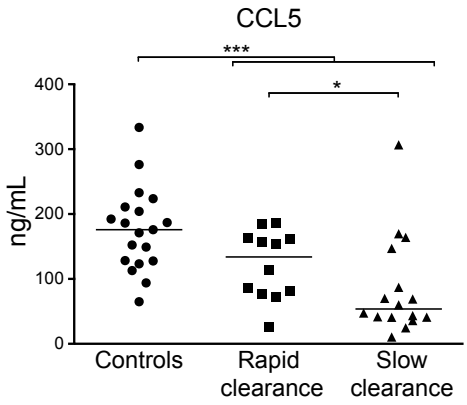


*Figure 14. Serum levels of CCL5 (chemokine (C-C-motif) ligand 5, previously called RANTES) in patients with rapid vs. slow clearance of norovirus, and healthy controls (n=20). One outlier in the control group with very high value (>800 ng/mL) was omitted for clarity. Horizontal lines denote median values. ***, p<0.001; *, p<0.05.*

Cytokines have multiple and overlapping effects in vivo. The interpretation of this kind of findings, from non-experimental settings, should be made cautiously. In this study we measured the serum cytokine levels, which may not be relevant at the site of norovirus replication. The cohort included a limited number of patients, who were elderly and hospitalised. For these reasons, we consider the association between low CCL5 and slow clearance a preliminary finding that needs to be confirmed in further studies.

## 4.3 Rectal swab samples for the diagnosis of norovirus (paper IV)

While rectal swab samples are more readily available than stool samples, the diagnostic value of swab samples for the detection of enteric viruses had not been evaluated systematically before 2010. We compared the PCR results for stool samples and rectal swab samples, obtained simultaneously from 69 patients admitted with suspected viral gastroenteritis. At least one of the sample types was positive, for any virus, in 40 of the sample pairs. In 38 pairs we detected virus in both samples. One pair was positive in the stool sample only and one pair in the rectal swab only. In both cases the Ct value of the positive was very high (>40). When the gold standard was set as a positive result in either (or both) of the sample types, the overall sensitivity was 97.5% (39/40), with a 95% CI of 93-100%. For NoV GII the sensitivity was 100% (27/27).

The average Ct was lower in stool samples, with a mean difference of 2 cycles. In one-quarter of the sample pairs, however, the Ct was lower in swab sample.

*Table 6. Detection rates for norovirus with qPCR, in pairs of rectal swab and stool samples from hospitalised patients with gastroenteritis. ViGGo=the viral gastroenteritis in Gothenburg study*

| | Paper IV *n*=69 | *ViGGo* study *n*=69 | Both studies combined *n*=138 |
|---|---|---|---|
| Either or both samples + | 27 (39) | 34 (49) | 61 (44) |
| Rectal swab sample + | 27 (39) | 31 (45) | 58 (42) |
| Stool sample + | 27 (39) | 33 (48) | 60 (43) |

Data shown as *n* (%)

We also compared parallel samples, collected within one 24-hour period, from patients in the prospective *ViGGo* study (not included in the published version of paper IV). The combined results are presented in Table 6. The detection frequency for norovirus remained high when we added these additional sample pairs. When all samples were considered together swab sample sensitivity was 95% (58/61) with a 95% CI of 90-100%. The sensitivity of stool samples was 98% (60/61; 95%CI 95-100)

In paper IV, we demonstrated an equal clinical usefulness of rectal swab samples and stool samples for the diagnosis of norovirus infections in a small pilot study. We obtained a similar result in the *ViGGo* study. Since the publication of paper IV, its conclusions have been confirmed in larger series of samples. One study collected paired samples from 100 children aged <5 years with diarrhoea in Guatemala and found equal detection rates for both sample types [137]. Another study, conducted in Rwanda, analysed 326 paired samples from children <5 years [80]. Here, 119 patients constituted a control group of children without gastroenteritis. Again the detection rates were almost identical in rectal swabs and stool samples, *i. e.* sensitivity and specificity were equal. Kabayiza *et al* also confirmed that the average Ct is 1-3 cycles higher in swab samples. This indicates that swab samples contain approximately 10-20 times less virus than stool samples

Despite the recently published evidence that supports the use of rectal swab samples, stool samples are still considered optimal and are recommended for norovirus detection in the CDC guidelines [26]. The performance of rectal swab samples is less clear for other types of assays than qPCR [138]. There is a risk of only limited volumes of faeces binding to a rectal swab, and this risk is likely increased with watery stools. When watery stools are present, during the acute phase of norovirus gastroenteritis, large amounts of virus are excreted (typically $10^7$-$10^{11}$ copies/g faeces [139]). The concentration of virus in the supernatant will be well above the limit of detection of qPCR assays, even if less stool is bound to the swab. There are also several examples of swab+/stool- sample pairs, in our studies as well as in the subsequent studies. This indicates that faeces volume is not the only important factor for successful amplification. Dilution of faecal inhibitory factors in the swab samples may even promote amplification efficiency [140, 141]. An inherent risk with swab samples is that incorrect sampling is likely to occur more often than with stool samples. This risk could be addressed by adding some marker of the faecal flora to the assay, as the absence of faecal bacteria in a swab sample would indicate faulty sample collection.

