Antiviral Research 161 (2019) 100–107

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Progress in human picornavirus research: New findings from the AIROPico consortium

Katja C. Wolthers[a,*], Petri Susi[b], Dirk Jochmans[c], Janne Koskinen[d], Olfert Landt[e], Neus Sanchez[f], Kaia Palm[g], Johan Neyts[c], Sarah J. Butcher[h]

[a] Department of Medical Microbiology, Laboratory of Clinical Virology, Amsterdam University Medical Centers, Location AMC, Amsterdam, the Netherlands
[b] Institute of Biomedicine, University of Turku, Turku, Finland
[c] Laboratory of Virology and Chemotherapy, Department of Microbiology and Immunology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium
[d] Research and Development Department, ArcDia International Ltd, Turku, Finland
[e] TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany
[f] AbBnc, S.L. (AntibodyBcn), Bellaterra, Spain
[g] Protobios LLC, Tallinn, Estonia
[h] HiLIFE -Institute of Biotechnology, and Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland

ARTICLE INFO

Keywords:
Picornavirus
Enterovirus
Point of care (POC) diagnostics
Pathogenesis
Antiviral therapy

ABSTRACT

Several research groups in Europe are active on different aspects of human picornavirus research. The AIROPico (Academia-Industry R&D Opportunities for Picornaviruses) consortium combined the disciplines of pathogenesis, diagnostics and therapy development in order to fill the gaps in our understanding of how picornaviruses cause human disease and how to combat them. AIROPico was the first EU consortium dedicated to human picornavirus research and development, and has largely accelerated and improved R&D on picornavirus biology, diagnostics and therapy. In this article, we present the progress on pathogenesis, diagnostics and treatment strategy developments for human picornaviruses resulting from the structured, translational research approach of the AIROPico consortium. We here summarize new insights in protection against infection by maternal or cross-protective antibodies, the visualisation of interactions between virus and neutralizing antibodies by cryoEM structural imaging, and the outcomes from a picornavirus-infected human 3D organoid. Progress in molecular detection and a fast typing assay for rhinovirus species are presented, as well as the identification of new compounds potentially interesting as therapeutic compounds.

1. AIROPico consortium overview

AIROPico, an interdisciplinary research network of four European academic institutions and four companies, was run during 2014–2018 under the FP7 Marie Curie Industry-Academia Partnership and Pathways (IAPP) program. The main aim was to promote research and development on human picornaviruses by exchange of knowledge, experience and new research techniques between academy and companies (Fig. 1). The network was built upon long-standing academic and academic-industry collaborations in the field of human picornavirus research and/or diagnostic and antiviral development, with newly acquired partners bringing in expertise on antibody production and profiling. In this manner, a sustainable, interdisciplinary, integrated academia-industry consortium was established with experts in molecular biology, structural biology, diagnostics, antibody production, antibody profiling and antiviral development, to unravel mechanisms of human picornavirus pathogenesis and to develop fast diagnostics, novel treatment options and additional therapy strategies.

During 4 years, 22 fellows in different career stages (from technicians to senior staff members) were exchanged between academic and industry partners (Fig. 1) for periods ranging from 2 months to 2 years. A total of 117 person months divided over 30 secondments were executed. In addition, 6 post-doctoral researchers were recruited to AIROPico projects for periods ranging from 12 to 24 months. Together they have executed a pre-designed plan, which included work plans for fellows and recruits, and descriptions of tasks with deliverables, divided over 5 work packages. The fellows were part of the projects in WP 1–3 which aimed to unravel pathogenesis and accelerate detection and treatment developments for human picornavirus infections.

* Corresponding author.
E-mail address: k.c.wolthers@amc.uva.nl (K.C. Wolthers).

https://doi.org/10.1016/j.antiviral.2018.11.010
Received 12 November 2018; Accepted 17 November 2018
Available online 23 November 2018

Exchanged Fellows	
Female/male	14/8
Early Career	6
Postdoc	8
Technician	4
Staff/senior	4

Fig. 1. Principle of the AIROPico network: *(red)* 4 companies and *(white)* 4 academic groups, *(right)* exchanging 22 fellows in total.

2. Scientific results from the AIROPico consortium

2.1. Prevalence and protection against disease

As global efforts to eradicate poliovirus are successfully on their way, other picornaviruses are still circulating worldwide (Table 1). Large outbreaks of enterovirus A71 (EV-A71) in Asia, EV-D68 in the US, Europe and Asia and human parechovirus 3 (PeV-A3) in Australia (Solomon et al., 2010) (Pons-Salort et al., 2015) (Khatami et al., 2015), all associated with severe disease, show the importance of the non-polio picornaviruses as threat to human health (Harvala et al., 2018). The host response to infection is mainly humoral: neutralizing antibodies (nAbs) are elicited against a specific virus type leading to virus clearance. The protective nAb response is often type-specific and does not necessarily provide protection against other types of viruses from the same geno-group (Anastasina et al., 2017; Karelehto et al., 2017). The role of genotype-specific neutralizing in controlling the neurotropic picornaviruses EV-A71, EV-D68, and PeV-A-3 was reviewed recently in (Anastasina et al., 2017).

Outbreaks of EV-A71 in Asia are related to high illness and death rates among children, but up to now such outbreaks have not occurred in Europe (Chang et al., 2016). To gain insight into the potential threat for the population of Europe, we determined the neutralizing activity in batches of pooled immunoglobulins (IVIg) and individual serum samples from donors in the Netherlands against EV-A71 strains isolated in Europe and in Asia (Van der Sanden et al., 2016). All IVIg batches and 41%, 79%, and 65% of serum samples from children ≤5 years of age, women of childbearing age, and HIV-positive men, respectively, showed high neutralizing activity against a Dutch C1 strain, confirming widespread circulation of EV-A71 in the Netherlands. Asian B3-4 and C4 strains were efficiently cross-neutralized, predicting possible protection against extensive circulation and associated outbreaks of those types in Europe. However, C2 and C5 strains that had few mutations in the capsid region consistently escaped neutralization, emphasizing the importance of monitoring antigenic diversity among circulating EV-71 strains.

We showed previously that the *Parechovirus (PeV)* group is as prevalent as the EV group, being the second leading cause of viral CNS infections in neonates (Wolthers et al., 2008). Despite their apparent clinical significance, less is known about their epidemiology and pathogenesis than for the other picornaviruses. PeV-A1 is the most commonly found followed by PeV-A3. While PeV-A1 is associated with mild disease in children > 6 months, PeV-A3 is associated with severe disease in neonates (Benschop et al., 2006). We conducted a prospective case-control study of Dutch mother-infant pairs to investigate if maternal nAbs against HPeV-A1, 3 and 4 (the most prevalent PeVs in the Netherlands), protect young Dutch infants from severe disease related to PeV infection (Karelehto et al., 2018b). No differences in nAb seroprevalence against PeV-A1 or PeV-A4 could be detected between case and control infants or mothers. In contrast, the PeV-A3 nAb seroprevalence was significantly lower in PeV-A3-infected infants and their mothers. Our results suggest that young Dutch infants are

Table 1
Background on the human picornavirus infections addressed in AIROPico.

