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To my family

You can shine
no matter what you are made of.
-Mr. Bigweld

ABSTRACT

Pirjo Merilahti

Cell surface interactions of coxsackievirus A9 and human parechovirus 1

University of Turku, Faculty of Medicine, Department of Virology, Turku Doctoral Programme of Molecular Medicine
Annales Universitatis Turkuensis, Turku, 2017

Coxsackievirus A9 (CV-A9) and human parechovirus 1 (HPeV-1) belong to family *Picornaviridae*, genera *Enterovirus* and *Parechovirus* respectively. CV-A9 has been associated with aseptic meningitis, myocarditis in addition to other mild and/or severe clinical manifestations. HPeV-1 infection most commonly induces mild gastrointestinal and respiratory symptoms, but also more severe manifestations such as myocarditis and transient paralysis may occur. HPeV-1 infection is very common in children and neonates; likewise, CV-A9 infection occurs most often in children. Vaccines, antivirals or drugs against these viruses do not exist.

CV-A9 and HPeV-1 harbor an arginine-glycine-aspartic acid (RGD) –motif on their capsids, which is the binding motif of some integrins, a group of heterodimeric cell adhesion receptors. Integrins participate in many cellular functions, for example in cell signaling and organization of the intracellular cytoskeleton. The RGD-binding integrins are hypothesized to act as cell surface receptors for CV-A9 and HPeV-1. In this study, the receptor tropism of CV-A9 and HPeV-1 was extensively analyzed *in vitro*. Different mammalian cell lines for the receptor studies were used, and applied methods such as blocking experiments with neutralizing antibodies and receptor antagonists were utilized. The results were mainly analyzed with fluorescence microscopy.

The *in vitro* studies suggest that CV-A9 can penetrate into the cells without integrins. Instead, CV-A9 binds to heat shock protein family A member 5 (HSPA5) on the cell surface with heparan sulfate (HS) and β2-microglobulin (β2M) acting as accessory receptors. However, the results suggest that HPeV-1 utilizes αVβ1 integrin as its primary receptor, but HS and β2M act as accessory receptors for HPeV-1 similarly to CV-A9.

Keywords: coxsackievirus A9, human parechovirus 1, receptor, integrin, heparan sulfate, RGD motif, HSPA5, β2-microglobulin

TIIVISTELMÄ

Pirjo Merilahti

Coxsackievirus A9:n ja ihmisen parechovirus 1:n reseptorivuorovaikutukset

Turun yliopisto, Lääketieteellinen tiedekunta, Virusoppi, Turun molekyylilääketieteen tohtoriohjelma
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Coxsackievirus A9 (CV-A9) ja ihmisen parechovirus 1 (HPeV-1) kuuluvat *Picornaviridae*-heimoon ja tarkemmin määriteltynä *Enterovirus*- ja *Parechovirus*- sukuihin. CV-A9 on yhdistetty muun muassa aseptiseen aivokalvontulehdukseen ja sydänlihastulehdukseen ja lisäksi myös muihin vaikeusasteeltaan vaihteleviin infektioihin. CV-A9-infektio on yleinen lapsilla. HPeV-1 aiheuttaa useimmiten lieviä vatsaoireita ja hengitystieinfektioita, mutta sen on todettu aiheuttavan myös monia vakavampia sairauksia, kuten sydänlihastulehdusta ja tilapäisiä halvausoireita. HPeV-1 on CV-A9:n tapaan myös erittäin yleinen lapsilla ja vastasyntyneillä. Kumpaakaan virusta vastaan ei ole olemassa lääkitystä tai rokotetta.

CV-A9:n ja HPeV-1:n pintaproteiinissa on arginiini-glysiini-asparagiinihappo (RGD) -alue, jonka on todettu sitoutuvan integriineihin. Integriinit ovat ryhmä heterodimeerisiä soluadheesioreseptoreita, joilla on useita tehtäviä muun muassa solujen viestintään ja solunsisäisten rakenteiden järjestelyyn liittyen. RGD-alueeseen sitoutuvien integriinien on ehdotettu toimivan CV-A9:n ja HPeV-1:n reseptoreina. Tämän väitöskirjatyön tarkoituksena oli tutkia yksityiskohtaisesti CV-A9:n ja HPeV-1:n reseptorivuorovaikutuksia. Työn kokeellisessa toteutuksessa käytettiin hyväksi soluviljelmiä sekä molekyylibiologian menetelmiä; reseptoriproteiinien toimintoja estettiin muun muassa vasta-aineilla ja inhibiittoreilla, ja virusinfektion etenemistä seurattiin immunofluoresenssi- ja konfokaalimikroskopialla.

Soluviljelmäkokeiden perusteella CV-A9 pystyy kulkeutumaan isäntäsoluun integriineistä riippumatta. Integriinien sijaan CV-A9 sitoutuu HSPA5-proteiiniin solun pinnalla käyttäen apunaan β2-mikroglobuliini (β2M)-proteiinia ja heparaanisulfaattia (HS). Sen sijaan HPeV-1 käyttää αVβ1-integriiniä ensisijaisena reseptorinaan, minkä lisäksi HS ja β2M voivat toimia viruksen avustavina reseptoreina.

Avainsanat: coxsackievirus A9, ihmisen parechovirus 1, reseptori, integriini, heparaanisulfaatti, RGD-alue, HSPA5, β2-mikroglobuliini

TABLE OF CONTENTS

ABBREVIATIONS

A549	human epithelial lung carcinoma cell line
ADP	adenosine diphosphate
AF	Alexa Fluor®
Arf6	ADP-ribosylated factor 6
ATCC	American Type Culture Collection
β2M	β2-microglobulin
BiP	binding immunoglobulin protein
BSA	bovine serum albumin
CAR	coxsackie and adenovirus receptor
CAV1	caveolin-1
CDC	Centers for Disease Control and Prevention
CDHR3	cadherin-related family member 3
CHO	chinese hamster ovary cell line
CIE	clathrin-independent endocytosis
CLIC	clathrin-independent carrier
CME	clathrin-mediated endocytosis
CNS	central nervous system
CSF	cerebrospinal fluid
CSGAG	chondroitin sulfate glycosaminoglycan
CV	coxsackievirus
cVDPV	circulating vaccine-derived polio
DAF	decay-accelerating factor
DMEM	Dulbecco's modified Eagle's media
E	echovirus
ECM	extracellular matrix
EE	early endosome
EM	electron microscopy
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
EV	enterovirus
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum

FDA	Food and Drug Administration
FMDV	foot-and-mouth disease virus
FRET	fluorescence resonance energy transfer
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GE11	mouse epithelial cell line
GEEC	GPI-anchored protein enriched compartment
GPI	glycosylphosphatidyl inositol
HAV	hepatitis A virus
HAVcr-1	hepatitis A virus cellular receptor 1
HeLa	human cervical cancer cell line
HFMD	hand-foot-and-mouth disease
HLA	human leukocyte antigen
HPeV	human parechovirus
HS	heparan sulfate
HSGAG	heparan sulfate glycosaminoglycan
HSPA5	heat shock protein family A member 5
HSPG	heparan sulfate proteoglycan
ICAM-1	intracellular adhesion molecule
ICTV	International committee on taxonomy of viruses
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IL	interleukin
IRES	internal ribosome entry site
kDa	kilodalton
KO	knock-out
LDLR	low-density-lipoprotein receptor
LE	late endosome
LRP	LDL-related protein
MOI	multiplicity of infection
MVB	multivesicular body
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PKC	protein kinase C
PolyA	polyadenylation
PSGL-1	P-selectin glycoprotein ligand-1
PV	poliovirus
PVR	poliovirus receptor
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RE	recycling endosome
RV	rhinovirus
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
SA	sialic acid
SCARB2	scavenger receptor class B member 2
SCR	short consensus repeats
ss	single-stranded
SVDV	swine vesicular disease virus
SW480	human colon adenocarcinoma cell line
T1D	type 1 diabetes
TIM-1	T-cell immunoglobulin and mucin domain
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
UTR	untranslated region
VCAM-1	vascular cell adhesion molecule type 1
WHO	World Health Organization
VLDLR	very low density lipoprotein receptor
VP	viral protein
VPg	genome linked viral protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals **I-III**:

I 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. Virology Journal 13: 171. doi:10.1186/s12985-016-0619-y.

II **Merilahti P**, Tauriainen S, Susi P (2016) Human parechovirus 1 infection occurs via αVβ1 integrin. PLoS ONE 11(4): e0154769. doi:10.1371/journal.pone.0154769

III **Merilahti P**, Karelehto E, Susi P (2016) Role of heparan sulfate in cellular infection of integrin-binding coxsackievirus A9 and human parechovirus 1 isolates. PLoS ONE 11(1): e0147168. doi:10.1371/journal.pone.0147168

1 INTRODUCTION

Viruses are part of our everyday life. They are very small pathogens, which are not able to survive without a host organism; human, animal, plant or even bacteria. The family *Picornaviridae* consists of large group of virus types, which infect mainly mammals and birds, however, new picornaviruses are continually discovered from different host animals. Picornaviruses are small, non-enveloped viruses that contain single-stranded RNA genome. The route of virus transmission is mainly via fecal-oral or respiratory routes. Many well-known viruses belong to this group of viruses, including entero-, polio- and rhinovirus, and the disease spectrum caused by these viruses is very broad from common cold to paralysis (reviewed e.g. by Tuthill et al. 2010). With few exceptions, like poliovirus, there are no vaccines or approved drugs against picornavirus infections. In addition to the well-known picornaviruses, the virus family include many other significant pathogens, which cause severe infections, especially in children. Coxsackievirus A9 (CV-A9) belongs to *Enterovirus B* species and it causes diverse range of infections from common cold –like illnesses to infections of the central nervous system (Blomqvist et al. 2008). Human parechovirus 1 (HPeV-1) belongs to *Parechovirus A* species. Like CV-A9, also HPeV-1 causes a wide range of symptoms varying from mild respiratory or gastrointestinal infections, but HPeV-1 can also cause neurological symptoms especially in neonates (Harvala and Simmonds 2009).

The cells of the host organism offer a platform for the invading virus to reproduce and to spread into the neighbouring cells. However, before that, the virus needs to access the cell interior. This may occur via several routes, but herein is focused on the entry via host cell receptors. Receptors are specific proteins that locate on the cell surface, and to which the invading virus binds before internalization. Integrins are transmembrane proteins that act as connectors between cell interior and the extracellular matrix (ECM) (Hynes 2002), and integrins bind ligands, such as collagen, vitronectin and fibronectin, presented in the ECM (Humphries et al. 2006). In addition to its natural ligands, some viruses, including picornaviruses, have been adapted to utilize integrins as its receptors.

Although some examples of extensively described receptor-virus interactions exist, the receptors of many viruses remain unknown or poorly studied. The aim of my thesis was to study the receptor usage of CV-A9 and HPeV-1 by utilizing methods such as blocking and binding assays in combination with advanced microscope imaging in different cell lines. The identification of binding receptors of these viruses would expand the understanding of properties of picornaviruses thus alleviating the development of antivirals.

2 REVIEW OF LITERATURE

2.1 Taxonomy of picornaviruses

The family *Picornaviridae* belongs to the order *Picornavirales*, and currently it consists of 54 species grouped into 31 genera. Additionally, four new genera and 24 new species have been proposed by The Picornavirus Study Group, member of the International Committee on Taxonomy of Viruses (ICTV), to be included in the *Picornaviridae* family (Adams et al. 2016) (**Table 1**). Classification in virus taxonomy includes the following levels: order, family, genus and species. However, picornaviruses are also named at type level, under which each isolate/strain has its specific identifier. For example, human parechovirus 1 (HPeV-1) is classified as follows: Order: *Picornavirales* family: *Picornaviridae*, genus: *Parechovirus*, species: *Parechovirus A*, type: human parechovirus 1*,* isolate or strain: Harris (and identifier: GenBank acc no L02971). All taxonomic levels are capitalized and in italics.

The ultimate aim of taxonomy is to construct a hierarchical classification that mimics phylogeny. Earlier ICTV classification of picornaviruses was based on either serology or pathogenesis induced in cell culture or in laboratory animals. However, according to the modern definition picornavirus species comprise a group of closely related strains that have virtually identical genome maps. The individual virus types and isolates are divided within the genera based on morphology, physicochemical and biological properties, antigenic structures, genome, and the mode of replication. However, the isolates within the same species may vary significantly in genome sequence, antigenicity, and even in host range. (Hyypia et al. 1997, Knowles et al. 2010)

Within the past ten years, the taxonomy of picornaviruses has changed considerably. As a result, many species lost their host defining names, for example *Human enterovirus A* was renamed to *Enterovirus A*. Also new virus genera have been introduced, e.g. parechoviruses that originally belonged to the genus *Enterovirus* now form genus *Parechovirus*. Some genera have merged to another genus, for instance all rhinoviruses now belong to genus *Enterovirus*. In some cases, the names of both genus and species have changed, e.g. *Encephalomyocarditis virus* is now *Cardiovirus A* (Adams et al. 2016). The changes are mainly due to adoption of genetic methods in virus typing and increased knowledge of the viruses. In addition to the recognized *Picornaviridae* species, more than thirty new picornaviruses from cattle, pigs, cats, dogs, sea lions, tortoises, birds, and bats await classification. The recent expansion in the number of picornavirus genera and ongoing characterization of future candidate genera suggests that picornaviruses could form a higher taxonomic level (subfamily) in the future (Zell et al. Conference abstract D08, Europic 2016).