One difficulty with the routine use of qPCR for the diagnosis of viral gastroenteritis is that the detection rate is high in asymptomatic patients [12]. To overcome this problem, cut-off Ct values for stool samples have been proposed [84, 142]. These are not directly applicable to swab samples and specific swab sample Ct cut-offs have to be established. In a recently published study Elfving *et al* presented an attempt to define such cut-offs for a variety of diarrhoeic agents [143]. However, no cut-off Ct was found for norovirus. A positive rectal swab sample implicated norovirus disease, regardless of the Ct. Results from this study, performed in Zanzibar, may not

be directly applied to Europe or North America. In tropical and sub-tropical areas norovirus is endemic [39], and inhabitants are continuously exposed to the virus. It is possible that long-term viral shedding, which reduces the predictive value of a positive sample, is more common in an area with annual epidemics and intervening antigen drift.

In summary, rectal swab samples can be obtained conveniently without delay. If collected by trained staff, rectal swab samples are a reliable alternative to stool samples, despite the lower amount of virus in the sample.

# 5 CONCLUSIONS

Norovirus gastroenteritis, with community onset of symptoms, is associated with increased short-term all-cause mortality in elderly patients. Annual epidemics of norovirus infections may contribute to excess mortality in vulnerable groups.

An elevated venous lactate on arrival to the hospital is associated with higher mortality for patients with community onset of norovirus gastroenteritis. In elderly patients presenting to the emergency department with suspected viral gastroenteritis, venous lactate measurements can help to identify patients at risk for complications and a severe course of disease.

The duration of viral shedding in norovirus infection is highly variable, ranging from less than one week to over one month. Patients with a long duration of shedding appear to have low systemic concentrations of the chemokine CCL5 during the acute phase of gastroenteritis. This suggests a role for this chemokine in the clearance of norovirus infections.

Rectal swab samples are a reliable alternative to stool samples for the diagnosis of viral gastroenteritis.

# 6 FUTURE PERSPECTIVES

The most important clinical issue that remains to be addressed regarding norovirus infections is whether an effective vaccine can be developed. At present the outlook is cautiously optimistic. An experimental vaccine, consisting of Norwalk virus (GI.1) virus-like particles (VLP) administered nasally, was immunogenic and protected against re-challenge with the corresponding virus [92]. Phase I trials with intramuscular administration of multivalent GII.4 VLPs gave promising results [144] but, disappointingly, the first challenge study failed to show protective efficacy [145]. A possible explanation is that the VLPs lack the minor structural protein, VP2, which is a major determinant of protective immunity induction in animal studies [146]. Other groups use the P particle, a peptide multimer of a key antigenic part of the virus capsid, which appears to elicit a stronger cell-mediated immune response compared to VLPs [89]. If further studies are successful we have for the first time a realistic possibility to prevent norovirus infections. A commercially available norovirus vaccine may well appear in a not too distant future.

When a vaccine is available and its efficacy has been demonstrated in large trials on broad patient groups, the task of weighing potential benefit against side effects and cost remains. For this judgment, robust data on the disease burden of norovirus is needed. Recent epidemiological studies have provided improved estimates of the mortality and hospitalisations caused by norovirus. These results need to be reproduced as well as confirmed in other settings. Considering that norovirus is a mild, self-limiting infection in the vast majority of cases, a vaccination strategy targeted at the general population will be problematic. Identification of key risk groups can help guide future interventions to those patients who have the greatest benefit. Possible target populations include patients awaiting transplantation and elderly nursing-home residents. Based on the findings in this thesis and present knowledge, an effective vaccine may save lives in vulnerable groups.

The hopes of effective vaccination are tempered by the fact that even following natural infection immunity is short-term. Cross-protection between related strains is not certain. In the successful trials that have been performed so far vaccine efficacy was around 50% (and only 26% for preventing asymptomatic infection). Although these figures should improve with improved formulations, the vaccine effectiveness in risk groups, such as the elderly and patients with immune deficiencies, will likely be rather low. Effective prevention will still require adherence to infection control measures

and prompt identification and isolation of suspected cases. Annual or bi-annual vaccination with antigens tailored to the projected dominant GII.4 strains may become a valuable adjunct in this work. Improved herd immunity among risk groups and caregivers should decrease the occurrence of large and lingering outbreaks.