Genus	Enterovirus		Parechovirus
Species	Human Enterovirus A-D (EV-A/D)	Human Rhinovirus A-C (RV-A/C)	Human Parechovirus A (PeV-A)
Genotypes	113 types: poliovirus 1–3, coxsackieviruses A and B, echovirus 1–34, enterovirus 68-119	167 types	19 types
Disease association	Paralytic disease, meningitis, encephalitis, myocarditis, neonatal sepsis, hand-foot-mouth disease, hepatitis, conjunctivitis, respiratory disease, fever.	Common cold, rhino-sinusitis, lower respiratory disease, exacerbation asthma/COPD.	Mild respiratory and gastrointestinal disease, neonatal sepsis, meningitis, encephalitis, myocarditis.
Epidemiology	Fecal-oral transmission, circulation late Summer-Fall. Frequent worldwide circulation, leading causes of viral meningitis (US/Europe) and HFM disease outbreaks (Asia).	Respiratory transmission. Frequent worldwide circulation throughout year, responsible for > half of all 'common cold' cases.	Fecal-oral transmission, circulation Summer-Fall. Among the most frequently circulating picornaviruses in children in Europe/US. Worldwide less data than for EVs but circulation observed in all continents.
Detection	5′UTR EV RT-qPCR from patient samples: respiratory swab, stool, CSF, vesicle swab. Genotyping by VP1 sequencing Virus culture still done but far less sensitive	5′UTR RV RT-qPCR from respiratory samples (swab, aspirates, BAL) Typing by VP2/4 sequencing but mostly not performed. EV and RV 5′UTR PCRs can cross react. Virus culture obsolete.	5′UTR PEV-specific RT-qPCR from patient samples: respiratory swab, stool, CSF, vesicle swab. Genotyping by VP1 sequencing. Many PeVs uncultivatable
State-of-the-art	EV-A71 outbreaks in Asia and current local vaccine development. EV-D68 outbreaks US/Europe associated with paralytic disease. No treatment available. Standard care supportive, IVIG can be considered	Association of RV-C with more severe disease under debate. Association of RV with asthma development. No treatment available.	PeV-A3 associated with neonatal sepsis and meningitis. Outbreaks of PeV-A3 in Australia with a recombinant strain. No treatment available. Standard care supportive, IVIG can be considered.

protected against severe disease related to PeV-A1 and PeV-A4 by maternal nAbs, but less against PeV-A3, explaining the distinct age distributions and disease severity profiles of children infected with these PeV genotypes.

Recent PeV-A3 outbreaks in Australia suggest lower population immunity compared to regions with endemic PeV-A3 circulation. However, a serosurvey among Dutch, US and Australian populations prior to and after the Australian outbreak in 2013 showed high PeV-A3 neutralizing antibody prevalence across all regions and time periods (68.9%), indicating long-term widespread PeV-A3 circulation (Karelehto et al., 2018b). This shows that the outbreaks were not caused by a lack of nAb protection against the outbreak strain.

We previously observed apparent discrepancies between seropositivity rates for PeV-A3 nAbs in Japan (high) and in the Netherlands and Finland (both low) (Tanaka et al., 2016; Westerhuis et al., 2013). This was explored further by determining the neutralizing activity of Japanese and Dutch IVIg, aPeV-A3 polyclonal Abs (pAb), and a human aPeV-A3-specific monoclonal Ab (mAb), against the PeV-A3 A308/99 prototype strain and clinical isolates from Japan, the Netherlands and Australia (Karelehto et al., 2017). All PeV-A3 isolates were highly neutralized by the polyclonal pAb (pAb) whereas neutralization titers of IVIG varied; the mAb exclusively neutralized the A308/99 strain (Fig. 2). Mapping of the amino acid variation among a subset of the PeV-A3 strains on a PeV-A3 capsid structure revealed that the majority of the surface-exposed amino acid variation was located in viral protein (VP)1. Furthermore, amino acid mutations in a variant resistant to neutralization by the mAb indicated the location for potential antigenic determinants. Virus aggregation and the observed antigenic diversity in PeV-A3 can thus explain the varying levels of nAb seropositivity reported in previous studies (Tanaka et al., 2016; Westerhuis et al., 2013).

2.2. PeV structures and interactions with human monoclonal antibodies

Knowledge of the molecular structure of viruses is pivotal in understanding structure-function relationships, virus entry and assembly. By the synergistic combination of X-ray crystallography, three-dimensional cryo-electron microscopy (3DEM), homology modelling and interaction studies with Abs we were able to investigate the mechanism of action of neutralizing and broadly specific Abs (Karelehto et al., 2017; Shakeel et al., 2016, 2017; Shakeel et al., 2015) (Domanska et al., 2018). As viruses evolve, in reaction to the host immune response, the capsid structures helped us to map the antigenic variability of emergent isolates (Karelehto et al., 2017). We developed a high-throughput next generation phage display method (MVA) to delineate PeV-specific immunoprofiles. As a result of the pilot study of infected Dutch mothers, several potential epitopes were identified on structural and non-structural proteins of HPeV1 and HPeV3 (Anastasina et al., unpublished). This work is important for predicting how well diagnostic and therapeutic tools based on recognition of that surface will function.

Three PeV structures were solved by 3DEM: two PeV-A3 and one PeV-A1. By binding human mAb fragments to the viruses and repeating the structure determination we could also determine the viral epitopes and Ab paratopes (Kalynych et al., 2015; Seitsonen et al., 2010; Shakeel et al., 2016, 2017; Shakeel et al., 2015) (Domanska et al., 2018). Both PeV-A1 and PeV-A3 have the conserved picornavirus capsid structure containing 60 copies each of VP0, VP1 and VP3 arranged in a T = 1 capsid. Unlike other picornaviruses, VP0 is not cleaved into VP2 and VP4. The PeV structure has three distinct features: firstly, 25% of the PeV RNA genome is highly ordered, interacting with conserved regions of the capsid proteins VP1 and VP3. Secondly, the VP0 N terminus stabilizes the capsid inner surface, in contrast to other picornaviruses where on expulsion as VP4, it forms an RNA translocation channel. Lastly, VP1's hydrophobic pocket, the binding site for the anti-picornaviral drug pleconaril, is blocked and thus inappropriate for antiviral development. Together, these results suggest a direction for