Table 1: Classification of picornaviruses. *Proposed new genera and species, proposed by Picornaviridae Study Group in August 2016, are in brackets.*

Genus 31 (+4)	Number of species 54 (+24)	Example of Species	Types used in this study
(Ampivirus)	(1)	*(Ampivirus A)*	
Aphtovirus	4	*Foot-and-mouth disease virus*	
Aquamavirus	1	*Aquamavirus 1*	
Avihepatovirus	1	*Avihepatovirus 1*	
Avisivirus	1 (+ 2)	*Avisivirus A*	
Cardiovirus	3	*Cardiovirus A*	
Cosavirus	1 (+ 4)	*Cosavirus A*	
Dicipivirus	1	*Cadicivirus A*	
Enterovirus	12 (+ 1)	*Enterovirus B*	coxsackievirus A9
Erbovirus	1	*Erbovirus A*	
Gallivirus	1	*Gallivirus A*	
(Harkavirus)	(1)	*(Harkavirus A)*	
Hepatovirus	1 (+ 8)	*Hepatovirus A*	
Hunnivirus	1	*Hunnivirus A*	
Kobuvirus	3 (+ 3)	*Aichivirus A*	
Kunsagivirus	1	*Kunsagivirus A*	
Limnipivirus	3	*Limnipivirus A*	
Megrivirus	1 (+ 2)	*Melegrivirus A*	
Mischivirus	1 (+ 2)	*Mischivirus A*	
Mosavirus	1	*Mosavirus A*	
Oscivirus	1	*Oscivirus A*	
Parechovirus	2 (+ 2)	*Parechovirus A*	human parechovirus 1
Pasivirus	1	*Pasivirus A*	
Passerivirus	1	*Passerivirus A*	
Potamipivirus	1	*Potamipivirus A*	
(Rabovirus)	(1)	*(Rabovirus A)*	
Rosavirus	1	*Rosavirus A*	
Sakobuvirus	1	*Sakobuvirus A*	
Salivirus	1	*Salivirus A*	
Sapelovirus	3	*Sapelovirus A*	
Senecavirus	1	*Senecavirus A*	
Sicinivirus	1	*Sicinivirus A*	
Teschovirus	1	*Teschovirus A*	
(Torchivirus)	(1)	*(Torchivirus A)*	
Tremovirus	1	*Tremovirus A*	

*Source: International Committee on Taxonomy of Viruses, ICTV: http://www.ictvonline.org, ICTV Master Species List 2015 v1.

2.2 Picornaviruses as human pathogens

The *Picornaviridae* family includes viruses that cause a variety of human and animal diseases including poliomyelitis, common cold, aseptic meningitis, hepatitis, hand-foot-and-mouth disease and many more (Tuthill et al. 2010). In addition, many picornaviruses are associated with chronic disease. Currently, antivirals against picornaviruses do not exist, and a vaccine is available only against few virus types. Poliovirus (PV) is one of the most lethal virus in human history. The development of effective polio vaccines by Jonas Salk in 1955 and by Albert Sabin in 1961 marks a major milestone in the history of human health. Economic modelling has found that the eradication of polio would save at least 40 to 50 billion dollars between 1988 and 2035 mostly in low-income countries (WHO). Rhinoviruses (RVs), first discovered in the 1950s, are the most common viruses circulating within population worldwide and they are responsible for more than half of common cold cases. It has been studied in the Northern America, that RV infections cause expenses of billions of dollars annually via medical visits and missed workdays (Jacobs et al. 2013). Enteroviruses (EVs), including coxsackieviruses (CV), are common causative agents of aseptic meningitis, otitis and other infections. Enterovirus A71 (EV-A71) is the main causative agent of hand-foot-and-mouth disease (HFMD) and it is associated to severe neurological diseases (Ooi et al. 2010). In December 2015, China's Food and Drug Administration (FDA) approved two inactivated EV-A71 vaccines (Mao et al. 2016). EVs, especially coxsackie B viruses (CVBs), have also been associated with Type 1 diabetes (T1D) (Hyoty 2016). Although vaccine and/or drug development has advanced significantly, there is no cure for picornavirus-induced disease, and the treatment is limited to supportive care (van der Linden et al. 2015).

2.2.1 *Enteroviruses*

EVs and RVs belong to the genus *Enterovirus* according to the current taxonomy. EVs and RVs are the most common human pathogens, but they also infect other primates and many domestic animals. Currently, the genus *Enterovirus* comprise 12 species (and a putative species; *Enterovirus I*), seven of which present human pathogens (ICTV, www.picornaviridae.com). These species are *Enterovirus A* to *D,* and *Rhinovirus A* to *C*. EV infections manifest from asymptomatic infections and life-threatening systemic infections of newborns to poliomyelitis or other neurological diseases with potentially persisting sequelae (Knowles et al. 2010, Pallansch et al. 2013). RVs cause common cold, and the infection is restricted to respiratory mucosa with local disease symptoms (Knowles et al. 2010). RV infections are associated with exacerbations of asthma in children (Steinke and Borish 2016), and they can trigger severe episodes of lower-airway dysfunctions in asthmatic patients (Gern and Busse 1999).

Enterovirus A (EV-A) species consists of 25 types, including several types of coxsackievirus A (CV-A) and enterovirus A71 (EV-A71). Typically, the members of EV-A including CV-A6, -A10, -A16, as well as EV-A71 cause hand-foot-and-mouth disease (HFMD) (Grist et al. 1978, Osterback et al. 2009, Repass et al. 2014). HFMD is a common febrile illness occurring mainly in children, which manifests as skin rash involving palms and soles, and ulcers on oral mucosa (Grist et al. 1978). EV-A71 causes a broad range of neurological diseases, which include aseptic meningitis, acute flaccid paralysis, brainstem encephalitis, and neurogenic pulmonary edema. EV-A71 may also cause long-term neurological sequelae, mainly in infants and young children making it the most important enterovirus nowadays (Alexander et al. 1994, McMinn 2002, Modlin 2007). In 2015, the FDA of China approved two EV-A71 vaccines to prevent epidemics (Mao et al. 2016). Some other EV-A types, including CV-A and -B serotypes, are commonly associated with herpangina (Nishimura and Shimizu 2012, Pallansch et al. 2013). Herpangina is a febrile illness with relatively sudden onset of symptoms such as fever and sore throat. The illness is common among the young, is usually self-limited, and disappears within a few days. Occasionally the disease is associated with more severe clinical manifestation e.g. meningitis (Pallansch et al. 2013).

Enterovirus B (EV-B) species comprise 63 types, including CV-B1 to B6, CV-A9, over 30 types of echoviruses (E) and over 20 EV-B serotypes. Viruses of the EV-B species cause a vast number of diseases with varying severity. Infection by CV-B type viruses can induce severe inflammation in secondary tissues, leading to, for example atherosclerosis and viral myocarditis (Roivainen et al. 1998). CV-A9 has been associated for example with aseptic meningitis and myocarditis (Jadrnickova-Volakova et al. 1967, Eisenhut et al. 2000, Whitton et al. 2005, Blomqvist et al. 2008, Cui et al. 2010). EV-B types, as well as CV-A9, are suspected also to cause persistent infections and to contribute to the development of chronic diseases such as T1D (Hyoty et al. 1995, Roivainen et al. 1998, Roivainen and Klingel 2009, Tauriainen et al. 2011, Laitinen et al. 2014).

Enterovirus C (EV-C) species comprise 23 types, including several CV-A types, EV-C types, and the most extensively studied EV, poliovirus (PV). PV causes poliomyelitis, an acute human disease of the central nervous system (CNS). PV work has had a significant impact to molecular virology, since PV was the first animal virus completely cloned and sequenced (Kitamura et al. 1981, Racaniello and Baltimore 1981a). PV was also the first animal RNA virus, for which an infectious clone was constructed (Racaniello and Baltimore 1981b), and the first human virus that had its three-dimensional structure solved by x-ray crystallography (Hogle et al. 1985). On average 1 out of 200 PV infections in a fully susceptible population results in the paralytic disease known as poliomyelitis (WHO). In 5 to 10% of poliomyelitis cases, death occurs due to paralysis of the respiratory center (WHO). Common PV infection manifests as a mild febrile illness with or without gastrointestinal signs, but PV may also cause aseptic meningitis (Pallansch et al. 2013). In 2015, 74 cases of PV infection were reported (wild type PV-1 and circulating vaccine-derived

polio (cVDPV)), mostly in Pakistan and Afghanistan. PV-2 has been concluded to be eradicated 20th September 2015, and PV-3 has last been detected in 10th November 2012 (WHO).

Enterovirus D (EV-D) species consists of five types; EV-D68 (previously known as human rhinovirus 87 (HRV-87)), EV-D70, EV-D94, EV-D111, and EV-D120. EV-D68 has caused several outbreaks within the past ten years (Centers for Disease Control and Prevention (CDC) 2011, Midgley et al. 2014). The infections have induced severe respiratory and CNS symptoms and even acute flaccid myelitis (Oberste et al. 2004, Rahamat-Langendoen et al. 2011, Kreuter et al. 2011, Aliabadi et al. 2016). In the autumn of 2016, EV-D68 was prevalent in the region of Finland Proper, affecting particularly asthmatic children and leading to hospitalizations (National Institute for Health and Welfare, Finland). EV-D70 infection associates with acute hemorrhagic conjunctivitis (Mirkovic et al. 1973) and EV-D94 infection with acute flaccid paralysis (Junttila et al. 2007).

Rhinovirus A (RV-A), *Rhinovirus B* (RV-B) and *Rhinovirus C* (RV-C) species respectively comprise 80, 32, and of 55 virus types. RVs, first detected in the 1950s, included only RV-A and RV-B types until the establishment of RV-C in 2006 after identification of novel RVs from respiratory samples of patients in Queensland and New York City (Lau et al. 2010). RVs cause about half of common cold cases in children and adults year-round (Makela et al. 1998, Toivonen et al. 2016), but RVs are also closely associated with acute otitis media episodes (Blomqvist et al. 2002, Toivonen et al. 2016). However, while earlier associated mostly with benign upper respiratory tract illness, increasing evidence suggests RVs to cause more severe illnesses. RV infection is associated with e.g. exacerbations of chronic pulmonary disease, asthma development, cystic fibrosis, bronchiolitis in infants and children, and fatal pneumonia in elderly and immunocompromised adults (Papadopoulos et al. 2002, Kotaniemi-Syrjanen et al. 2003, Hershenson 2010, Jacobs et al. 2013, Muller et al. 2015, Martin et al. 2015, Steinke and Borish 2016). The newly discovered RV-C circulates worldwide and is an important cause of febrile wheeze (Lau et al. 2007). RV-C also associates strongly with exacerbation of asthma resulting in hospitalization of children (Lau et al. 2007, Bizzintino et al. 2011). However, RV-C infection also associates with bronchitis, bronchiolitis, pneumonia, otitis media, sinusitis and systemic infections complicated by pericarditis (Lau et al. 2010). In adults, RV-C infection may cause more severe disease such as pneumonia and exacerbation of chronic obstructive pulmonary disease (Lau et al. 2010). The inability to culture RV-C type viruses in standard cell lines hampers the study of their pathogenesis.

2.2.2 *Parechoviruses*

The genus *Parechovirus* includes two species, *Parechovirus A* (HPeV-A) and *Parechovirus B* (HPeV-B, formerly known as *Ljungan virus*), and two proposed species; *Parechovirus C* (HPeV-C) and *Parechovirus D* (HPeV-D) (www.picornaviridae.com). Only viruses of HPeV-A are known to infect humans (Hyypia et al. 1992, Niklasson et al. 1999, Joffret et al. 2013, Smits et al. 2013). Parechoviruses belonged originally to echoviruses (HPeV-1 and HPeV-2 as E-22 and E-23, respectively) (Wigand and Sabin 1961), but more thorough characterization showed them to be distinct from echoviruses and other picornavirus groups, due to which they formed a new genus, *Parechovirus* (Stanway et al. 1994, Stanway and Hyypia 1999). HPeV-1 and HPeV-2 were discovered in USA from children with diarrhea in 1956 (Wigand and Sabin 1961). Currently, nineteen HPeV-A types exist, and they cause variable clinical symptoms (Zhao et al. 2016). While HPeV-1 to 6 grow in normal cell lines (Westerhuis et al. 2013), the lack of virus samples or cell culture model hampers studies with HPeV-7 to 19.

HPeV transmission occurs most likely via fecal-oral or respiratory route. Like EVs, HPeV infections cause for instance mild gastrointestinal or respiratory symptoms, but most of all HPeVs are the second most important cause of viral sepsis-like illness and meningitis in infants (Abed and Boivin 2006, Benschop et al. 2006, Wolthers et al. 2008, Harvala et al. 2009). The main HPeV serotypes in childhood and adulthood are HPeV-1, followed by HPeV-3, HPeV-4 and HPeV-6 (Benschop et al. 2008, van der Sanden et al. 2008, Harvala and Simmonds 2009). The prevalence of other HPeVs varies among different populations (Westerhuis et al. 2013). The prevalence of HPeV infections have been underestimated, but it has been shown that HPeV infections are at least as common as EV infections (Benschop et al. 2008). Based on serology, over 90% of children suffer at least one HPeV infection by the age of two years (Joki-Korpela and Hyypia 1998, Tauriainen et al. 2007, Harvala et al. 2010). HPeV infections are rare in older children and adults (Esposito et al. 2014). Several studies show that the median age of children infected with HPeV-1 is significantly higher than that of HPeV-3, and severe HPeV-3 infections occur almost exclusively in children under the age of three months (Benschop et al. 2006, Abed and Boivin 2006, Harvala et al. 2009). This could be due to lower frequency of past HPeV-3 infections as compared to other HPeV infections in adults. Because HPeV-3 seroprevalence in adults is low, neonates and young infants are not protected against HPeV-3 by maternal antibodies (Harvala et al. 2009, Harvala et al. 2010, Westerhuis et al. 2013).

HPeV-1 is the most studied parechovirus. HPeV-1 infection causes most often mild gastrointestinal and respiratory diseases, but manifests occasionally more severely in young children causing bronchiolitis, encephalitis, encephalomyelitis, transient paralysis, and aseptic meningitis (Berkovich and Pangan 1968, Grist et al. 1978, Figueroa et al. 1989, Koskiniemi et al. 1989, Ehrnst and Eriksson 1993, Legay et al. 2002, Abed and Boivin 2006, Harvala and Simmonds 2009, Wildenbeest et al. 2013, Esposito et al. 2014). HPeV-

1 infection can also cause myocarditis (Maller et al. 1967, Russell and Bell 1970), haemolytic uraemic syndrome (O'Regan et al. 1980) and necrotizing enterocolitis (Birenbaum et al. 1997). Additionally, HPeV-1 infection links to acute otitis media (Tauriainen et al. 2008), but in contrast to EVs, it has not been associated with T1D (Tauriainen et al. 2007).

HPeV-3 is the most pathogenic HPeV type (Harvala et al. 2009, van der Linden et al. 2015). HPeV-3 infection associates with paralysis, myositis, meningitis, neonatal sepsis-like illness, and encephalitis in infected infants (Boivin et al. 2005, Verboon-Maciolek et al. 2008, Harvala et al. 2009, Wildenbeest et al. 2010, Sainato et al. 2011, Selvarangan et al. 2011, Walters et al. 2011, Schuffenecker et al. 2012, Pariani et al. 2014, Khatami et al. 2015, Bissel et al. 2015). Few cases of fatal encephalitis caused by HPeV-3 have been reported (van Zwol et al. 2009, Sedmak et al. 2010, Fischer et al. 2014, Bissel et al. 2015). As shown, HPeV-3 is very neuropathogenic, which may reflect its ability to replicate rapidly in neuronal cells (Westerhuis et al. 2013).