There might also be more to norovirus vaccination than protective immunity. Here, the results from paper III open for future studies. The finding that low viral load and rapid clearance of virus is related to CCL5 levels suggests that immune mechanisms affect these variables. If these underlying mechanisms can be described it might aid vaccine development, as well as other therapeutic options. Given that low viral load decreases the risk of transmission, which is likely but has yet to be proven, a vaccine formulation that is able to incorporate stimulation of these putative "rapid clearance mechanisms" may be more effective in reducing the frequency of outbreaks.

The diagnostics of gastrointestinal infections is evolving rapidly. Whole-genome sequencing offers new possibilities for norovirus research, and has potential clinical applications [147]. For instance, replacing primer-and-probe-based PCR with the detection of full-length norovirus genomes might enhance the specificity of molecular assays. In the meantime, fully automated, cartridge-based, PCR technology has become available for norovirus, and for other agents with risk for nosocomial spread, such as *Clostridium difficile*. The use of swab samples in combination with this type of point-of-care tests, supplying reliable results within one to two hours, could potentially eliminate the need to isolate patients with a low clinical suspicion of norovirus. Even if these tests are 100% accurate, the question whether isolation facilities are used more efficiently with access to rapid testing for norovirus still has to be answered [148]. Another route of development is to create increasingly complex multiplex qPCR systems. Virtually all pathogens of interest, be it viruses, bacteria or protozoans, can be included. A correct diagnosis is made quickly, with other possible diseases excluded simultaneously. However, these multiplex systems need to be evaluated thoroughly in relation to current standard methods. Many gastrointestinal infections are regulated under the Swedish Communicable Diseases Act, based on positive stool cultures, and before alternative methods for detection are implemented these issues have to be addressed in clinical settings.

Improved diagnostics will perhaps not help us get rid of norovirus. But hopefully it will free us from something else – norovirus phobia. And with it, the fear of fear itself.

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# APPENDIX

*Table I. Primers, probes and target regions for the multiple real-time PCR assay used throughout this thesis*

| Pathogen | Mixture | Forward primer | Reverse primer | Probe | Target region | Reference |
|---|---|---|---|---|---|---|
| Norovirus GI | 1 | TGGCAGGCCATGTTCCGCT | TTTGKTGGGGCGTCCTTAGAC | ATTGCGATCTCCTGTCCA | pol-capsid junction | Nenonen *et al*, 2009 |
| | 1 | | CGCTTGATGTAGCGTCCTTAGAC | | | |
| Norovirus GII | 2 | TGGAYTTTTAYGTGCCCAG | CGACGCCATCTTCATTCAC | AGCCAGATTGCGATCGCCC | pol-capsid junction | Nenonen *et al*, 2009 |
| Rotavirus | 2 | AACCATCTACACATGACCCTCTATGA | GGTCACATAACGCCCCTATAGC | CAATAGTTAAAAAGCTAACACTGTCAAA | non-structural protein 3 | Pang *et al*, 2004 |
| | 2 | AACCATCTTCACGTAACCCTCTATGA | | | | |
| Astrovirus | 3 | GACTGCWAAGCAGCTTCGTGA | GCTAGCCATCACACTTCTTTGGTCCT | TCACAGAAGAGCAACTCCATCGCATTTG | pol-capsid junction | Gustavsson *et al*, 2011 |
| Sapovirus | 3 | TTGGCCCTCGCCACCTAC | CCCTCCATYTCAAACACTA | CCRCCTATRAACCA | pol-capsid junction | Oka *et al*, 2006 |
| | 3 | GAYCASGCTCTCGCYACCTAC | | | | |
| Adenovirus | 4 | GCCACGGTGGGGTTTCTAAACTT | GCCCCAGTGGTCTTACATGCACATC | TGCACCAGACCCGGGCTCAGGTACTCCGA | hexon | Heim *et al*, 2006 |
| Clostridium difficile | 4 | ATCATTACTTCATCTTTGGGGATAGC | ATCTCGAAGTACAAGTTCATTGTTTACTAA | CAGGAATTTCAGCAGGTATA | toxin B | v d Berg *et al*, 2006; modification |

GI, genogroup I; GII, genogroup II; pol, polymerase.

*Table II. Primers for the nested PCR used for genotyping (paper III)*

| | Strand | Sequence | Name | Reference |
|---|---|---|---|---|
| Primary PCR | + | ATACCACTATGATGCAGAYTA | JV12Y | Vennema *et al*, 2002 |
| | - | CCRCCNGCATRHCCRTTRTACAT | G2SKR | Kojima *et al*, 2002 |
| Nested PCR | + | CARGARBCNATGTTTYAGRTGGATGAG | CoG2F | Nishida *et al*, 2003 |
| | - | CCRCCNGCATRHCCRTTRTACAT | G2SKR | Kojima *et al*, 2002 |