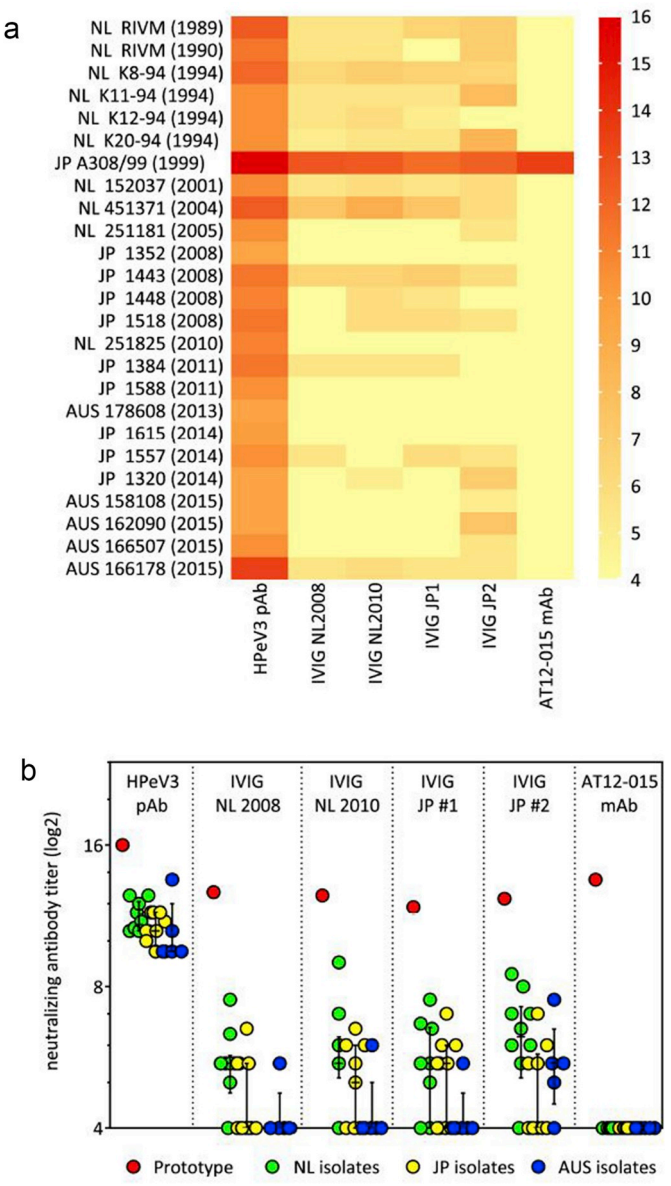

Fig. 2. Neutralization capacity of a polyclonal rabbit PeV-A3 antiserum (PeV-A3 pAb), Dutch (NL) and Japanese (JP) IVIG batches and a human AT12-015 monoclonal antibody (PeV-A3 mAb) against a panel of 25 PeV-A3 isolates collected between 1989 and 2015. (A) Heat map representation of log2-transformed nAb titers against individual PeV-A3 isolates arranged based on year of isolation. (B) Comparison of prototype PeV-A3 A308/99 strain nAb titers against the median nAb titers (with interquartile range) of the clinical PeV-A3 isolates. Samples were grouped by the geographical location of isolation (Dutch isolates; NL, Japanese isolates; JP and Australian isolates; AUS). Positivity was defined as a titer ≥5 log2. PeV-A3 isolate group median nAb titers with interquartile range were compared by Kruskal-Wallis test with Dunn's post hoc analysis (significance level $p < 0.05$). Taken from (Karelehto et al., 2017) with permission.

development of nAbs, antiviral drugs based on targeting the RNA–protein interactions and dissection of virus assembly on the basis of RNA nucleation. This work was followed by analysis of the repeating RNA motif in the PeV genome that binds to the capsid, where we revealed that the capsid assembly is driven by the RNA-protein interactions and by intrinsically disordered regions of the capsid (Shakeel et al., 2017) (Shakeel et al., 2018) (Domanska et al., 2018).

We identified two potential therapeutic human mAbs with the capacity to neutralize PeV-A1. Strikingly, these mAbs have the capacity to

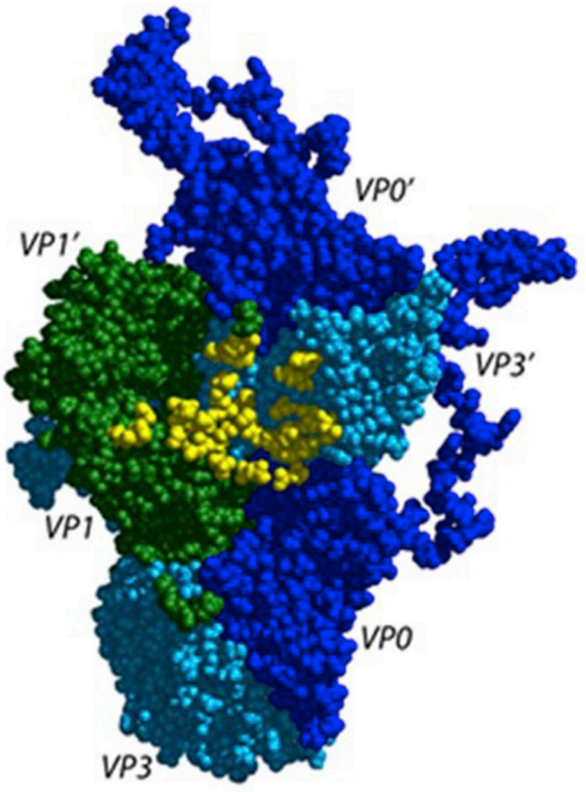

Fig. 3. Interactions between Fab AT12-015 and PeV-A3. The Fab binds to an epitope extended across neighboring asymmetric units in the assembled virion. Two asymmetric units are shown. VP0, VP1, VP1′, and VP3′ residues participating in Fab heavy and light chain binding are highlighted in yellow. Blue VP0, green VP1, and light blue VP3. Taken from (Domanska et al., 2018) with permission. PDB entry ID 6GV4 and EMDB entry ID EMD-0069 (Deposition ID D_1200010538).

neutralize other PeVs as well (Westerhuis et al., 2015). We now know exactly which viral structures are targeted by the two mAbs, and the mechanism through which these mAbs neutralize the virus. One mAb binds at exactly the same spot that is used by the virus to attach to human target cells via integrins (Merilahti et al., 2016). Hence, binding of the mAb to the virus renders the virus unable to attach to the human target cells resulting in a neutralized virus. The second mAb binds to a conformational epitope stabilizing the capsid so that the expansion required to release the genome into the cell, is inhibited (Shakeel et al., 2015). For PeV-A3, the mAb (AT12-015) that neutralized the prototype strain A308/99 but none of the recent clinical isolates tested (Karelehto et al., 2017; Shakeel et al., 2016) was used (Domanska et al., 2018). A high-resolution structure of PeV-A3 A308/99 in complex with the Fab fragments was determined using 3DEM to 2.8 Å resolution. The conformational epitope for mAb AT12-015 is shown in Fig. 3 in yellow on the surface of the virus. We showed that Mab decoration blocks binding of PeV-A3 A308/99 to cultured human intestinal cells (Domanska et al., 2018). The structure revealed that the binding energy of the interaction is stabilized by multiple hydrogen bonds and salt bridges, which were readily destroyed by the amino acid changes in the capsid proteins found in escape mutants, and by changes in the C-terminus of VP1 that extrudes from the capsid surface just next to the epitope.

This is a first step towards understanding the molecular basis of the specificity of binding of (therapeutic) mAbs to emergent viruses, and optimizing this computationally in conjunction with conventional screening methods is imperative, to drive the design for lead compounds.

2.3. Human organoid cultures to study infection

Human picornaviruses are transmitted either via the fecal-oral route or by respiratory transmission. Replication starts in the respiratory tract and/or the gastro-intestinal tract, followed by spreading via the blood to infect various target organs such as skin, heart, or brain as summarized in Table 1. Except for the rhinoviruses (RVs), the human picornaviruses have a broad tissue tropism, and while several cellular receptors have been identified and studied in cell models (Baggen et al., 2018; Heikkilä et al., 2016; Merilahti et al., 2016), mechanisms of entry events and the role of previously identified receptors in native tissues are largely unknown.

Advances in 3D cell culture or organoid technology (Clevers, 2016) make it possible to culture human mini-organs, e.g. gut or respiratory epithelial tissue, in a dish (Fig. 4A and B). These organoid models open up a new way for studying virus-host interactions without the use of immortalized cell lines or animal models.