HPeV-2 cause relatively mild infections like HPeV-1 (Stanway et al. 2000). However, HPeV-4 associates with lymphadenitis (Watanabe et al. 2007), TORCH syndrome (Schnurr et al. 1996), and neonatal sepsis-like syndrome (Jaaskelainen et al. 2013, Kolehmainen et al. 2014). HPeV-5 causes gastrointestinal symptoms, fever, joint ache and rash (van der Sanden et al. 2008), whereas HPeV-6 infections manifests as gastroenteritis, rash, respiratory infections, flaccid paralysis and Reye syndrome (Watanabe et al. 2007). HPeV-7 and HPeV-8 respectively associate with non-polio acute flaccid paralysis and enteritis, (Li et al. 2009). The number of HPeV-9 to 19 patient cases described in the literature is low or lacking.

Scientific community has neglected HPeVs in the past, but increasing evidence on global HPeV circulation and severe HPeV infections have increased the awareness on their clinical significance (de Crom et al. 2016). Nowadays HPeVs have been described as clinically relevant viruses and HPeVs play a significant role in various severe pediatric manifestations, particularly in neonatal sepsis and severe CNS infections. In the case of CNS infections, routine screening for HPeVs in cerebrospinal fluid (CSF) and blood have been suggested, especially within the patients under three months old children (Sainato et al. 2011, Schuffenecker et al. 2012). No data exists on the prognosis and long-term effects of HPeV meningitis in children (de Crom et al. 2016). However, follow-up studies concerning children with HPeV encephalitis show neurodevelopmental sequelae, such as epilepsy, cerebral palsy, central visual impairment or difficulties in gross motor development (Verboon-Maciolek et al. 2008, Britton et al. 2016).

2.2.3 Other human picornaviruses

The genus *Hepatovirus* comprise a single species, *Hepatovirus A*, and its only serotype is hepatitis A virus (HAV). Natural HAV infection usually follows ingestion of virus from material contaminated with feces, mostly water and seafood. The most common clinical manifestation of HAV infection is acute hepatitis, which typically presents with rapid onset of nausea, loss of appetite, fever, abdominal pain, dark urine, and jaundice (Lemon 1985). Almost all acute hepatitis A cases are self-limited and clinically resolve within a few weeks, but a small proportion of infections result in fulminant hepatitis with liver failure (Feng and Lemon 2010). In the absence of effective sanitation and clean potable water HAV is a major public health hazard, especially in the developing countries, despite the fact that an effective vaccine (Havrix) exists. In contrast, in developed countries, HAV infection is increasingly rare, although intravenous drug use and increased travelling induce outbreaks (Leino et al. 1997, Feng and Lemon 2010). HAV vaccines are the safest and most effective viral vaccines ever manufactured (Feng and Lemon 2010, Matheny and Kingery 2012).

In addition to *Entero-, Parecho-* and *Hepatovirus*, few other picornavirus genera contain virus types that infect humans. These include *Aphtho-, Cardio-, Cosa-*, *Kobu-* and *Salivirus*. Although *Aphtho-, Cardio-,* and *Kobuvirus* contain mainly animal viruses, they all include viruses that can infect humans. Foot-and-mouth disease virus (FMDV) within the genus *Aphtovirus* is a zoonotic virus (Bauer 1997). However, the risk of FMDV transmission to humans is supposedly minor (Prempeh et al. 2001). Saffold virus (SAFV), genus *Cardiovirus*, associates with fever in humans (Jones et al. 2007).Viruses from *Cosavirus* and *Salivirus* link to diarrhea (Holtz et al. 2008, Holtz et al. 2009). Yamashita and coworkers discovered Aichi virus (a kobuvirus), which caused an oyster-associated nonbacterial gastroenteritis in man in 1989 (Yamashita et al. 1991). Some of the animal-infecting picornaviruses, like Seneca Valley virus (*Senecavirus*), can cause asymptomatic infection in humans, and they are employed in oncolytic virotherapy virus (Burke 2016).

2.3 Structure of picornaviruses

Picornaviruses are non-enveloped, small RNA viruses. The name of the virus family refers to the size of the virion (pico, a small unit of measurement) and the type of genome (rna). Picornavirus studies have contributed significantly to our understanding of virus evolution, assembly, host-cell interaction, host adaptation, and antigenic variation (Ehrenfeld et al. 2010). The findings provide the basis for novel therapeutic strategies. Picornaviruses hold an important role in the development of modern virology, because they were the first animal viruses whose structure was determined in atomic detail (Hogle et

al. 1985). PV was crystallized already in 1955 (Schaffer and Schwerdt 1955). X-ray crystallography and the knowledge of the complete protein sequence enabled the determination picornavirus structure in 1985 (Hogle et al. 1985). In addition to PV, also the structure of RV-B14 was visualized in atomic detail in 1985 (Rossmann et al. 1985). Nowadays, cryo-electron microscopy (cryo-EM) provides high-resolution structures for viruses and virus-receptor complexes. High-resolution structures are available for several picornaviruses (206 by March 2017 (http://viperdb.scripps.edu/)), including CV-A9 (Hendry et al. 1999, Shakeel et al. 2013) and HPeV-1 (Seitsonen et al. 2010, Kalynych et al. 2015) used in this study.

2.3.1 Particle structure

Typical picornavirus capsid is 27-30 nm in diameter and composed of 60 protomers with icosahedral symmetry. Most picornaviruses comprise four structural viral proteins (VP), VP1-VP4 in protomers. Five protomer units form a pentagon-shaped pentamer, and twelve pentamers form the complete icosahedral capsid of the virion (**Figure 1**) (Hogle et al. 1985, Rossmann et al. 1985). On the surface of the capsid protomers (with the exceptions of kobu-, avihepato- and parechoviruses; see below), the VP1 proteins locate around the five-fold axes, and VP2 and VP3 alternate around the two- and three-fold axes. The smaller, N-terminally myristoylated, VP4 locates to the inner surface of the particle and is possibly in contact with the viral genome (Racaniello 2013).

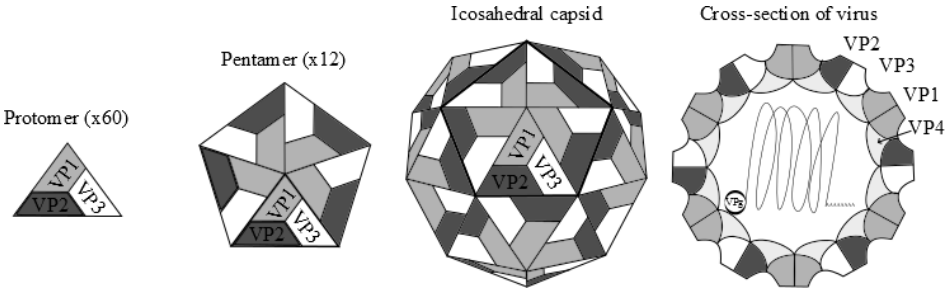

Figure 1: Schematic structure of picornaviruses. *A protomer consists of three to four VPs. A pentamer consists of five protomers, and the icosahedral capsid of twelve pentamers. The viral RNA (vRNA) locates inside the capsid. Genome linked viral protein (VPg) is covalently linked to the 5´-end of the RNA.*

During particle maturation, VP0 is autocatalytically cleaved to VP2 and VP4 in the final maturation step of the virion (Rossmann et al. 1985, Arnold et al. 1987, Curry et al. 1997, Hindiyeh et al. 1999). In avihepato-, kobu- and parechoviruses, the VP0 protein remains non-cleaved, and the mature virions contain only three structural proteins (VP0, VP1 and

VP3) (Stanway et al. 1994, Yamashita et al. 1998, Stanway and Hyypia 1999, Stanway et al. 2000, Kim et al. 2006).

VP1, VP2 and VP3 share no sequence homology, yet all three proteins adopt a fold found widely in RNA viruses; they form a wedge-shaped eight-stranded antiparallel β-barrel (also called jelly roll fold). VP4 differs significantly from the other three proteins in that it has an extended conformation (Racaniello 2013). The arrangement of the capsid proteins within the virion defines also the receptor-binding specificity of the virus as well as its antigenic properties. VP1 usually contributes most to the accessible surface area, while VP3 contributes most towards capsid stability (Fry and Stuart 2010). In viruses of the genus *Enterovirus* (rhinoviruses and enteroviruses), there is a deep depression encircling the fivefold axes below VP1. The depression, referred to as "canyon", is the site of interaction with cellular receptors in many picornaviruses (Colonno et al. 1988, Olson et al. 1993, Plevka et al. 2012). The canyon is partially filled in the cardioviruses to leave a series of depressions that span the twofold axes (sometimes referred to as "pits"), which are also involved in receptor binding (Luo et al. 1987, Toth et al. 1993, Grant et al. 1994, Hertzler et al. 2000). The minor group RV receptor (low-density lipoprotein) binds close to the fivefold axis, on the star-shaped plateau surrounded by the canyon (Rankl et al. 2008). Such binding enables multiple low-affinity interactions to combine resulting in a high-avidity virus-receptor-complex. The canyon also presents a target of interest for making antivirals against picornaviruses. A drug compound known as pleconaril, developed against RVs in the 1980s, is FDA approved for clinical use (Hayden et al. 2003). Currently no antivirals against picornaviruses are in clinical use, since pleconaril was banned due to side effects in early 2000.

2.3.1.1 Detailed structures of CV-A9 and HPeV-1

HPeV-1 virion with an average diameter of 24.7 nm is the smallest picornavirus structurally characterized (Kalynych et al. 2015). In comparison, the diameter of CV-A9 is 28 nm, close to the average diameter of picornaviruses As shown in **Figure 2**, the arrangement of CV-A9 and HPeV-1 VPs is different on the surface as well as on the inside of the capsid. This is as because of the VP0 cleavage in CV-A9, as described above. The surface of HPeV-1 is smoother than that of CV-A9 (Figure 2, right panel), and there are no canyons that are typical for picornaviruses on the cell surface (Seitsonen et al. 2010). The smoothness of HPeV-1 is explained by the shorter surface loops in VP1 and deletions in other loops compared to CV-A9 (Seitsonen et al. 2010, Kalynych et al. 2015). A hydrophobic pocket, the target for capsid-binding antiviral compounds in many other picornaviruses, is not present in HPeV-1 (Kalynych et al. 2015). In CV-A9, there are five distinct depressions rather than a continuous circular canyon around each fivefold protrusion. Depressions also exist on the icosahedral twofold axis (Hendry et al. 1999).

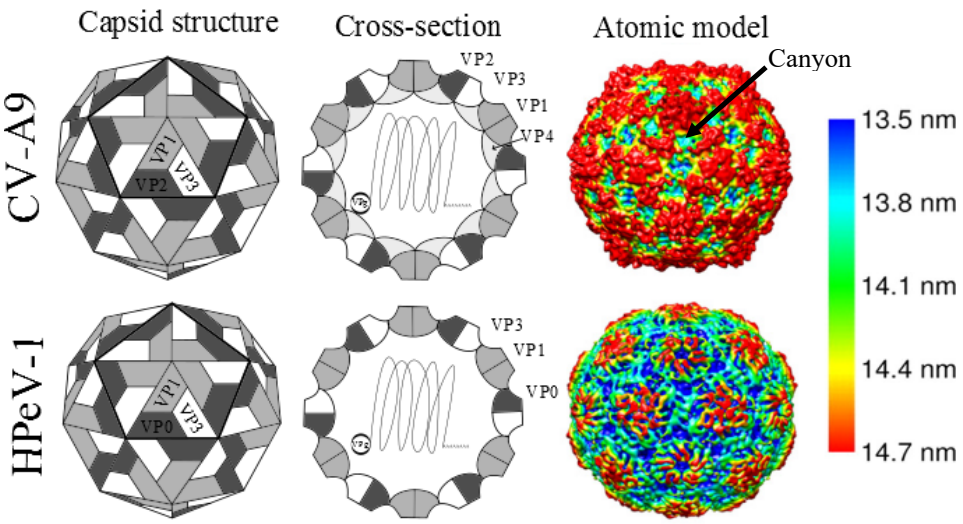

Figure 2: Schematic and atomic structures of CV-A9 and HPeV-1. *The upper panel shows capsid structure, cross-section and atomic model of CV-A9, and the lower panel of HPeV-1. In the atomic model peaks are shown in red, and pits in blue. Canyon, to which picornavirus receptors often binds, is shown within CV-A9 atomic model. (Modified from Seitsonen et al. 2010)*

2.3.2 Picornavirus genome

The genome of picornaviruses is positive-sense, single-stranded RNA (ssRNA). The viral RNA (vRNA) is infectious because it functions similarly to messenger RNA, and is translated after cell entry (Racaniello 2013). The genome organization of picornaviruses is canonical, highly conserved, and a defining characteristic of the virus family. The known genomes vary in length from approximately 7000 to 8800 nucleotides, with a median of about 7600 nucleotides (Palmenberg et al. 2010). VPg (genome linked viral protein) is a small peptide covalently linked to the 5′-end of the RNA genome (Flanegan et al. 1977, Lee et al. 1977). VPg, encoded by a single viral gene, varies in length from 22 to 24 amino acid residues among different picornaviruses, except in FMDV, which has three VPg genes (Forss and Schaller 1982). VPg acts as a protein primer for the initiation of vRNA synthesis (Paul et al. 1998). The 3′-end of the genome is polyadenylated (PolyA) in all picornaviruses but the length varies among virus species. The polyA tail is genetically encoded and partially replicated, and then extended during genome synthesis (Palmenberg et al. 2010).

The single open reading frame (ORF) for the polyprotein occupies 85 to 90% of the theoretical coding capacity of the RNA. The genome translates into a single polyprotein; the

cleavage of which by virally encoded proteases yields mature protein products. The structural proteins locate to the N-terminus of the polyprotein. The remainder of the polyprotein includes proteins involved in modifying the cellular environment to optimize virus replication and the proteins directly responsible for replication. The ORF encoding both viral capsid (VP1-4) and replication proteins (2A-C and 3A-D) resides between the untranslated regions, 5´-UTR and 3´-UTR. An example of CV-A9 genome represents a typical picornavirus genome in **Figure 3**. The middle region of the polyprotein contains nonstructural peptides 2A, 2B and 2C. The 5′-UTR is especially long (500 to 1500 nucleotides) and highly structured. The 5´-UTR also contains a number of important replication and translation control elements, including an internal ribosome entry site (IRES) that directs translation of the mRNA. The 3´-UTR of picornaviruses is short, ranging from 40 to 330 nucleotides in length. A remarkable series of co- and posttranslational reactions, catalyzed by viral proteases and characteristic of each genus, processes the polyprotein into the full cohort of precursors and mature proteins necessary to establish and maintain a replicative cycle (Palmenberg et al. 2010, Tuthill et al. 2010, Racaniello 2013).

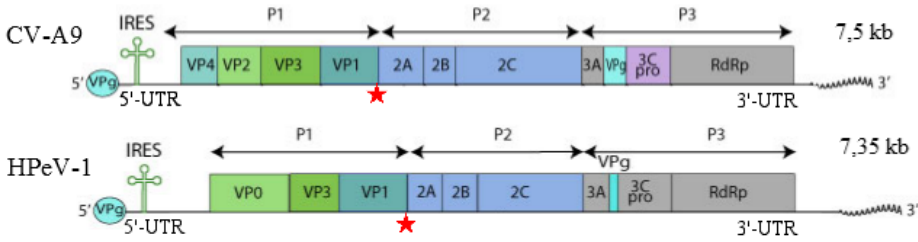

Figure 3: Typical picornavirus genome (CV-A9), and genome of HPeV-1. *Median length of picornavirus genome is 7,5 kb. VPg is covalently linked to 5´-UTR, and polyA tail is located in the 3´-UTR. The red star indicates the Arginine-Glycine-Aspartic acid (RGD) motif.*

HPeV-1 has a smaller genome than CV-A9, and it packs with higher density to the capsid than that of EVs such as CV-A9 (Kalynych et al. 2015). The genome organizations of CV-A9 and HPeV-1 are rather similar.

2.4 Picornavirus infection cycle

Picornaviruses initiate infection of cells by first attaching to a receptor (or receptors) on the plasma membrane of the host cell (**Figure 4**). Picornaviruses utilize a wide variety of receptor types, and there are almost 20 picornavirus receptors known at present. The picornavirus receptor specificity varies depending on both genera and species. The receptor specificity may determine the cell and tissue tropism and therefore pathogenesis of the

virus (Rossmann et al. 2002, Tuthill et al. 2010). Virus-receptor interaction usually triggers viral uptake into the cell through a specific endocytic vesicle that depends on the virus type, receptor specificity and cell line (Tuthill et al. 2010). The release of picornaviral RNA genome to the cell cytoplasm occurs in a process called uncoating during vesicular transport. The vRNA translates to form a polyprotein, which by autocatalytic cleavage yields the structural and non-structural proteins. The synthesis of positive- and negative-sense RNA strands occurs in the membrane-associated replication complexes, and once produced, the viral components drive the formation of progeny virions that subsequently escape the cell.

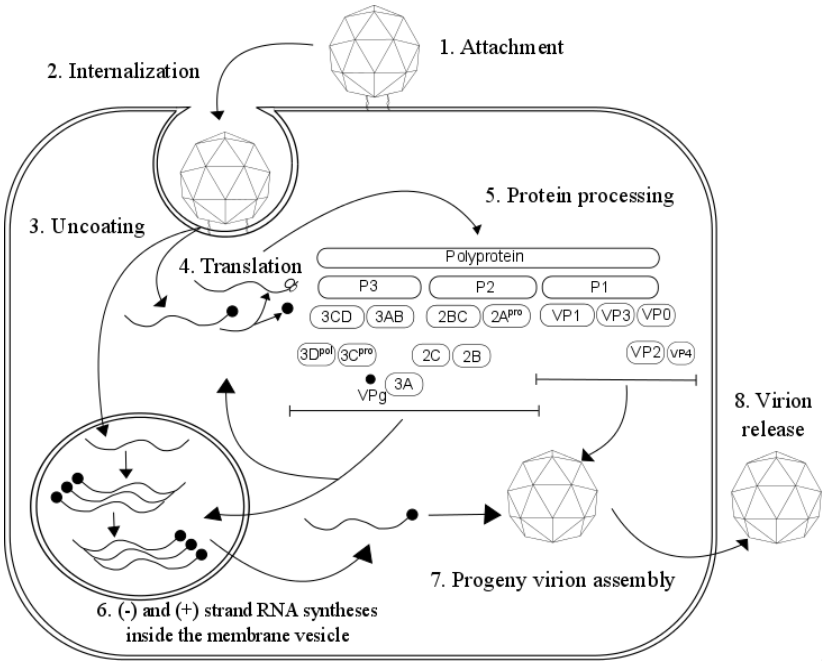

Figure 4: The life cycle of picornaviruses. *(1) particle attaches to the host cell surface via a cellular receptor (receptors), (2) internalization of the virus (3) uncoating and RNA genome release, (4) viral genome translation, (5) protein processing into the nonstructural and structural proteins, (6) viral RNA replication inside the membrane vesicle, (7) virion assembly, (8) particle release.*

2.4.1 Receptor interactions

Cell surface receptors are located on the cell surface participating in cell signalling by binding to extracellular molecules, such as fibronectin (Humphries et al. 2006). Some viruses may use more than a single receptor for infectious entry, allowing viruses to expand their host range. Some molecules act as "sticky surface" – the first site of attachment on the cell surface. When the virus reaches the cell surface, one or more accessory molecules or attachment factors may be required before the actual protein receptor enables virus internalization into the cell. In some cases, a single receptor molecule may participate in all steps from attachment to the cell surface to synthesis and release of new virions (Rossmann et al. 2002, Racaniello 2013). However, according to recent findings, the use of multiple receptors is more a rule than an exception (Bergelson 2010).

Usually virus life cycle starts with the attachment to the (protein) receptor on the cell surface. The nature of picornavirus receptors remained obscure until the identification of the receptors for PV and the major group RVs in 1989 (Greve et al. 1989, Staunton et al. 1989, Mendelsohn et al. 1989). Since then the receptors for many other family *Picornaviridae* members were identified, including relatively recent findings of EV-71 receptors (Nishimura et al. 2009, Yamayoshi et al. 2009, Yang et al. 2009a) and *Rhinovirus C* receptor (Bochkov et al. 2015) discussed more below. In general, picornaviruses utilize receptors from the immunoglobulin-like family (poliovirus receptor (PVR), intracellular adhesion molecule 1 (ICAM-1), coxsackie- and adenovirus receptor (CAR) and vascular cell adhesion molecule type 1 (VCAM-1)), the low-density lipoprotein receptor (LDLR) family, decay-accelerating factor (DAF) and the integrin family, in addition to individual receptor molecules including hepatitis A virus cellular receptor 1 (HAVcr-1) and β2-microglobulin (β2M) (Evans and Almond 1998, Tuthill et al. 2010, Racaniello 2013) (**Table 2, Figure 5**).

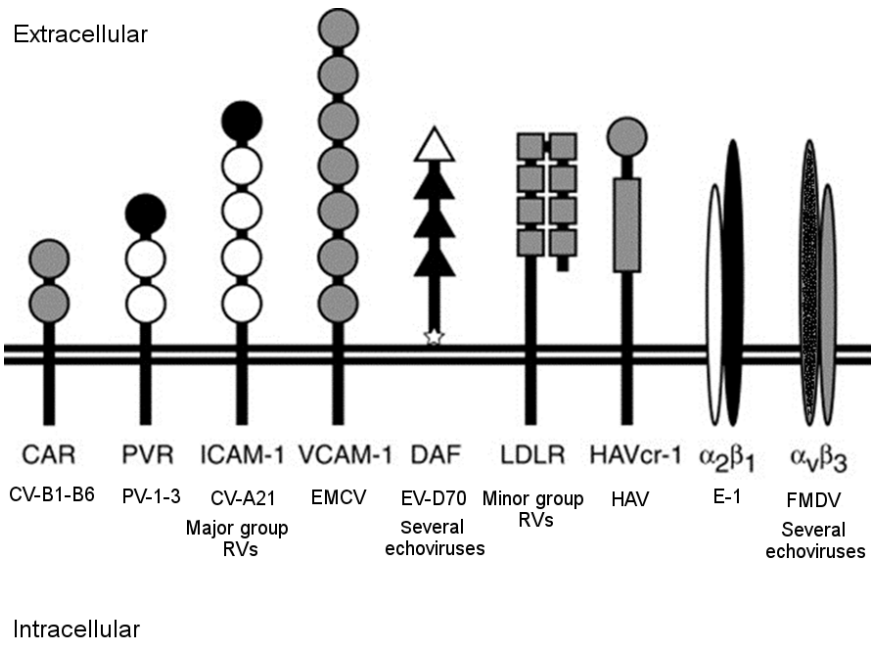

Figure 5: Picornavirus receptors. *Picornaviruses have adapted to use multiple receptors. In the figure is shown some of the picornavirus receptors and example(s) of virus(es) utilizing the receptors. (Modified from Evans and Almond 1998)*

Virus–receptor interactions of the viruses representing the more established picornavirus genera have been extensively characterized. However, there are a large number of picornaviruses, for which the receptors are unknown (Rossmann et al. 2002, Tuthill et al. 2010). The first and simplest function of a receptor, from viruses' point of view, is to permit attachment and accumulate the virus to the cell surface. Beyond attachment, receptors may also perform functions in viral replication. The receptors promote infection following mechanisms; by inducing the capsid disassembly immediately after internalization, by targeting the particle to a specific subcellular compartment (like intramembrane vesicles, wherein the disassembly and genome release can occur), or by inducing intracellular signaling, which mediates virus endocytosis. Virus may evolve to use a specific receptor not only because the receptor is expressed on particular target cells but also because the specific signalling capacity of the receptor provides the best cellular properties for entry and infection (Rossmann et al. 2002, Tuthill et al. 2010, Bergelson 2010). Some viruses bind to different cell surface receptors, depending on the virus isolate or the cell line. For example, clinical FMDV isolates bind to several different integrin receptors, but integrin αVβ6 is likely the native receptor for the virus (Monaghan et al. 2005). Adaptation to cell culture leads to selection virus variants that bind to and use heparan sulfate (HS), a sulfated glycan, in the absence of integrins (Jackson et al. 1996, Martinez et al. 1997, Sa-Carvalho et al. 1997). Furthermore, cell culture passaging may produce a virus

that infects cells independent of HS and integrins (Baranowski et al. 2000). As demonstrated by several CV-B isolates, variation in receptor usage exists among individual virus isolates and variants with altered receptor specificity may arise during infection in humans as well as in cell culture (Bergelson et al. 1997b). Some viruses, e.g. CV-Bs, are adapted to enter the cell via specialized sites, such as tight junctions, that may not be present in normal cell culture systems. This emphasizes the need for caution in interpreting data obtained from different experimental systems (Coyne and Bergelson 2006, Tuthill et al. 2010).

Table 2: Known picornavirus receptors

Picornavirus receptor	Virus	References
Immunoglobulin superfamily:		
Poliovirus receptor (PVR, CD155)	PV-1-3	Mendelsohn et al. 1989
Intracellular adhesion molecule 1 (ICAM-1, CD54)	RV (major group)	Greve et al. 1989, Staunton et al. 1989, Tomassini et al. 1989
	CV-A13, CV-A18, CV-A21	Colonno et al. 1986
	CV-A15, CV-A20	Pulli et al. 1995
	CV-A24v	Baggen et al. Conference abstract B11, Europic 2016
Coxsackie- and adenovirus receptor (CAR, CXADR)	CV-B1-6	Bergelson et al. 1997, Tomko et al. 1997, Martino et al. 2000
Vascular cell adhesion molecule type 1 (VCAM-1)	EMCV	Huber 1994
Integrins:		
α2β1	E-1	Bergelson et al. 1992
αVβ1, αVβ3, αVβ6	HPeV-1	Roivainen et al.1994, Pulli et al. 1997, Seitsonen et al. 2010, Triantafilou et al. 2000a
αVβ1, αVβ3, αVβ6	CV-A9	Roivainen et al. 1991, Roivainen et al. 1994, Pulli et al. 1997, Triantafilou et al. 1999, 2000b, 2000c, 2001, 2003, Williams et al. 2004, Heikkilä et al. 2009, Shakeel et al. 2013
αVβ3	E-9	Nelsen-Salz et al. 1999
αVβ3	E-7, E-11, E-25, E-30, E-32	Ylipaasto et al. 2010
αVβ1, αVβ3, αVβ6, αVβ8, α5β1	FMDV	Berinstein et al. 1995, Jackson et al. 2000a, Jackson et al. 2000b, Jackson et al. 2002, Jackson et al. 2004
αVβ6	CV-B1	Agrez et al. 1997
Glycoproteins:		
Sialic acid (SA)	EMCV	Allaway et al. 1987, Jin et al. 1994
	Bovine enterovirus	Zajac&Crowell 1965
	EV-A71	Yang et al. 2009

Picornavirus receptor	Virus	References
	EV-D68	Liu et al. 2015 , Uncapher et al. 1991, Thibaut et al. Conference abstract B10, Europic 2016
	EV-D70	Alexander&Dimock 2002
	CV-A24v	Nilsson et al. 2008, Mistry et al. 2011
	Equine rhinitis A Virus	Stevenson et al. 2004
	Theiler's murine encephalomyelitis virus	Lipton et al. 2006, Guy et al. 2009
Heparan sulfate (HS)	FMDV	Jackson et al. 1996
	E-6	Goodfellow et al. 2001
	CV-B3	Zautner et al. 2003
	Theiler's murine encephalomyelitis virus	Lipton et al. 2006
	E-5	Israelsson et al. 2010
	RV-A89	Vlasak et al. 2005
	RV-A54	Khan et al. 2007
	CV-A9	McLeish et al. 2012
	SVDV	Escribano-Romero 2004
Other:		
Decay-accelerating factor (DAF, CD55)	CV-A13, CV-A18	Colonno et al. 1986 x 2
	CV-A21	Shafren et al. 1997
	CV-B1, CV-B3, CV-B5	Shafren et al. 1995, Bergelson et al. 1995
	E-3, E-6, E-7, E-11, E-12, E-20, E-21, E-24, E-29, E-33	Bergelson et al. 1994, Ward et al. 1994, Ward et al. 1998
	EV-D70	Karnauchow et al. 1996
Low-density-lipoprotein receptor (LDL-R, LDLR)	RV (minor group)	Hofer et al. 1994
Cadherin-related family member 3 (CDHR3)	RV-C	Bochkov et al. 2015
Heat shock protein family A member 5 (HSPA5, GRP78)	CV-A9	Triantafilou et al. 2002
Annexin II	EV-A71	Yang et al. 2011
P-selectin glycoprotein ligand-1 (PSGL-1)	EV-A71	Nishimura et al. 2009
	CV-A24v	Mistry et al. 2011
Scavenger receptor class B, member 2 (SCARB2)	EV-A71	Yamayoshi et al. 2009, Yamayoshi et al. 2012
	CV-A7, CV-A14, CV-A16	Yamayoshi et al. 2009, Yamayoshi et al. 2012
Hepatitis A virus cellular receptor 1 (HAVcr-1, TIM-1)	HAV	Kaplan et al. 1996
β2-microglobulin (β2M)	CV-A9	Ward et al. 1998, Triantafilou et al. 1999, Heikkilä et al. 2010
	E-1 to E-9, E-11 to E-19, E-21, E-24 to E-29, E-33	Ward et al. 1998
	E-11	Chevaliez et al. 2008
	EV-D70	Ward et al. 1998
Heat shock protein family A member 5 (HSPA5, GRP78, BiP)	CV-A9	Triantafilou et al. 2000b, 2002, 2003

2.4.1.1 Immunoglobulin superfamily receptors (PVR, ICAM-1, CAR, VCAM-1)

Immunoglobulin superfamily receptors include several well-characterized receptors for viruses of the EVs: Intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), poliovirus receptor (PVR), and coxsackie- and adenovirus receptor (CAR). These receptors consist of a series of immunoglobulin superfamily (IgSF) domains, and the amino-terminal domain binds the canyon on the virion while the carboxy-terminus mediates attachment to the host cell's plasma membrane (Rossmann et al. 2002). All three serotypes of PV bind to PVR, which is a transmembrane protein with four Ig-like extracellular domains (Mendelsohn et al. 1989). The PVR N-terminal Ig-like domain binds within the canyon in the virion (Belnap et al. 2000, He et al. 2000, Xing et al. 2000). The interaction between PVR and the virus occurs in two steps: after initial reversible attachment, conformational changes permit the receptor to make contacts deeper within the canyon, which cause destabilization of the virion (Tsang et al. 2001, Zhang et al. 2008). Normal cellular function of PVR is in the establishment of intracellular junctions between epithelial cells, and it is involved in intestinal humoral immune responses (Maier et al. 2007).