It has been suggested that infection via the respiratory tract instead of the gut may predispose for infection of the CNS (Zhang et al., 2011). In EV-A71 infections, pulmonary edema has been described as a deadly complication. Therefore, the human airway seems an important site for human picornaviruses. We infected a newly developed human lung organoid model (Sachs et al., 2018) with EV-A71 to test susceptibility and infection kinetics in human respiratory tissue (Van der Sanden et al., 2018). We could now for the first time show in a human model that EV-A71 replication kinetics are strain dependent, while the infection kinetics of the different strains was comparable in unrelated Caucasian adult donors. The glutamine (Q) at position 145 of the VP1 capsid protein was identified as a key determinant of infection efficacy. Remarkably, this is in agreement with the observation that presence of the EV-A71 VP-145Q strain in patients was associated with more severe disease. These promising results obtained with organoids underline their potential to translate to the human *in vivo* situation is higher than for cell lines or animal models.

To study PeV entry in the respiratory epithelium, we infected upper human airway epithelial (HAE) cultures (Karelehto et al., 2018a) (Fig. 4C) . In contrast to what was expected, both PeV-A1 and PeV-A3 genotypes infected HAE preferentially from the basolateral surface, while the progeny virus was shed towards the apical side, arguing against the respiratory tract as a primary entry site for PeV. Confocal microscopy revealed the target cells to be the $p63^{+}$ basal cells for both viruses. Blocking the known receptors for PeV-A1, αV integrins (ITG) and heparan sulfate (HS) had no effect on the replication of either virus; this was expected for PeV-A3, which lacks the RGD motif necessary for integrin binding (Merilahti et al., 2012; Seitsonen et al., 2010). However, ineffective blocking of the known receptors for PeV-A1 indicates that unidentified receptors play a role in entry of primary replication sites *in vivo*.

In summary, these results show the potential of 3D cell cultures to study virus-host interaction for human picornaviruses.

2.4. Rapid generation of picornaviral cDNA clones

With the advent of Next Generation Sequencing methods and the interest to analyze novel pathogenic picornavirus isolates, it is important to have less time-consuming and resource-saving methods to generate templates for sequencing and mutagenesis studies. In prior work a pre-existing cDNA clone of coxsackievirus A9 (CVA9) was used as a template to develop a method for rapid generation and recovery of T7 promoter-tagged full-length CVA9 clones (Heikkilä et al., 2011). Within AIROPico we further developed the method and tested either alone or in combination high fidelity PCR enzymes and reverse transcriptases to identify the key enzymes for rapid and sensitive amplification of full-length viral amplicons. We found two PCR enzymes that were superior in their ability to amplify 7.5 kb fragments; such enzymes were able to multiply picornaviral cDNA clones with sensitivity of 1000

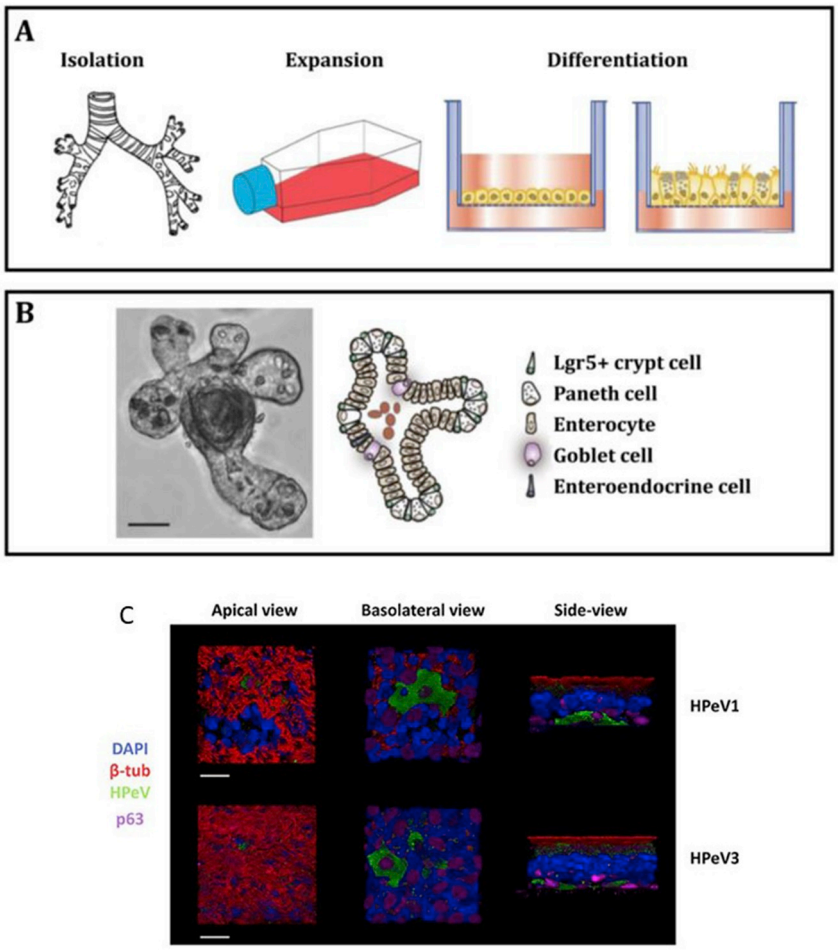

Fig. 4. Organotypic cell culture models recapitulate characteristics of *in vivo* tissues. (A) Human airway epithelium (HAE) culture model. Image adapted from STEMCELL Technologies Inc. All rights reserved, image copyright © 2018 by STEMCELL Technologies Inc. (B) Intestinal organoids from (O'Rourke et al., 2016) (C) PeV target p63 positive basal cells in the airway epithelium. Confocal image stacks of PeV1- and PeV3-infected HAE cultures triple-stained by ciliated cell marker β-tubulin (*red*), basal cell marker p63 (*purple*) and HPeV antibody (*green*); adapted from (Karelehto et al., 2018a).

cDNA copies. In combination with RT enzyme, *in vitro* transcribed, purified viral RNA was converted to PCR with highest sensitivity of 10,000 RNA copies (manuscript in preparation). This easily corresponds to the copy number of most cell cultivated picornaviruses and many clinical specimens suggesting that it will be feasible to try recovery of picornaviruses directly from clinical specimens. To enable cloning and conversion of picornaviral cDNA clones into viable virus we tested different strategies and optimized a method that allows direct generation of infectious picornavirus variants in a few weeks instead of using slow and tedious subcloning strategies.

2.5. Human picornavirus detection

We aimed to develop rapid molecular diagnostics assays for multiplexing and typing of human picornaviruses and to develop and test a point of care (POC) assay utilizing picornavirus-specific Abs. The aim was also to evaluate the performance of assays in a clinical setting and by using clinical specimens. While there are several POC tests for the detection of selected respiratory viruses such as respiratory syncytial virus (RSV) and influenza virus (Bruning et al., 2017b), there are also test systems for a panel of respiratory viruses. AIROPico partner ArcDia has developed an assay technique (mariPOC®), which is a rapid and fully automated POC test system for multi-analyte testing of pathogens from swab samples within 20 min (Sanbonmatsu-Gámez et al., 2015). This assay depends on Abs for capture and complexing of viral particles for their detection by separation-free two photon excitation fluorescence technology (Koskinen et al., 2007). The easy-to-use system allows random-access analysis of patient samples and can easily be operated by lab technicians or nurses in polyclinic departments. Within AIROPico, a clinical study was set up in the general practitioner's office to assess the value of viral diagnostics in the GPs office and to gain more insight into the epidemiology of respiratory viruses in a non-hospital patient population. It was shown that fast diagnostics with the mariPOC® for selected respiratory viruses contributed to a precise and evidence-based diagnosis of respiratory tract infections, which could have impact on prescription of antibiotics by GPs, but that its impact on clinical decision making should be further assessed (Bruning et al., 2017a) In addition, the studies showed that the majority of the respiratory tract infections were caused by human picornaviruses (particularly RVs) (Bruning et al., 2018) suggesting that there is a need for a specific picornaviral POC assay.