Major-group rhinoviruses from RV-A and -B species (totally 91 types) use ICAM-1 for recognition and attachment to the cells (Staunton et al. 1989, Tomassini et al. 1989, Greve et al. 1989, Evans and Almond 1998). ICAM-1 has five extracellular Ig-like domains and, as in the case for the PVR, the N-terminal ICAM-1 domain inserts into the RV canyon (Olson et al. 1993, Kolatkar et al. 1999). Several CV-A isolates bind to ICAM-1 (Colonno et al. 1986, Pulli et al. 1995). All coxsackie B viruses (CVBs) bind to coxsackie- and adenovirus receptor (CAR), a 46-kDa protein with two extracellular Ig-like domains, which functions in cell-cell adhesion and as a barrier for the paracellular flow of solutes and macromolecules within the tight junctions in polarized epithelial cells (Bergelson et al. 1997a, Cohen et al. 2001). Like all other immunoglobulin superfamily receptors, CAR binds to the virus canyon and causes formation of an A-particle, which is a porous uncoating intermediate wherein VP4 is lost and an amphipathic sequence of VP1 is exposed for interaction with the lipid bilayer (He et al. 2001, Milstone et al. 2005). A similar molecule, the murine VCAM-1, is a receptor of encephalomyocarditis virus (EMCV) in rodents (Huber 1994).

2.4.1.2 Decay-accelerating factor (DAF)

The decay-accelerating factor (DAF, or CD55), is a member of the complement control family. DAF is composed of four homologous extracellular short consensus repeat modules attached to the plasma membrane by a glycosylphosphatidyl inositol (GPI) anchor,

and its function is to protect cells from lysis by autologous complement (Lublin and Atkinson 1989). DAF serves as a receptor for several coxsackie A and B viruses, as well as echoviruses (Colonno 1986, Colonno et al. 1986, Ward et al. 1994, Bergelson et al. 1994a, Shafren et al. 1995, Bergelson et al. 1995, Karnauchow et al. 1996, Shafren et al. 1997, Ward et al. 1998). The mode of DAF binding to viruses varies, and different viruses have diverse binding sites on both capsid and receptor (Powell et al. 1999, Lea 2002). For example, with CV-B3, short consensus repeats (SCR) of DAF are in contact with residues near two-fold axis of symmetry (Hafenstein et al. 2007). For most of these viruses, however, interaction with DAF is not sufficient for infection; this molecule is an attachment receptor and it collects virus particles onto the cell surface, but binding to DAF alone does not lead to virion uncoating (Racaniello 2013). For example, CV-A21, CV-B1 and CV-B3 bind to DAF, but infection does not occur unless ICAM-1, αVβ6 integrin or CAR, respectively, binds to the virus and triggers uncoating (Shafren et al. 1997, Agrez et al. 1997, He et al. 2001).

2.4.1.3 Low-density lipoprotein receptor (LDLR) family and Cadherin-related family member 3 (CDHR3)

Low-density lipoprotein receptor (LDLR) family includes LDLR itself, as well as the very low-density lipoprotein receptor (VLDLR) and the LDLR-related protein (LPR). These receptors consist extracellular domain of seven (LDLR), eight (VLDLR), or 31 (LRP) ligand-binding repeats, a transmembrane domain, and a cytoplasmic domain. The structure of these molecules enables these molecules to bind effectively with multiple capsid proteins, which stabilizes the virion (Nicodemou et al. 2005), as well as to bind effectively to multiple virus serotypes (Tuthill et al. 2010). The receptor binds to the star-shaped prominence at the five-fold axis of the virus, not with the canyon as many other receptors do (Hewat et al. 2000). The minor group RVs (13 types) bind to members of the LDLR family (Hofer et al. 1994, Vlasak et al. 2005b). Contact with the LDLR does not appear to induce conformational changes in the capsid or initiate RNA release (Marlovits et al. 1998). The receptor function is to deliver the virus to the endosomal compartment, where uncoating is initiated instead of RNA release into the cytoplasm (Prchla et al. 1994, Brabec et al. 2003). The receptor of RV-C types is cadherin-related family member 3 (CDHR3) (Bochkov et al. 2015). The biological function of CDHR3 is yet unknown.

2.4.1.4 HAV cellular receptor 1 (HAVcr-1, TIM-1)

The T-cell immunoglobulin mucin (TIM) gene family, particularly TIM-1, contains cell surface receptors that are important in T-cell regulation and T-cell differentiation. A human homolog of TIM-1, HAV cellular receptor 1 (HAVcr-1) (with 79% identity) was

identified in 1996 and shown to interact with cell culture-adapted HAV (Kaplan et al. 1996, Feigelstock et al. 1998). HAVcr-1 is a mucin-like class I integral membrane glycoprotein of 451 amino acids and its extracellular domain contains four putative N-glycosylation sites and two distinctive regions (Kaplan et al. 1996, Thompson et al. 1998). HAVcr-1 is widely distributed in different tissues and its natural ligand is immunoglobulin A (IgA) (Feigelstock et al. 1998, Tami et al. 2007). The N-terminal cysteine-rich immunoglobulin-like region of the HAVcr-1 ectodomain is sufficient for binding HAV, but both this region and the mucin-like region are required for viral particle conformational changes leading to HAV uncoating (Thompson et al. 1998, Silberstein et al. 2003). It has been speculated, that TIM-1/HAVcr-1 may not be the only molecule involved in HAV binding and entry (Feng and Lemon 2010), but other putative receptors have not been identified yet.

2.4.1.5 P-selectin glycoprotein ligand-1 (PSGL-1) and Scavenger receptor class B, member 2 (SCARB2)

In 2009, two EV-A71 receptors, P-selectin glycoprotein ligand-1 (PSGL-1) and Scavenger receptor class B, member 2 (SCARB2), were found (Yamayoshi et al. 2009, Nishimura et al. 2009). PSGL-1 is a sialomucin leukocyte membrane protein expressed as a homodimer of disulphide-linked subunits and binds to different selectins (Nishimura and Shimizu 2012). SCARB2 is a heavily N-glycosylated type III transmembrane protein that comprise 478 amino acids (Fujita et al. 1992, Calvo et al. 1995). In addition to PSGL-1 and SCARB2, EV-71 uses Annexin II and sialic acid as its receptors (Yang et al. 2009b, Yang et al. 2011). PSGL-1 also acts as a receptor for CV-A24 variant (Mistry et al. 2011), and SCARB2 is a receptor of CV-A16 (Yamayoshi et al. 2009) and many other EV-A species coxsackieviruses (Yamayoshi et al. 2012).

2.4.1.6 Glycoprotein subfamilies (Heparan sulfate, Sialic acid)

Proteoglycans are glycoproteins with one or more glycosaminoglycan (GAG) chains attached to the core protein (Turnbull et al. 2001). GAGs are long linear polysaccharides composed of disaccharide repeat units. Based on core disaccharide structures, GAGs divide into four groups: HSGAGs (heparan sulfate glycosaminoglycan), CSGAGs (chondroitin sulfate glycosaminoglycan), keratin sulfate and hyaluronic acid (Sasisekharan et al. 2006). HSGAGs often covalently attach to various core proteins, and these HSGAG-protein –conjugates are termed heparan sulfate proteoglycans (HSPGs) (Sasisekharan et al. 2002). HSPGs are abundant on cell surfaces, and they are essential in development and homeostasis. HSPGs also participate various pathological processes. The functions of HSPGs are largely utilized via interaction of the heparan sulfate (HS) side chains,

which normally attach to core proteins through serine residues (Turnbull et al. 2001, Li and Spillmann 2012). Members of glycosaminoglycan family receptors often act as co-receptors or receptors, which viruses utilize in the absence of their primary receptor. Many cells express these molecules and they may act as a "sticky surface" on the cell surface. After attachment onto cell surface via GAG family molecules, a virus finds its primary receptor (Sasisekharan et al. 2006). For example, the major group RVs, RV-A89 and RV-A54, bind to HS in the absence of ICAM-1 (Vlasak et al. 2005a, Khan et al. 2007), and CV-A9 have been suggested to have interaction with HS via specific heparin binding site (McLeish et al. 2012). FMDV also binds to HS (Jackson et al. 1996). The binding site for HS on cell culture-adapted FMDV is formed of the three major capsid proteins VP1, VP2, and VP3 (Fry et al. 1999).

In addition to HSPGs, the other proteoglycans can interact with viruses. Sialic acid (SA) is a generic term for a monosaccharide with a nine-carbon backbone, and SAs act as components of oligosaccharide chains of glycoproteins. SAs are widely distributed in animal tissues (Varki and Varki 2007, Varki et al. 2009). SA has been suggested to interact with CV-A24v (Nilsson et al. 2008), EV-A71 (Yang et al. 2009a) and EV-D70 (Alexander and Dimock 2002).

2.4.1.7 Integrins

The integrin family comprise 24 combinations of heterodimeric complexes formed of α and β subunits. Integrins participate in specialized cell-cell interactions and mediate the attachment of cells to the extracellular matrix (ECM) (**Figure 6**) (Hynes 2002, Barczyk et al. 2010). Each subunit (18 α and 8 β subunits) crosses the cell membrane once. The subunits are glycoproteins that include a large extracellular domain, transmembrane helix and short cytoplasmic domain (**Figure 7**) (Hynes 2002). Integrins can mediate bidirectional signals (outside-in and inside-out) across the plasma membrane. Upon ligand (e.g. virus) binding integrins undergo conformational changes, which lead to outside-in signaling and potentially result in virus intake. Eight of the integrin heterodimers (five αV-, two β1- and αIIbβ3-integrins) recognize the arginine-glycine-aspartic acid (RGD) sequence in the ligand (for example virus), while the other integrins are associated with collagen, laminin or leukocyte-specific functions (Hynes 2002). The RGD motif, identified in the late 1980s, mediates binding of many integrin ligands including fibronectin and vitronectin (Humphries et al. 2006).

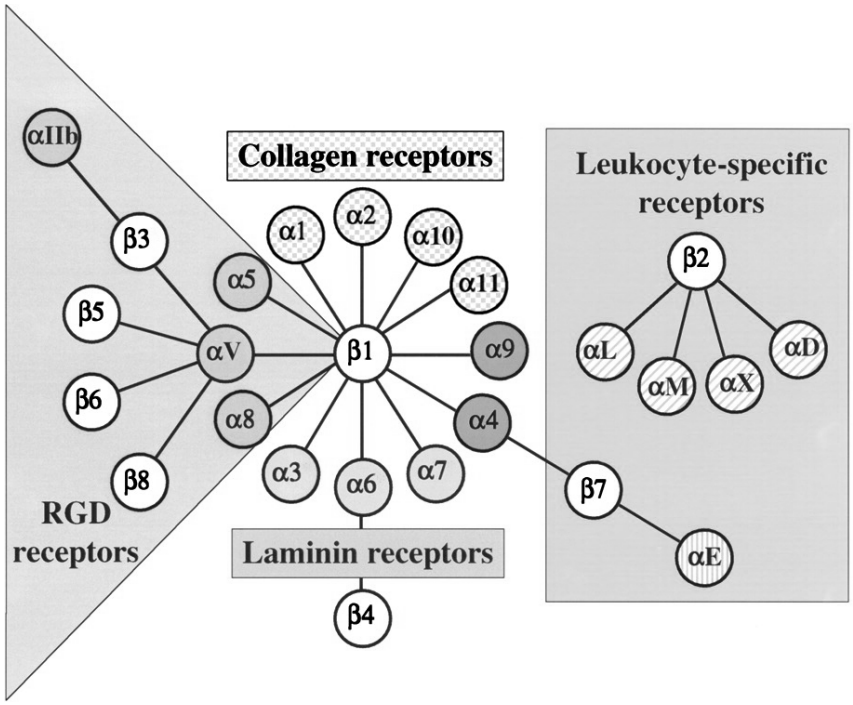

Figure 6: Integrins. *Integrin heterodimers consist of 18 α and 8 β subunits. Integrins are grouped to four groups; RGD-, collagen-, laminin- and leukocyte-specific receptors. One collagen receptor (α2β1 integrin) and several RGD receptors act as picornavirus receptors. (Modified from Hynes 2002).*

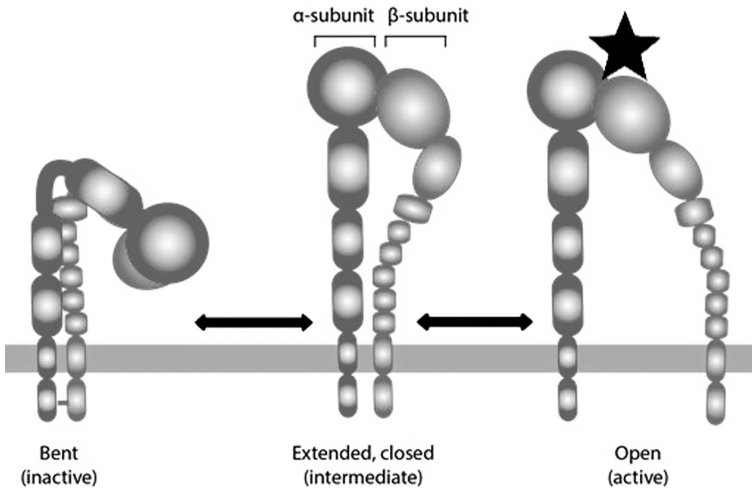

Figure 7: Different conformations of integrins. *Integrins have three different conformations; inactive, intermediate and active conformation. Usually ligands bind to active conformation of integrins (shown as star), however, E-1 binds to inactive conformation of α2β1 integrin (Jokinen et al. 2010). (Modified from www.ivaskalab.com)*

Several picornaviruses bind to integrins both *in vitro* and *in vivo*. These viruses include CV-A9 (Roivainen et al. 1991, Roivainen et al. 1994, Triantafilou et al. 1999, Williams et al. 2004, Heikkila et al. 2009), HPeV-1 (Triantafilou et al. 2000a, Triantafilou and Triantafilou 2001, Seitsonen et al. 2010), E-1 (Bergelson et al. 1992), E-9 (Nelsen-Salz et al. 1999), E-7, E-11, E-25, E-30 and E-32 (Ylipaasto et al. 2010), CV-B1 (Agrez et al. 1997), and FMDV (Berinstein et al. 1995, Jackson et al. 2000a, Jackson et al. 2000b, Jackson et al. 2002, Jackson et al. 2004). Only four of them (HPeV-1, CV-A9, E-9 and FMDV) bind integrin receptors via the viral RGD motif, although in total ten picornaviruses contain RGD motif in its capsid (Merilahti et al. 2012). In human picornaviruses, RGD motif resides in the flexible site on the viral capsid near the C-terminus of VP1 (**Figure 8**) (Hendry et al. 1999). As an interesting exceptions, the RGD motif in E-5 resides in VP3, and E-9 contains two RGD motifs in its VP1 (Merilahti et al. 2012). However, it is likely that the virus in natural infection uses only one or a few of these receptors. For example, αVβ6 integrin has been proposed to act as the natural receptor of FMDV (Monaghan et al. 2005, Dicara et al. 2008, Tuthill et al. 2010). However, the presence of the RGD motif in the virus capsid does not necessary signify that the virus utilizes RGD-binding integrins as its receptors. For instance, CV-A9-RGDdel mutant, in which the RGD motif is deleted, exemplifies this. The mutated virus is fully capable to infect certain cell lines (Roivainen et al. 1991, Hughes et al. 1995, Roivainen et al. 1996). In contrast, it is also possible that a virus lacking the RGD motif uses RGD-binding integrins as its receptor. For example, several echoviruses lacking the RGD motif bind to αVβ3 integrin (Ylipaasto et al. 2010).

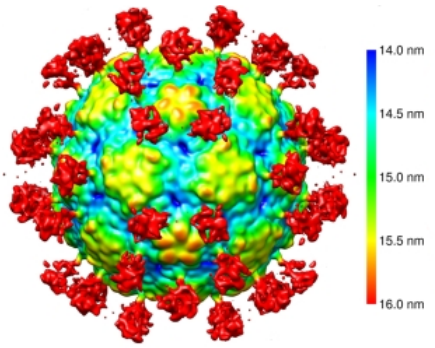

Figure 8: ***Reconstruction of HPeV-1 complexed with αVβ6 integrin.*** *αVβ6 integrin (shown red) binds in vitro to RGD motif of HPeV-1 located on the VP1 on the virus capsid. Since there is 60 copies of VP1, there is also 60 possible binding sites of integrin. (Modified from Seitsonen et al. 2010).*

As mentioned, both CV-A9 and HPeV-1 capsid contains the RGD motif. Thus, the use of integrins as cellular receptors by CV-A9 and HPeV-1 seems logical and the hypothesis has been confirmed in several studies (Ruoslahti and Pierschbacher 1987, Chang et al. 1989, Roivainen et al. 1991, Dickinson et al. 1994, Stanway et al. 1994, Humphries et al.

2006, Hussein et al. 2015). Analyses of virus samples spanning several decades confirms, that RGD sequence in the C-terminus of CV-A9-VP1 is highly conserved, but most of the surrounding residues are variable (Chang et al. 1989, Chang et al. 1992, Santti et al. 2000). In contrast, there are clinical variants of HPeV-1 and E-9, which lack the RGD motif (Zimmermann et al. 1995, Nelsen-Salz et al. 1999, Benschop et al. 2008, Westerhuis 2014). Thus, the significance of RGD motif in virus life cycle is debatable.

In CV-A9 and HPeV-1, the RGD sequence is located in the C-terminus of VP1 (Roivainen et al. 1991, Chang et al. 1992, Stanway et al. 1994). Due to the location and structural flexibility, the RGD motif is not clearly visible in the electron density map of CV-A9 or HPeV-1 (Hendry et al. 1999, Kalynych et al. 2015). However, the motif locates in the middle the icosahedral 5-fold and 3-fold axes (HPeV-1) or next to the icosahedral 5-fold axis (CV-A9) (Shakeel et al. 2013, Kalynych et al. 2015). However, CV-A9 may use an RGD motif independent receptor-binding site (Hughes et al. 1995).

2.4.1.8 β2-microglobulin (β2M)

β2-microglobulin (β2M) is a 12-kDa protein that associates with class I heavy chains to form class I human leukocyte antigen (HLA) complexes, which presents foreign antigens to T cells (Kvist and Levy 1993). Ward et al. (1998) indicated that β2M plays a critical role in infection of several echoviruses as well as EV-D70. In addition, E-7, which normally binds DAF, can infect DAF-negative cells using CD59 or β2M as co-receptors (Ward et al. 1998, Goodfellow et al. 2000). The precise role of β2M in virus infection remains unclear, but the interaction between virus and β2M supposedly occurs after attachment but before RNA translation and replication (Ward et al. 1998, Triantafilou et al. 1999).

2.4.2 Endocytosis mechanisms

After picornaviruses attach to their cellular receptor, the endocytic pathway internalizes the viral capsid followed by genome release into the cytoplasm, wherein replication occurs (Racaniello 2013). During the recent years, the knowledge of the complexity of endocytic mechanisms has increased. For instance, viruses can tolerate and adapt to use alternative entry pathways, which are also dependent of the cell lines used (**Figure 9**) (Mercer and Greber 2013). The most studied endocytosis mechanisms for picornaviruses are clathrin- (CME) and caveolin-1- (CAV1) mediated pathways. However, there is variation even within these classical pathways. In addition, several less well-characterized CME- and CAV1-independent endocytic mechanisms exist (Tuthill et al. 2010, Mercer et al. 2010, Levy et al. 2010). FMDV as well as minor and major group RVs have been

suggested to use CME (Grunert et al. 1997, Snyers et al. 2003, O'Donnell et al. 2005), while E-1 and CV-B viruses have been shown to use macropinocytosis (Coyne and Bergelson 2006, Liberali et al. 2008). However, E-1 and CV-B3 have been shown to endocytose also via caveolin-1 containing vesicles (Marjomaki et al. 2002, Coyne et al. 2007). However, the current classification of entry pathways relies on molecules and structures (called endocytic markers) involved in different steps of the entry route, without specific naming of the pathways. An example of the less-characterized pathway is the endocytosis of RV-B14. This pathway combines characteristics of classical macropinocytosis with unusual features such as involvement of dynamin (Khan et al. 2010).

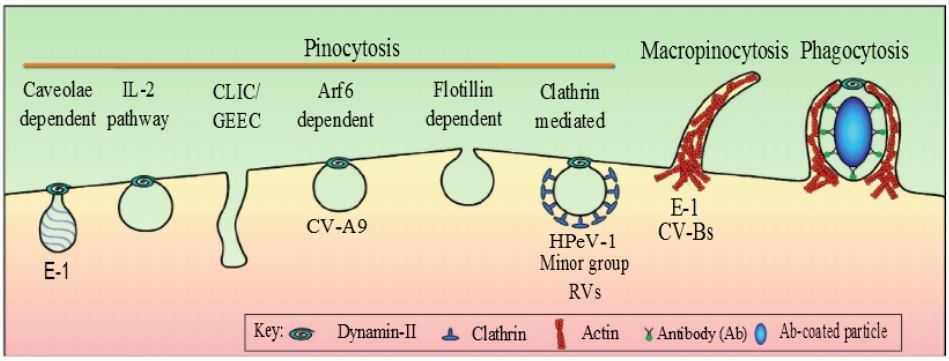

Figure 9: Endocytic mechanisms used by viruses. *The endocytic mechanisms include pinocytic, macropinocytic, or phagocytic mechanisms. Pinocytic mechanisms include caveolae, ADP-ribosylation factor 6 (Arf6) and flotillin dependent pathways, as well as IL-2 pathway, clathrin mediated pathway, and a pathway where clathrin-independent carriers (CLICs) which matures into glycosylphotidylinositol-anchored protein enriched compartments (GEECs). The classification of pinocytic mechanisms depends on their cellular requirements, such as dynamin or actin. Only a few detailed examples of picornavirus endocytosis pathways exists. (Modified from Mercer and Greber 2013).*

Detailed endocytosis pathways of CV-A9 and HPeV-1 remain unknown. Heikkilä et al. (2010) showed that CV-A9 endocytosis is not dependent on macropinocytosis and it does not utilize typical CME or CAV1 mediated pathways. Instead, CV-A9 internalization is mediated by Arf6 and dynamin (Heikkila et al. 2010). HPeV-1 has been studied even less, but HPeV-1 has proposed to use CME pathway (Joki-Korpela et al. 2001).

2.4.2.1 Integrins and endocytosis

Integrins internalize via different entry mechanisms, via either clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIE). CME is the best-characterized route, and CIE routes include less well-understood routes such as caveolae-mediated endocytosis, clathrin-independent carriers (CLICs) and macropinocytosis (Bridgewater et al. 2012). Different integrins utilize different routes: β1 integrins are found within CLICs (Howes et al. 2010), and α2β1, α5β1 and αVβ3 integrins are associated with caveolin-1 (Bridgewater et al. 2012). A large and diverse array of proteins regulate integrin trafficking at multiple levels enabling a particular integrin to follow specific routes in different cells and conditions (Bridgewater et al. 2012). The general pathway to all cellular receptors, with or without their ligands, is via early endosomes (EE) to late endosomes (LE), multivesicular bodies (MVB), lysosomes or back to plasma membrane vie recycling endosomes (RE) (Sorkin and von Zastrow 2009, Alanko 2016). β1 integrin life cycle is well-characterized, and its internalization involves a large number of regulators, including protein kinase Cα (PKCα), Arf6 and several Rabs (Ng et al. 1999, Powelka et al. 2004, Pellinen and Ivaska 2006, Caswell et al. 2009). Inactive β1 integrin is recycled back to the cell surface while active β1 integrin mainly remains intracellular (Arjonen et al. 2012).

Integrins can act as co-receptor or primary receptor in the entry process. Virus attachment to integrins triggers host-cell signaling pathways and the mechanisms that regulate virus entry overlap significantly with the known endocytic pathways of integrins (Pellinen and Ivaska 2006). The most studied picornavirus in respect to integrin receptor binding and endocytosis is E-1 (Pietiainen et al. 2004). E-1 recognizes the α2 I-domain of the inactive α2β1 integrin on the cell surface (Bergelson et al. 1994b, Jokinen et al. 2010). This leads to uptake of the virus-receptor complex via caveolae/raft-dependent endocytosis into α2 integrin -enriched MVBs (α2-MVBs) (Marjomaki et al. 2002, Upla et al. 2004, Karjalainen et al. 2008). Integrin-induced signaling events and other cellular markers strictly regulate internalization (Pietiainen et al. 2004). In contrast to the normal inactive β1 integrin, E-1-clustered α2β1 integrins do not recycle back to the plasma membrane but instead undergo down-regulation (Rintanen et al. 2012).

2.4.3 From uncoating to virus release

Virus uncoating occurs during vesicular transport leading to the release of picornaviral RNA genome into the cytoplasm. The precise mechanism of picornavirus uncoating is unknown. For most picornaviruses, the uncoating initiates by interaction of the VP4 myristoyl groups with the host cell membrane, which leads to the loss of VP4. This exposes the VP1 N-terminus, followed by genome injection into the cytoplasm from the acidic endosomes, in which the virus is internalized (Smyth and Martin 2002).

Once the positive-stranded vRNA enters the cytoplasm, it must be translated because picornaviruses do not utilize cellular RNA polymerases and no viral enzymes are included to the viral capsid. VPg cleavage from the vRNA occurs upon infection of the host cell (Ambros and Baltimore 1980), after which the host cell ribosomes recognize the internal ribosome entry site (IRES) in the vRNA, resulting in initiation of translation (Rivera et al. 1988, Skinner et al. 1989). Picornavirus protein synthesis occurs via translation of the vRNAs single ORF, followed by cleavage of the polyprotein by virus-encoded proteinases (Racaniello 2013). The translation and subsequent protein processing occurs in the cell cytoplasm, but the genome replication and RNA synthesis occurs on small membranous vesicles. The synthesis is induced by several virus-produced proteins (Racaniello 2013). The polyprotein cleavage by viral-encoded proteases gives rise to P1, P2 and P3 (Holland and Kiehn 1968, Jacobson and Baltimore 1968, Summers and Maizel 1968, Rueckert and Wimmer 1984), and these proteins are further cleaved as shown in **Figure 10**.

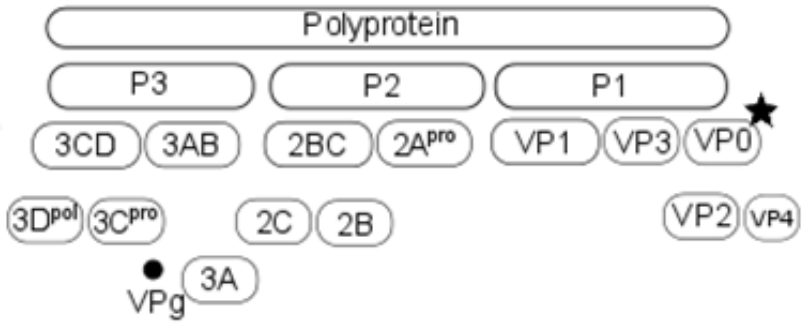

Figure 10: Protein processing. *The polyprotein is cleaved by viral-encoded proteases into three precursor proteins, P1, P2 and P3, which are further cleaved into smaller polypeptides. In certain picornaviruses, e.g. in parechoviruses VP0 (marked as star), VP0 is not cleaved into VP2 and VP4.*

When the capsid protein pool is sufficiently large, encapsidation begins. Cleavage of the coat proteins precursor, P1, produces an immature protomer, which then assembles into pentamers with direct interaction with RNA to form genome-filled capsids (Nugent and Kirkegaard 1995). A single replication cycle takes from five to ten hours, depending on the particular virus, temperature, pH, host cell, and multiplicity of infection (moi). The release of several picornaviruses occurs upon host cell lysis, however, the release of some picornaviruses (e.g. hepatitis A virus) occurs without cytopathic effect (Racaniello 2013).