One of the most demanding tasks within AIROPico was to develop specific picornavirus rapid POC assays based on antigen detection. There were two approaches used to identify specific pan-picornavirus Abs: 1) identification and testing of commercial Ab preparations and 2) design and generation of new Abs. A review of the commercially-available Abs resulted in the identification and testing of several commercial pan-entero Ab candidates for use in antigen testing and paved the way for identification of common epitopes.

For in-house Ab development we used *in silico* analysis of picornaviral sequences by means of bioinformatics and chose several target proteins to be generated recombinantly in *E. coli*. In parallel,

representative viruses were purified by gradient ultracentrifugation to be used as immunogens. While most attempts to generate mono-specific Abs failed due to insufficient immune response, we can report one successful case. Based on prior studies and bioinformatics analysis PeV VP0 protein was chosen as an immunogen to develop mono-specific Abs to PeVs. PeV-VP0-immunized mice gave positive responses on both ELISA and an immunofluorescence assay. Hybridoma cell lines were generated from PeV-A1-VP0 immunized animals, and one positive monoclonal cell line was rescued (BCN4823, AntibodyBcn). In parallel to animal immunizations, we used synthetic scFv antibody libraries to screen Abs against recombinant PeV-VP0. Synthetic scFv PeV-VP0 antibodies also gave good response on ELISA and immunofluorescence assays. Ab epitopes on the viral surface were mapped by cryoEM. The aPeV-Abs were further tested at ArcDia using the mariPOC platform but the Abs did not enable the development of a wash-free sandwich immunoassay. This suggested that anti-parechoviral Abs likely had affinities too low to give sufficient sensitivity, even though they gave good response in ELISA and IFA. Nevertheless, these data lay a solid groundwork for future Ab and assay optimization projects.

Molecular diagnostics and more specifically reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) has become the state-of-art technique in detection of pathogens. RT-qPCR has largely replaced cell culture and microscopical methods because it is highly sensitive in detecting picornaviruses in clinical samples. In addition, molecular diagnostics can be used to detect genotypes such as RV-C that cannot be cultured. For molecular diagnostics, we worked on both typing and multiplexing methods. Fast typing of picornaviruses in conditions such as neonatal disease or CNS infections would influence clinical decision making and furthermore provide epidemiological data for outbreak management. While in many hospital laboratories PCR is the current method-of-choice for picornavirus diagnostics (Harvala et al., 2018), picornavirus typing is not routinely performed because typing protocols have not yet been standardized.

In AIROPico we implemented both sequence-specific detection and sequencing strategies for typing the viruses. A Chipron array utilizing several sets of type-specific probes to detect and distinguish between RV-A, RV-B and RV-C, reduces the time to results considerably. The developed assays were validated in a clinical setting (Westerhuis et al., 2018). In parallel, we developed a low cost workflow for entero- and rhinoviral typing using RT-qPCR, which should expedite the identification of positive samples after PCR amplification and subsequent Sanger sequencing. Similarly to cDNA cloning, this method is dependent on long distance PCR enzymes that are capable of amplification the target picornaviral sequences used for typing (Chansaenroj and Susi, 2018. Unpublished). In addition to typing procedures, TIB MOLBIOL developed novel multiplex respiratory panels including specific entero- and rhinovirus assays or a panPicorna test followed by running specific assays in order to improve productivity and differentiation between entero- and rhinovirus. The assays have been validated in two typing laboratories against their reference assay with repeat testing of diagnostic samples.

2.6. Antiviral strategies to fight enterovirus infection

Today, more than 20 different drugs for the treatment of HIV infections are approved; further potent antivirals for treatment of infections with herpesviruses, hepatitis B and C viruses, and influenza viruses are available or in clinical development (Debing et al., 2015). For human picornaviruses, small molecules that block *in vitro* replication have been reported. Although these compounds were designed with the aim to treat infected patients, none of them reached the market. In many cases, toxicity or poor pharmacokinetics posed major issues (Bauer et al., 2017) (van der Linden et al., 2015).

However, the pressure to develop anti-picornavirus drugs has increased and hence, the constraints for such drugs in terms of tolerability may be reconsidered. Especially in Asia, EVs have become a major threat in the young population and clinicians urgently call for an efficient and selective treatment. Likewise, the need for a treatment to block RV infections has also increased since it is now widely recognized that these viruses are implicated in exacerbations of chronic lung diseases (Zwaans et al., 2014).

RV infections do not only cause common colds, but may also trigger severe exacerbations of asthma and chronic obstructive pulmonary disease (COPD). Even though RVs have been the focus of extensive drug development efforts in the past (such as pleconaril, which has been withdrawn), no anti-rhinoviral drug has made it to market. In the past, VP1 has been shown to be an important target for the development of antiviral molecules. Furthermore, many different chemical scaffolds appear to possess the properties that are required to inhibit virus replication by this mechanism of action. An analogue of the RV inhibitor pirodavir, ca603, a molecule with a modified linker structure, showed antiviral activity against a panel of RVs and EVs. The compound binds to VP1 in lipid factor pocket and thus stabilizes the viral capsid (Lacroix et al., 2015).

We initiated an antiviral development program against picornaviruses. To start this antiviral program, a diverse set of several thousand chemical compounds was tested in an antiviral screening against Coxsackie B viruses (CVB). We identified several small molecules that had pan-enterovirus activity, affecting CVB3, CVB1, CVB6, CVB4, CVB5. A time-of-drug addition, experiment revealed that the most interesting compound targets CVB3 replication at the entry stage of the viral cycle as most of the antiviral activity was lost when the compound was added 2h post-infection. The structure determination of CVB3 with the compound bound by 3DEM proved that it has a unique antiviral profile with a different binding mode to those compounds that bind in the lipid factor pocket like ca603 described above. Further, we could predict which other picornaviruses would be inhibited. This work has led to a very promising small hit explosion with several more interesting leads (Ma et al., 2017) (Abdelnabi et al.,submitted).

In addition to CVB inhibitors, an effort was undertaken to find antivirals against PeV infections. Convenient, robust and reproducible assays systems were elaborated, which were validated for antiviral screening purposes. This will now allow to run screenings campaigns to identify inhibitors of PeV replication and hence to initiate drug development programs against this important human pathogen.

We found that tryptophan dendrimers that inhibit HIV replication (by binding to the envelope glycoproteins gp120 and gp41), unexpectedly, are also potent, specific, and selective inhibitors of the replication of the unrelated EV-A71. A consensus compound that was synthesized on the basis of the structure-activity relationship analysis of a series of dendrimers inhibited a large panel of clinical isolates in the low-nanomolar/high-picomolar range (Rivero-Buceta et al., 2016).