2.5 The receptor tropism of CV-A9 and HPeV-1

Table 3 summarizes the known interactions of CV-A9 and HPeV-1 between integrins and other receptors. Receptor interactions have been analyzed using various methods and cell lines, and thus there are differences in conclusions between the studies.

Table 3: Receptors of CV-A9 and HPeV-1. *Detected interaction (+), no detected interaction (-).*

	CV-A9	HPeV-1	Reference	Method(s) used
αVβ1	+	+	Pulli et al. 1997	Phage display library, blocking assay
		+	Triantafilou et al. 2000a	Blocking and binding assays
	-		Triantafilou et al. 2000c	Blocking and binding assays
		-	Joki-Korpela et al. 2001	Blocking assay, co-localization
αVβ3	+		Roivainen et al. 1994	Blocking and binding assays
	+	-	Pulli et al. 1997	Blocking and binding assays
	+		Triantafilou et al. 1999	Plaque titration, binding assay, flow cytometry analysis
		+	Triantafilou et al. 2000a	Blocking assay
	+		Triantafilou et al. 2000c	Blocking and binding assays
		+	Joki-Korpela et al. 2001	Blocking assay, co-localization
	+		Triantafilou et al. 2001	Flow cytometry analysis
	+		Triantafilou et al. 2002	Immunoprecipitation, FRET analysis, blocking and binding assays
	+		Triantafilou et al. 2003	FRET analysis
		+	Seitsonen et al. 2010	Blocking and binding assays, neutralization assay, cryo-EM
αVβ6	+		Williams et al. 2004	Blocking and binding assays, neutralization assay
	+		Heikkilä et al. 2009	Antibody blocking, binding assay, neutralization assay
		+	Seitsonen et al. 2010	Binding studies, blocking assays, neutralization assays, cryo-EM
	+		Shakeel et al. 2013	Cryo-EM
αVβ5	-		Pulli et al. 1997	Blocking assay
		-	Triantafilou et al. 2000a	Blocking assay
α5β1	-	-	Pulli et al. 1997	Phage display library, blocking assay
α2β1		-	Triantafilou et al. 2000a	Blocking and binding assays
	-		Triantafilou et al. 2001	Flow cytometry analysis
		-	Joki-Korpela et al. 2001	Blocking assay, co-localization

	CV-A9	HPeV-1	Reference	Method(s) used
β2M	+		Triantafilou et al. 1999	Binding assay
	+	-	Joki-Korpela et al. 2001	Blocking assay
		-	Boonyakiat et al. 2001	Blocking assay
	+		Heikkilä et al. 2010	siRNA assay, confocal imaging
HS		-	Boonyakiat et al. 2001	Plaque titration, binding assay
	+/-		McLeish et al. 2012	Plaque titration, mutation, binding assay
HSPA5	+		Triantafilou et al. 2000b	Immunoprecipitation
	+		Triantafilou et al. 2002	Immunoprecipitation, FRET analysis, blocking and binding assays
	+		Triantafilou et al. 2003	FRET analysis
RGD-dependent		+	Stanway et al. 1994	Blocking and binding assays
		+	Boonyakiat et al. 2001	RGD-mutation
RGD-independent	+		Roivainen et al. 1991	Trypsin-cleavage of RGD, RGD blocking assay
	+		Hughes et al. 1995	RGD-mutation
	+		Roivainen et al. 1996	Trypsin-cleavage of RGD
	+		Triantafilou et al. 2000c	RGD-mutation

As Table 3 indicates, several studies concerning the receptor usage of CV-A9 and HPeV-1 exist. There has been the relative consensus and many studies concerning of utilization of integrins in CV-A9 infection. However, in addition to αV integrins, CV-A9 also interacts with β2M and the role of the interaction has been shown to occur at the post-attachment stage and in connection with integrins (Triantafilou et al. 1999, Joki-Korpela et al. 2001, Heikkila et al. 2010). Heat shock protein family a member 5 (HSPA5, also referred to as binding immunoglobulin protein, BiP, or glucose-regulated protein of 78 kDa, GRP78) is also involved in CV-A9 infection (Triantafilou et al. 2000b, Triantafilou et al. 2002, Triantafilou and Triantafilou 2003), but like with β2M, it's function as receptor is linked to integrins. HSPA5 is traditionally regarded as a major endoplasmic reticulum (ER) chaperone, which has many critical roles in protein processing, ER stress signaling, as well as in features of tumor progression (Ni et al. 2011). HSPA5 also has a role also in EV-71 infection, but rather in virus replication than as a receptor (Jheng et al. 2016). In addition, heparan sulfate (HS) has also recently been shown to mediate CV-A9 infection (McLeish et al. 2012). McLeish et al. proposed that there is a special HS-binding site in CV-A9 VP1 protein (VP1-T132R), which mediates binding of some CV-A9 isolates to HS. However, according to literature HS does not associate with HPeV-1 infection (Boonyakiat et al. 2001). HPeV-1 entry is undisputedly RGD-dependent (Stanway et al.

1994, Boonyakiat et al. 2001), but the exact role of the RGD motif in CV-A9 entry is unclear (Roivainen et al. 1991, Hughes et al. 1995, Berinstein et al. 1995, Roivainen et al. 1996, Triantafilou et al. 2000c). αVβ1, αVβ3 and αVβ6 integrins have all been suggested to mediate HPeV-1 infection (Roivainen et al. 1996, Pulli et al. 1997, Triantafilou et al. 2000a, Joki-Korpela et al. 2001, Seitsonen et al. 2010). However, a few studies have not supported the roles of αVβ1 and αVβ3 in HPeV-1 infection (Pulli et al. 1997, Joki-Korpela et al. 2001), Triantafilou et al. concluded that HPeV-1 favors αVβ3 over αVβ1 integrin (Triantafilou et al. 2000a) and Seitsonen et al. stated that HPeV-1 binds more efficiently to αVβ6 than to αVβ3 (Seitsonen et al. 2010).

3 AIMS OF THE STUDY

CV-A9 and HPeV-1 belong to a small group of picornaviruses, which harbor the RDG motif in their capsid. The RGD motif mediates interactions with integrins, and the previous receptor studies of CV-A9 and HPeV-1 have mainly focused on integrins. As detailed above, the number of studies is extensive, but with conflicting results. Most studies rely on blocking and binding assays with results verified using plaque titrations. Nowadays with modern equipment and techniques, the research is more definite and sensitive providing new aspects for the virus-receptor interaction studies. All these facts point to a demand for an updated view on the receptor tropism of CV-A9 and HPeV-1.

The aim of this thesis was to elucidate the receptors of CV-A9 and HPeV-1 in different cell lines to obtain a clear and precise view on the virus-cell interactions.

The specific aims of this thesis were:

1. To elucidate the integrin-independent receptor interactions of CV-A9
2. To determine the primary integrin receptor of HPeV-1
3. To determine the role of HS in CV-A9 and HPeV-1 infection.

4 MATERIALS AND METHODS

4.1 Cell lines and cultures (I-III)

The human epithelial lung carcinoma (A549; I, II, III) and human cervical cancer (HeLa-Ohio; II) cells were from the ATCC (American Type Culture Collection). Human colorectal adenocarcinoma (SW480; I-II) cell lines were from ATCC and Dr. Stephen Nishimura (UCSF, USA). The β1 knock-out cell line, GE11-KO, and its derivative β1 overexpressing cell line, GE11-β1 (GE11-β1A) (II), were kind gifts from Arnoud Sonnenberg (The Netherlands Cancer Institute, The Netherlands) (Gimond et al. 1999). Propagation of A549, HeLa-Ohio, GE11-KO and GE11-β1 cells was with in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal calf serum (FCS) and gentamicin. Propagation of SW480 cells was with either Ham's F12 (I) or DMEM (II) containing 10% FCS and gentamicin. Culture medium supplemented with 1% FCS was used for virus infections. DMEM supplemented with 1 mM $MgCl_2$ (I, II) was used in experiments with antibodies.

4.2 Viruses and virus purification (I-III)

Wild-type HPeV-1 (Harris strain; II, III), wild-type CV-A9 (Griggs strain; I-III) and CV-A9 RGD-mutant CV-A9 RGDdel (I) were from laboratory collections (Chang et al. 1989, Hughes et al. 1995, Hendry et al. 1999, Joki-Korpela et al. 2001). Clinical CV-A9 isolates (III) were collected between 1959-2008 in Finland, the Netherlands and the United States of America. Clinical HPeV-1 samples (II, III) were from collections of Dr. Katja Wolthers (Academic Medical Center, The Netherlands) and Dr. Sisko Tauriainen (University of Turku, Finland). Viruses (HPeV-1 Harris, CV-A9 Griggs, and CV-A9 RGD-mutant) propagated in A549 were purified by sucrose gradient ultracentrifugation as described (Abraham and Colonno 1984). Once passaged clinical virus isolates were used for VP1 sequencing and subsequent in experimental work.

4.3 Proteins, antibodies and cellular markers (I-III)

Polyclonal rabbit antisera against CV-A9 and HPeV-1 were from laboratory collections (Chang et al. 1989, Pulli et al. 1998, Joki-Korpela et al. 2001). The primary monoclonal antibodies specific to different integrins were against β1 (sc-53711, Santa Cruz, and Mab 2253, Millipore), αV (L230, ATCC), α5β1 (Mab 1969 and Mab 1999, Millipore), αVβ3 (Mab 1976 and Mab 1976z, Millipore), αVβ5 (Mab 1961z, Millipore), and αVβ6 (Mab

2077z, Millipore). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was from Sigma. Fluorescence conjugated antibodies against β1 (303015) and αV (327907) integrins were from BioLegend. The antibody to β2-microglobulin (sc-51509) and rabbit antibody to HSPA5 protein (sc-13968) were from Santa Cruz. Alexa Fluor (AF) 488-, 546-, 568- and 633-labeled anti-mouse and anti-rabbit secondary antibodies and AF 568-labeled phalloidin were from Life Technologies. Nuclei were stained with DAPI (Life Technologies) or Hoechst 33342 (Sigma-Aldrich). The horseradish peroxidase (HRP)-labelled anti-rabbit secondary antibody was from Pierce. Purified integrin αVβ3 was from BioMarket Ltd. (catalog item 01-INT-4). Integrin α5β1 and β2-microglobulin were from Millipore (catalog items CC1052 and CBL62020, respectively). Integrin αVβ6 was produced and purified in Chinese hamster ovary (CHO) cells as described (Weinacker et al. 1994).

4.4 Quantitation of integrin expression in A549 and SW480 cell lines with RT-qPCR (I)

Measurement of total mRNA levels of integrin subunits β3, β6, and β1 was by quantitative reverse transcription-PCR (RT-qPCR) (Heikkila et al. 2010).

4.5 Phage display screening (I)

Phage display screening with a peptide library displaying CX8C decapeptides (where X is any amino acid) was done as previously (Koivunen et al. 1999a, Koivunen et al. 1999b). Purified CV-A9 (50 μg ml^{-1} in PBS containing 0.5 mM $MgCl_2$) was coated on Nunc Maxisorp 96-well plates. Bound phages eluted from the wells were amplified in K91kan *Escherichia coli*. Two consecutive panning rounds were performed. Individual cell clones were colony sequenced, i.e. the cell mass was directly used as the template for PCR. PCR was done with primers flanking the peptide insertion site of the phage: forward primer 5′-TAA TAC GAC TCA CTA TAG GGC AAG CTG ATT AAC CGA TAC AAT-3′ and reverse primer 5′-CCC TCA TAG TTA GCG TAA CGA TCT-3′. PCR amplicons were sequenced using forward primer, and sequences translated to amino acids compared against SwissProt database using the FASTA program (Pearson and Lipman 1988).

4.6 Sequence analysis of CV-A9 clinical isolates (III)

The putative HS binding site was identified and sequenced from 54 clinical CV-A9 isolates. The representative regions were aligned by Clustal W (Thompson et al. 1994),

translated and trimmed by SeaView (Gouy et al. 2010) and visualized by GeneDoc (Nicholas et al. 1997) programs.

4.7 Virus internalization and infectivity assays (I-III)

4.7.1 Infectivity assays (I-III)

When analyzing the efficiency of virus infection, cells cultivated (A549, HeLa or SW480) on 96-well plates (Perkin Elmer) were used for fluorescence microscopy and Victor^3 multilabel counter (Perkin Elmer). After overlaying the cells with virus suspension, the cells were incubated 1 h on ice, unbound viruses removed, pre-warmed infection medium added, and cells incubated at 37°C and 5% CO_2. At 6 h post infection, the cells were washed with phosphate buffered saline (PBS), fixed (15 minutes with 4% formaldehyde in PBS), permeabilized (10 minutes with Triton-X100 in PBS), and stained as described below (4.7.2).

4.7.2 Staining of microscopical samples (I-III)

Fixed and permeabilized cells in 96-well plates were stained with virus-specific antiserum. Cells incubated for 1 h with virus-specific antiserum diluted in PBS with 3% bovine serum albumin (BSA) were washed with PBS, followed by 1.5 h incubation with AF-488-labeled secondary antibody. The nuclei were visualized with Hoechst or DAPI and infection efficiency was visualized by fluorescence microscope using Zeiss Axiovert 200M (10 or 20 × objective) (I-III) or Victor^3 multilabel counter (Perkin Elmer) (I). When staining confocal coverlips, the fixed and permeabilized or non-permeabilized cells were incubated for 1 h with primary antibodies, washed with PBS, incubated 30 min with secondary antibodies and washed again with PBS. Nuclei was stained with Hoechst 33342, and mounted with Mowiol 4-99 (Calbiochem-Novabiochem) containing Dabco (Sigma-Aldrich) (I) or with Prolong Gold Antifade Reagent containing DAPI (Life Technologies) (II). Samples were examined with a Zeiss LSM510 META (I) or Zeiss LSM780 (II) confocal microscope.

4.7.3 BioImageXD analysis (I-III)

The infection efficiency was determined with BioImageXD software (Kankaanpaa et al. 2012). Image datasets taken with Zeiss Axiovert 200M were used for quantification. The total cell count (blue channel, nuclei) and the number of infected cells (green channel, HPeV-1) were determined separately, after which the infection percentage was calculated manually. The system was set up with control cells, and image threshold was set to make all cells visible based on staining of nuclei (blue channel). The number of objects was then automatically calculated. The analysis was performed in the same way for the infected cells except green channel was used indicating only the cells positive for virus staining.