Next to the direct exploration of antiviral activity, several attempts were made to develop antiviral assay systems for RV-C. This is a highly relevant virus that remains challenging to grow in the lab, which limits the research towards therapies. The approach taken was to generate chimeric viruses with the RV-C 2C-gene or the RV-C polymerase-gene in a background of RV-A or RV-B. The work showed that chimeric RV-A/C or RV-B/C can be made, but currently they are hampered by an impaired viral replication. This impaired virus replication prohibited the study of antiviral activity. These results suggest that inter-typical exchanges of specific viral non-structural proteins generates chimeric constructs with defects in replication, thus not suitable models for antiviral studies (manuscript in preparation).

As the clinical relevance of picornaviruses has become more apparent, it is evident that the search for novel, potent and selective therapies to tackle picornavirus infections is one of the major challenges for the years to come in the field of antivirals.

3. Lessons learned from the experience of the AIROPico consortium

In the Marie Curie FP7 Industry-Academic Partnership consortia, partners need to exchange knowledge and human resources to obtain the research goals. The main tool is to bring employees from academia to industry and vice versa to obtain and transfer knowledge to their host institute. In addition, postdoctoral researchers can be recruited, but this is secondary to the secondments. While all AIROPico partners were willing to accept fellows on secondments for short (2–4 months) or long term fellowships (6–24 months), the main challenge was to have sufficient capacity to exchange fellows from the industry partners to the academic partners. Another challenge was to find suitable fellows for secondments, since the EU criteria for employees eligible for secondments are strict: while fellows are allowed to come from different career backgrounds, they have to be employed by the sending institution for at least one year, and employment after a secondment needs to be guaranteed for at least another year. Since many researchers are employed on short contracts in academia, it is a challenge to find employees who are eligible, *and* who are willing to go abroad and work and live in a different environment. The AIROPico partners were very good in identifying potential fellows, and motivating them to do secondments. The key to success in this is well-preparedness: projects and workplans were discussed and documented before starting a new project, and contacts between the sending institute, the hosting institute and the fellow was established at least 3 months before a secondment, if possible, by a site visit. Another important factor for a successful consortium is the monitoring of progress. Progression of secondments and well-being of the fellows was monitored by the supervisors as well as by the WP leader, who reported progression of the individual projects in monthly teleconferences with all WP leaders and the coordinator. End reporting was done in a written report by all fellows, and for longer secondments, progression reports from the fellow were mandatory every 3 months. Fellows presented the results as well as personal experiences of their secondment in the yearly consortium meetings. This way, the overall progression within the consortium was monitored and potential problems could be identified and dealt with rapidly.

The success of the consortium is reflected not only by the scientific output as described in the previous sections, but by non-scientific output as well (Wolthers and Geraets, 2016). Over 4 years, the majority of the secondments (96%) was performed as planned. AIROPico meetings, organized according to the original plan comprising of a Kick Off meeting, 4 workshops and an End meeting, were attended by the majority of consortium members and were valued highly. Importantly, most of the fellows that had been on secondments reported very positive experiences both professionally and personally. People liked the change of working environment, calling it inspirational and a good way to learn new techniques and new ways of working and learning. Fellows enjoyed their increased networks and, on several occasions, paid short informal visits to the hosting sites. Cultural differences (between countries, and between industry-academic environments) were experienced as very positive and refreshing, and most fellows liked living in another European city or town without experiencing many problems in adapting. The most reported problems were the financial complexity of the secondments, and finding an affordable spot to live.

Of the six recruited post-doctoral fellows (3 females, 3 males), 4 stayed in academic research, 1 stayed in industry, and 1 switched from academia to industry, indicating the successful career paths for Marie Curie fellows.

AIROPico partners valued the intensive collaborations and network formation, and recognized the positive effects on seconded employees in terms of knowledge transfer and broadened experience, as well as the gain of hosting fellows bringing in new ideas, techniques and experiences, all of which compensated for the 'loss of an employee' when an employee had to go on secondment.

In conclusion, the AIROPico consortium has resulted in intensive collaborations on research and development on human picornavirus biology, detection and antiviral compound development. This has led to long-lasting collaborations between partners that will be extended in projects on development of POC testing, exploitation of organoid models for virology, and combining antiviral compound development with structural biology.

Acknowledgements

For their wonderful work done within the consortium we would like to thank all AIROPico fellows (in alphabetical order): Heli Aatola, Maria Anastasina, Andrea Bruning, Alexander am Ende, Justin Flatt, James Geraets, Cornelia Götz, Eve Karelehto, Anri Kivil, Gerrit Koen, Ida Koho, Juha Koskinen, Pasi Laurinmäki, Urmas Liivas, Carmen Mirabelli, Toomas Neuman, Susan Pihelgas, Arno Pihlak, Sabine van der Sanden, Els Scheers, Laura Teixido, Hendrik Jan Thibaut, Lav Tripathi, Brenda Westerhuis, and Maryse Wiewel. We thank Monica Jara (as the first Antibody BCN representative) for her great contributions to the network. We thank our scientific advisors Prof Renate Fuchs and Prof Frank van Kuppeveld for their guidance throughout the project. Lastly we would like to thank our project managers Frank Groen, Stella Koppel, Ram Venkatachalam and Laurian Jongejan for their tremendous support during the project. This work was financially supported by the European Union's Seventh Framework Marie-Curie IAPP AIROPico grant [612308].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.11.010.

References

Anastasina, M., Domanska, A., Palm, K., Butcher, S., 2017. Human picornaviruses associated with neurological diseases and their neutralization by antibodies. J. Gen. Virol. 1145–1158. https://doi.org/10.1099/jgv.0.000780.

Baggen, J., Thibaut, H.J., Strating, J.R.P.M., Van Kuppeveld, F.J.M., 2018. The life cycle of non-polio enteroviruses and how to target it. Nat. Rev. Microbiol. 16, 368–381. https://doi.org/10.1038/s41579-018-0005-4.

Bauer, L., Lyoo, H., van der Schaar, H.M., Strating, J.R., Van Kuppeveld, F.J.M., 2017. Direct-acting antivirals and host-targeting strategies to combat enterovirus infections. Curr. Opin. Virol. 24, 1–8. https://doi.org/10.1016/j.coviro.2017.03.009.

Benschop, K.S.M., Schinkel, J., Minnaar, R.P., Pajkrt, D., Spanjerberg, L., Kraakman, H.C., Berkhout, B., Zaaijer, H.L., Beld, M.G.H.M., Wolthers, K.C., 2006. Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin. Infect. Dis. 42, 204–210. https://doi.org/10.1086/498905.

Bruning, A.H.L., de Kruijf, W.B., van Weert, H.C.P.M., Willems, W.L.M., de Jong, M.D., Pajkrt, D., Wolthers, K.C., 2017a. Diagnostic performance and clinical feasibility of a point-of-care test for respiratory viral infections in primary health care. Fam. Pract. 1–6. https://doi.org/10.1093/fampra/cmx019.

Bruning, A.H.L., Kruijf, W.B. De, van Weert, Henk, C.P., Vrakking, A., De Jong, M.D., Wolthers, K.C., Pajkrt, D., 2018. Respiratory viruses in a primary health care facility in Amsterdam, The Netherlands. Infect. Dis. Clin. Pract. 26, 211–215.

Bruning, A.H.L., Leeflang, M.M.G., Vos, J.M.B.W., Spijker, R., De Jong, M.D., Wolthers, K.C., Pajkrt, D., 2017b. Rapid tests for influenza, respiratory syncytial virus, and other respiratory viruses: a systematic review and meta-analysis. Clin. Infect. Dis. 2015, 1–7. https://doi.org/10.1093/cid/cix461.

Chang, P.-C., Chen, S.-C., Chen, K.-T., 2016. The current status of the disease caused by enterovirus 71 infections: epidemiology, pathogenesis, molecular epidemiology, and vaccine development. Int. J. Environ. Res. Public Health 13. https://doi.org/10.3390/ijerph13090890.

Clevers, H., 2016. Review modeling development and disease with organoids. Cell 165, 1586–1597. https://doi.org/10.1016/j.cell.2016.05.082.

Debing, Y., Neyts, J., Delang, L., 2015. The future of antivirals. Curr. Opin. Infect. Dis. 6, 596–602. https://doi.org/10.1097/QCO.0000000000000212.

Domanska, A. Flatt, J.W., Jukonen, J.J.J., Geraets, J.A., Butcher, S.J., 2018. 2.8 Å resolution cryo-EM structure of human parechovirus 3 in complex with Fab from a neutralizing antibody. J. Virol (in press).

Harvala, H., Broberg, E., Benschop, K., Berginc, N., Ladhani, S., Susi, P., Christiansen, C., McKenna, J., Allen, D., Makiello, P., McAllister, G., Carmen, M., Zakikhany, K., Dyrdak, R., Nielsen, X., Madsen, T., Paul, J., Moore, C., von Eije, K., Piralla, A., Carlier, M., Vanoverschelde, L., Poelman, R., Anton, A., López-Labrador, F.X., Pellegrinelli, L., Keeren, K., Maier, M., Cassidy, H., Derdas, S., Savolainen-Kopra, C., Diedrich, S., Nordbø, S., Buesa, J., Bailly, J.-L., Baldanti, F., MacAdam, A., Mirand, A., Dudman, S., Schuffenecker, I., Kadambari, S., Neyts, J., Griffiths, M.J., Richter, J.,

Margaretto, C., Govind, S., Morley, U., Adams, O., Krokstad, S., Dean, J., Pons-Salort, M., Prochazka, B., Cabrerizo, M., Majumdar, M., Nebbia, G., Wiewel, M., Cottrell, S., Coyle, P., Martin, J., Moore, C., Midgley, S., Horby, P., Wolthers, K., Simmonds, P., Niesters, H., Fischer, T.K., 2018. Recommendations for enterovirus diagnostics and characterisation within and beyond Europe. J. Clin. Virol. 101. https://doi.org/10.1016/j.jcv.2018.01.008.

Heikkilä, O., Kainulainen, M., Susi, P., 2011. A combined method for rescue of modified enteroviruses by mutagenic primers, long PCR and T7 RNA polymerase-driven in vivo transcription. J. Virol. Methods 171, 129–133. https://doi.org/10.1016/J.JVIROMET.2010.10.013.

Heikkilä, O., Merilahti, P., Hakanen, M., Karelehto, E., Alanko, J., Sukki, M., Kiljunen, S., Susi, P., 2016. Integrins are not essential for entry of coxsackievirus A9 into SW480 human colon adenocarcinoma cells. Virol. J. 13, 171. https://doi.org/10.1186/s12985-016-0619-y.

Kalynych, S., Pálková, L., Plevka, P., 2015. The structure of human parechovirus 1 reveals an association of the RNA genome with the capsid. J. Virol. 90 (3), 1377–1386. https://doi.org/10.1128/JVI.02346-15.

Karelehto, E., Cristella, C., Sridhar, S., Yu, X., Hulsdouw, R., Koekkoek, S.M., Pajkrt, D., de Jong, M.D., Wolthers, K.C., 2018a. Polarized entry of human parechoviruses in the human airway epithelium. Front. Cell. Infect. Microbiol. 22 (August).

Karelehto, E., Van Der Sanden, S.M.G., Geraets, J.A., Domanska, A., Van Der Linden, L., Hoogendoorn, D., Koen, G., Van Eijk, H., Shakeel, S., Beaumont, T., De Jong, M., Pajkrt, D., Butcher, S.J., Wolthers, K.C., 2017. Strain-dependent neutralization reveals antigenic variation of human parechovirus. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-12458-5.

Karelehto, E., Wildenbeest, J.G., Benschop, K.S.M., Koen, G., Rebers, S., Bouma-de Jongh, S., Westerhuis, B., de Jong, M.D., Pajkrt, D., Wolthers, K.C., 2018b. Human parechovirus 1, -3 and -4 neutralizing antibodies in Dutch mothers and infants and their role in protection against disease. Pediatr. Infect. Dis. J. Dec. 1304–1308. https://doi.org/10.1097/INF.0000000000001986.

Khatami, A., Mcmullan, B.J., Webber, M., Stewart, P., Francis, S., Timmers, K.J., Rodas, E., Druce, J., Mehta, B., Sloggett, N.A., Cumming, G., Papadakis, G., Kesson, A.M., 2015. Sepsis-like disease in infants due to human parechovirus type 3 during an outbreak in Australia. Clin. Infect. Dis. 60, 228–236. https://doi.org/10.1093/cid/ciu784.

Koskinen, J.O., Vainionpää, R., Meltola, N.J., Soukka, J., Hänninen, P.E., Soini, A.E., 2007. Rapid method for detection of influenza A and B virus antigens by use of a two-photon excitation assay technique and dry-chemistry reagents. J. Clin. Microbiol. 45, 3581–3588. https://doi.org/10.1128/JCM.00128-07.

Lacroix, C., Laconi, S., Angius, F., Coluccia, A., Silvestri, R., Pompei, R., Neyts, J., Leyssen, P., 2015. In vitro characterisation of a pleconaril/pirodavir-like compound with potent activity against rhinoviruses. Virol. J. 12, 10–15. https://doi.org/10.1186/s12985-015-0330-4.

Ma, Y., Abdelnabi, R., Delang, L., Froeyen, M., Luyten, W., Neyts, J., Mirabelli, C., 2017. New class of early-stage enterovirus inhibitors with a novel mechanism of action. Antivir. Res. 147, 67–74. https://doi.org/10.1016/j.antiviral.2017.10.004.

Merilahti, P., Koskinen, S., Heikkilä, O., Karelehto, E., Susi, P., 2012. Endocytosis of integrin-binding human picornaviruses. Adv. Virol., 547530. https://doi.org/10.1155/2012/547530.

Merilahti, P., Tauriainen, S., Susi, P., 2016. Human Parechovirus 1 Infection Occurs via αVβ1 Integrin. PloS One 11, e0154769. https://doi.org/10.1371/journal.pone.0154769.

O'Rourke, K.P., Ackerman, S., Dow, L.E., Lowe, S.W., 2016. Isolation, Culture, and Maintenance of Mouse Intestinal Stem Cells. Bio-protocol. https://doi.org/10.1037/emo0000122.Do.

Pons-Salort, M., Parker, E.P.K., Grassly, N.C., 2015. The epidemiology of non-polio enteroviruses. Curr. Opin. Infect. Dis. 1.

Rivero-Buceta, E., Sun, L., Martínez-Gualda, B., Doyagüez, E.G., Donckers, K., Quesada, E., Camarasa, M.J., Delang, L., San-Félix, A., Neyts, J., Leyssen, P., 2016. Optimization of a class of tryptophan dendrimers that inhibit HIV replication leads to a selective, specific, and low-nanomolar inhibitor of clinical isolates of enterovirus A71. Antimicrob. Agents Chemother. 60, 5064–5067. https://doi.org/10.1128/AAC.00626-16.

Sachs, N., Zomer-van Ommen, D.D., Papaspyropoulos, A., Inha, H., Böttinger, L., Dymph, K., Weeber, F., Huelsz-Prince, G., Iakobachvili, N., Viveen, M.C., Lyubimova, A., Teeven, L., Derakhshan, S., Begthel, J., Korving, H., Kumawat, K., Ramos, E., van Oosterhout, M.F.M., Olimpio, E.P., Ligt, J. de, Dijkstra, K.K., Smit, E.F., van der Linden, M., Voest, E.E., van Moorsel, C.H.M., van der Ent, C.K., Cuppen, E., van Oudenaarden, A., Coenjaerts, F.E., Meyaard, L., Bont, L.J., Peters, P.J., Tans, S.J., Zon, J.S. Van, Boj, S.F., Vries, R.G., Beekman, J.M., Clevers, H., 2018. Long-term Expanding Human Airway Organoids for Disease Modelling. bioRvix May 9. .

Sanbonmatsu-Gámez, S., Pérez-Ruiz, M., Lara-Oya, A., Pedrosa-Corral, I., Riazzo-Damas, C., Navarro-Marí, J.M., 2015. Analytical performance of the automated multianalyte point-of-care mariPOC® for the detection of respiratory viruses. Diagn. Microbiol. Infect. Dis. 83 (3), 252–256. https://doi.org/10.1016/j.diagmicrobio.2015.07.010.

Seitsonen, J., Susi, P., Heikkilä, O., Sinkovits, R.S., Laurinmäki, P., Hyypiä, T., Butcher, S.J., 2010. Interaction of alphaVbeta3 and alphaVbeta6 integrins with human parechovirus 1. J. Virol. 84, 8509–8519. https://doi.org/10.1128/JVI.02176-09.

Shakeel, S., Dykeman, E.C., White, S.J., Ora, A., Cockburn, J.J., Butcher, S.J., Stockley, P.G., Twarock, R., 2017. Genomic RNA folding mediates assembly of human parechovirus. Nat. Commun. 8 (1), 5. https://doi.org/10.1038/s41467-016-0011-z.

Shakeel, S., Evans, J.D., Hazelbaker, M., Kao, C.C., Vaughan, R.C., Butcher, S.J., 2018. Intrinsically-disordered N-termini in human parechovirus 1 capsid proteins bind encapsidated RNA. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-23552-7.

Shakeel, S., Westerhuis, B.M., Domanska, A., Koning, R.I., Matadeen, R., Koster, A.J., Bakker, A.Q., Beaumont, T., Wolthers, K.C., Butcher, S.J., 2016. Multiple capsid-stabilizing interactions revealed in a high-resolution structure of an emerging picornavirus causing neonatal sepsis. Nat. Commun. 7, 11387. https://doi.org/10.1038/ncomms11387.

Shakeel, S., Westerhuis, B.M., Ora, A., Koen, G., Bakker, A.Q., Claassen, Y., Wagner, K., Beaumont, T., Wolthers, K.C., Butcher, S.J., 2015. Structural basis of human parechovirus neutralization by human monoclonal antibodies. J. Virol. 89, 9571–9580. https://doi.org/10.1128/jvi.01429-15.

Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M.J., McMinn, P., Ooi, M.H., 2010. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect. Dis. 10, 778–790. https://doi.org/10.1016/S1473-3099(10)70194-8.

Tanaka, S., Aoki, Y., Matoba, Y., Yahagi, K., Itagaki, T., Matsuzaki, Y., Mizuta, K., 2016. Seroepidemiology of human parechovirus types 1, 3, and 6 in Yamagata, Japan. in 2014. Microbiol. Immunol. 60, 854–858. https://doi.org/10.1111/1348-0421.12456.

van der Linden, L., Wolthers, K.C., van Kuppeveld, F.J.M., 2015. Replication and inhibitors of enteroviruses and parechoviruses. Viruses 7, 4529–4562. https://doi.org/10.3390/v7082832.

Van der Sanden, S.M.G., Koen, G., Van Eijk, H., Koekkoek, S.M., De Jong, M.D., Wolthers, K.C., Eijk, H. Van, Koekkoek, S.M., Jong, M.D. De, Wolthers, K.C., 2016. Prediction of protection against Asian enterovirus 71 outbreak strains by cross-neutralizing capacity of serum from Dutch donors , The Netherlands. Emerg. Infect. Dis. 22, 1562–1569. https://doi.org/10.3201/eid2209.151579.

Van der Sanden, S.M.G., Sachs, N., Koekkoek, S.M., Koen, G., Pajkrt, D., Clevers, H., Wolthers, K.C., 2018. Enterovirus 71 infection of human airway organoids reveals VP1-145 as a viral determinant of infectivity. Emerg. Microb. Infect. 7. https://doi.org/10.1038/s41426-018-0077-2.

Westerhuis, B., Kolehmainen, P., Benschop, K., Nurminen, N., Koen, G., Koskiniemi, M., Simell, O., Knip, M., Hyöty, H., Wolthers, K., Tauriainen, S., 2013. Human parechovirus seroprevalence in Finland and The Netherlands. J. Clin. Virol. 58, 211–215.

Westerhuis, B.M., Benschop, K.S.M., Koen, G., Claassen, Y.B., Wagner, K., Bakker, A.Q., Wolthers, K.C., Beaumont, T., 2015. Human memory B cells producing potent cross-neutralizing antibodies against human parechovirus: implications for prevalence, treatment, and diagnosis. J. Virol. 89, 7457–7464. https://doi.org/10.1128/jvi.01079-15.

Westerhuis, B.M., Wiewel, M.A., Ende, A.A., van der Linden, L., Koekkoek, S.M., Wolthers, K.C., Landt, O., 2018. A chip-based rapid genotyping assay to discriminate between rhinovirus species A, B and C. J. Clin. Virol. 99–100. https://doi.org/10.1016/j.jcv.2017.12.004.

Wolthers, K.C., Benschop, K.S.M., Schinkel, J., Molenkamp, R., Bergevoet, R.M., Spijkerman, I.J.B., Kraakman, H.C., Pajkrt, D., 2008. Human parechoviruses as an important viral cause of sepsis like illness and meningitis in young children. Clin. Infect. Dis. 47, 358–363. https://doi.org/10.1086/589752.

Wolthers, K.C., Geraets, J.A., 2016. Picornaviruses go back under the research microscope. EU Res 10–11.

Zhang, Y., Cui, W., Liu, L., Wang, J., Zhao, H., Liao, Y., Na, R., Dong, C., Wang, L., Xie, Z., Gao, J., Cui, P., Zhang, X., Li, Q., 2011. Pathogenesis study of enterovirus 71 infection in rhesus monkeys. Lab. Invest. 91, 1337–1350. https://doi.org/10.1038/labinvest.2011.82.

Zwaans, W.A.R., Mallia, P., van Winden, M.E.C., Rohde, G.G.U., 2014. The relevance of respiratory viral infections in the exacerbations of chronic obstructive pulmonary disease-A systematic review. J. Clin. Virol. 61 (2), 181–188. https://doi.org/10.1016/j.jcv.2014.06.025.