4.8 Flow cytometry (I, II)

Monoclonal antibodies were used to detect αV, β1, αVβ3, αVβ6 and α5β1 integrins on A549, HeLa and SW480 cell lines, and the expression level of β1 integrin was determined on GE11-KO and GE11-β1 cells. The trypsin-detached cells were suspended in the buffer solution (PBS containing 0.5% BSA), which was used throughout the experiment. The receptor-specific primary antibodies were added into the buffer and incubated at 4°C. The cell pellets were washed and incubated with the secondary AF-labelled antibodies at 4°C, and after washing steps, the cell pellets were suspended in the buffer. Flow cytometry was done with FACS Calibur flow cytometer (Becton Dickinson) and in total 10 000 to 20 000 cells were analyzed in each experiment. The results were processed with a flow cytometry data analysis software (Flowing Software, www.flowingsoftware.com).

4.9 Blocking, activating, binding and inhibition assays (I-III)

4.9.1 Peptide blocking assay (I)

Synthetic ESPLSLVA and RRRGEL peptides were diluted into PBS with 1 mM $MgCl_2$. The RRRGEL-peptide was used as a negative control because it does not block CV-A9 infection (Stanway et al. 1994). CV-A9 was incubated 30 min with either ESPLSLVA or RRRGEL peptide (5 mM), the mix was put onto cells followed by 30 min incubation on ice. After incubation, infectivity assay was done as described above (4.7.1 and 4.7.2), the infection efficiency was determined with a Victor^3 multilabel counter (Perkin Elmer).

4.9.2 Antibody blocking (I, II)

Function-blocking antibodies were 15 µg/ml diluted in serum free DMEM with 1 mM $MgCl_2$, and 100 µl of antibody dilution per well was applied onto confluent A549 or SW480 cells in 96-well plates followed by 1 h incubation at RT with gentle shaking. Unbound antibodies removed by PBS washes and the cells were infected and stained as described above (4.7.1 and 4.7.2). Infection efficiency after antibody blocking was detected with a Victor^3 multilabel counter (I) or Zeiss Axiovert 200M microscope (II).

4.9.3 In vitro binding assay (I, II)

A 96-well plate (Costar High Binding or Corning Incorporated) was coated with 300 ng integrins or BSA (a negative binding control) in coating buffer (PBS with 1 mM $MgCl_2$). After coating, the wells were washed with PBS and blocked with BSA. Following the addition of the virus (0 to 200 ng in (I) and 200 ng in (II)), the plate was incubated at RT, unabsorbed virus removed, and virus-specific antibody added after washes. Following 1 h incubation at RT and washing, the wells were incubated 45 min at RT with the secondary antibody (anti-rabbit horseradish peroxidase conjugated antibody (HRP), Pierce). The staining was with TMB substrate and the absorbance was read at 450 nm using Victor^3 multilabel counter.

4.9.4 Activation of integrins (II)

SW480 cells on 96-well plates were treated 1 h at 37°C with β1 integrin activating antibody (TS2/16) and 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) at different concentrations (5 µg, 20 µg, or 50 µg /ml TS2/16, 10 nM or 100 nM TPA), followed by HPeV-1 infection and virus staining as described above (4.7.1 and 4.7.2). The samples were analyzed with a Zeiss Axiovert 200M microscope.

4.9.5 Treatments related to heparan sulfate (HS) (III)

Incorporation of sulfates into the proteoglycans was inhibited with sodium chlorate, $NaClO_3$ (Sigma-Aldrich). A549 cells grown on 96-well plates were incubated 72 h prior to the infectivity assay in plain DMEM or DMEM supplemented with 50 mM $NaClO_3$. Cell surface proteoglycans were digested by heparinase I (Sigma-Aldrich). The cells were incubated with 0.5, 2.5 or 5.0 U/ml of heparinase I in serum-free DMEM for 2 h at 37ºC

prior to the infectivity assay. Masking of the cell surface HS core was achieved using protamine sulfate (Sigma-Aldrich). The cells were incubated with 0.5 and 2.0 mg/ml protamine sulfate for 2 h at 37ºC prior to the infectivity assay. Soluble low molecular-weight heparin (Sigma-Aldrich) was used to saturate HS binding sites of the virion. Virus was incubated with 0.5 and 3.0 mg/ml heparin for 2 h prior to inoculation onto cells. After these treatments, the virus infectivity assays and staining were done as described above (4.7.1 and 4.7.2). Infection efficiency was determined with a Zeiss Axiovert 200M.

4.10 siRNA methods (I, III)

All small inhibitory RNAs (siRNAs) were from Qiagen (**Table 4**). Control cells were transfected with AllStars Negative control siRNA (Qiagen). Transfections of SW480 (I) and A549 (III) were done on 96-well plates. Briefly, 0.5 or 1.0 pmol of siRNA in 25 µl of H_2O was mixed with 0.2 or 0.4 µl of siLentFect (Bio-Rad) diluted in 25 µl of serum free medium followed by 30 min incubation at RT. The siRNA complexes were mixed with 25,000 to 30,000 cells in serum-supplemented medium, and cultured at 37°C for 48 h prior to the infectivity assay and staining done as described above (4.7.1 and 4.7.2). The infection efficiencies were measured with Victor^3 multilabel counter (I) or Zeiss Axiovert 200M (III). The transfection conditions were optimized by transfecting the cells with siRNA targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and measuring the GAPDH enzyme activity with a KDalert GAPDH Assay Kit (Applied Biosystems). The cell viability assay was performed with the dead-cell marker Sytox Orange nucleic acid stain (Molecular Probes) and Hoechst. Cells without CV-A9 served as negative control and positive controls included non-transfected, mock-transfected and scramble-transfected cells. The cells were incubated in a solution containing Sytox Orange (1.7 µM) and Hoechst (1 µg/ml) for 30 min at RT. The stain was removed, and fluorescence intensities were measured with a Victor^3 multilabel counter.

Table 4: siRNAs used in the study.

Gene	Protein, aliases	siRNA	Used in
	Negative control siRNA	AllStars	I, III
PVR	CD155, PVR	Hs_PVR_4 Hs_PVR_5	I
ICAM1	ICAM-1, Intracellular adhesion molecule-1, CD54	Hs_ICAM1_6 Hs_ICAM1_7	I
CXADR	CAR, Coxsackievirus-adenovirus receptor	Hs_CXADR_9 Hs_CXADR_11	I
HAVCR1	HAVcr-1; HAV cellular receptor	Hs_HAVCR1_8 Hs_HAVCR1_9	I
CD55	DAF, decay accelerating factor, CD55	Hs_CD55_2 Hs_DAF_3	I
ITGB1	Integrin β1	Hs_ITGB1_5 Hs_ITGB1_9	I
ITGB3	Integrin β3	Hs_ITGB3_1 Hs_ITGB3_5	I
ITGB6	Integrin β6	Hs_ITGB6_1 Hs_ITGB6_5	I
ITGB8	Integrin β8	Hs_ITGB8_5 Hs_ITGB8_6	I
ITGA2	Integrin α2	Hs_ITGA2_5 Hs_ITGA2_6	I
ITGA5	Integrin α5	Hs_ITGA5_5 Hs_ITGA5_7	I
LDLR	LDL-R, Low-density lipoprotein receptor	Hs_LDLR_3 Hs_LDLR_4	I
NANS	N-acetylneuraminic acid synthase (sialic acid synthase), SAS	Hs_NANS_2 Hs_NANS_5	I
EXT1	exostosin (multiple) 1; (an ER-resident type II transmembrane glycosyltransferase involved in the chain elongation step of heparan sulfate biosynthesis)	Hs_EXT1_1 Hs_EXT1_4	I, III I
B2M	β2-microglobulin	Hs_B2M_3 Hs_B2M_4	I

4.11 Confocal imaging (I-II)

4.11.1 Internalization and co-localization assays (I, II)

In all endocytosis assays, SW480 (I) and GE11 (II) cells were grown on coverslips in 24-well plates overnight at 37 °C after which the plate was transferred onto ice and cells were infected with CV-A9 (I-II) or HPeV-1 (prototype and clinical isolates) (II). The infections were followed up to 6 h at 37ºC, after which the cells were fixed and permeabilized. At 0-min time point, the cells were fixed without permeabilization. Samples were stained as described above (4.7.2). Samples were examined with a Zeiss LSM510 META confocal microscope using a Plan-Apochromat objective (63× oil) (I) or Zeiss LSM780 confocal microscope using Plan-Apochromat objectives (63× / 1.2 oil/water for HPeV-1 imaging, and 40×/ 1.2. oil/water for CV-A9 imaging) (II). Co-localization analyses (automatic thresholding after background subtraction, Costes P-value calculation with 100 iterations) of selected image stacks were performed with BioImageXD software.

4.11.2 Clustering assay (II)

SW480 cells were grown on coverslips in 24-well plates. The experimental procedure was performed as done earlier with E-1 (Upla et al. 2004, Jokinen et al. 2010). The samples included: 1) a negative control for clustering, where cells were incubated with primary β1 integrin antibody (TS2/16) only followed by fixing and staining with a secondary antibody and DAPI; 2) a positive control for clustering, where the cells were first incubated with β1 integrin antibody, after which the secondary antibody was added to induce β1 integrin clustering, followed by fixing and DAPI staining; and 3) a virus sample, where cells were incubated with HPeV-1 for 15 minutes followed by fixing and staining (anti-HPeV-1, anti-β1 integrin antibodies and DAPI for staining nuclei). The samples were examined with a Zeiss LSM780 confocal microscope using a Plan-Apochromat objective (63× / 1.2 oil/water).

5 RESULTS

Within the last decades, several studies have addressed the receptor usage of CV-A9 and HPeV-1. Despite the fact that almost 20 articles exist on this topic, the receptors required for successful infection remain unknown. HPeV-1 interacts with RGD-binding integrins αVβ1, αVβ3 and αVβ6, but the exact role of these receptor candidates is obscure. The receptor profile of CV-A9 is even more complex, since in addition to RGD-binding integrins, CV-A9 can infect cells RGD-independently via different receptor molecules, possibly involving β2M and HSPA5. The purpose of this work was to make a comprehensive study of receptor usage of HPeV-1, as well as to identify non-integrin receptors for CV-A9.

5.1 Independency of integrins in CV-A9 and HPeV-1 internalization (I)

5.1.1 Integrin-independent infection of CV-A9 (I)

To elucidate the role of integrins, cell lines with appropriate receptor expression profile were used. SW480 was chosen because it lacks the αVβ3 and αVβ6 integrins (Agrez et al. 1997, Williams et al. 2004, Berryman et al. 2005). The expression levels of β1, αVβ3 and αVβ6 integrins were determined with flow cytometry and RT-qPCR (**I / Fig. 1 A and B**), and the results confirmed that SW480 cells used expressed none or low levels of αVβ3 and αVβ6 integrins. In contrast to previous study by Williams et al. (2004), CV-A9 and CV-A9 RGDdel mutant infected SW480 cells efficiently (**I / Fig. 1 C**). To verify the independency of αV integrins in SW480 cells, an antibody-blocking assay was also performed. A549 cells were used as a control, because internalization of CV-A9 into A549 cells is integrin-dependent (Heikkila et al. 2009). αV and αVβ5 integrins were blocked with antibodies and as expected, αV integrin antibody blocked the CV-A9 infection in A549 but not in SW480 cells (**I / Fig. 2 B**). αVβ5 antibody did not block CV-A9 infection in SW480 cells (**I / Fig. 2 A**). The data suggest that CV-A9 infects SW480 cells independently of RGD/integrin interactions.

5.1.2 β2-microglobulin in CV-A9 and HPeV-1 infection (I)

An siRNA panel against known picornavirus receptors was developed to identify novel receptors that mediate CV-A9 infection in SW480 cells. The siRNA screen demonstrated that the most effective inhibition of the CV-A9 infection in SW480 cells occurred by β2-

microglobulin (β2M) siRNA (**I / Fig. 3 A**). This is in line with previous observations on A549 cells, wherein CV-A9 internalizes via β2M (Heikkila et al. 2010). To verify the role of β2M in CV-A9 infection, antibody-blocking experiment against β2M was performed in SW480 and A549 cell lines (**I / Fig. 3 C and Fig. S1**). The results indicate that reduction of CV-A9 infection by β2M blocking antibody is dose-dependent in both cell lines.

The role of β2M was also studied in HPeV-1 infection in SW480 cells. Previous studies (Joki-Korpela et al. 2001, Boonyakiat et al. 2001) propose that β2M does not have role in HPeV-1 infection. However, antibody blocking of β2M in SW480 cells reduced infectivity of HPeV-1 significantly (**Figure 11**), indicating that β2M may also play a role in HPeV-1 infection.

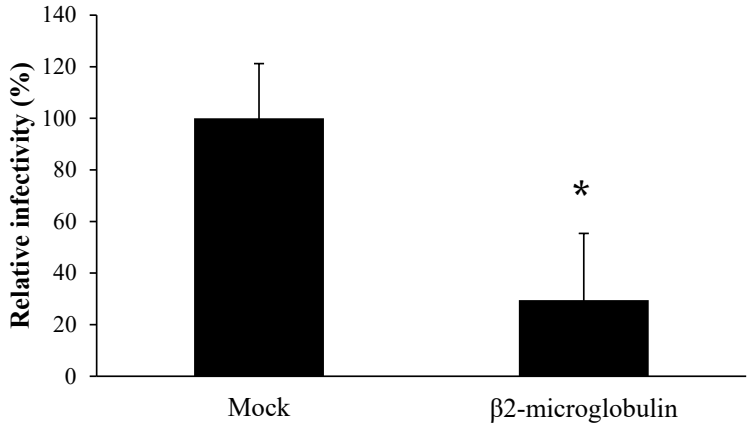

Figure 11: Effect of β2M blocking of HPeV-1 in SW480 cells. *SW480 cells were incubated with β2M blocking antibody prior HPeV-1 infection. Infection efficiency was analyzed with fluorescence microscopy after staining, and relative infectivities were calculated. Blocking of β2M with function-blocking antibody decreases HPeV-1 infection significantly. * indicates p<0,01.*

To further analyze the role of β2M in HPeV-1 infection, co-localization of β2M and HPeV-1 was studied in A549 cells. Heikkilä et al. (2010) showed that β2M and CV-A9 co-localize in A549 cells early in infection, therefore the early time point was used as a control. As shown in **Figure 12**, CV-A9 (upper panels) and β2M co-localize at 0 time point suggesting an interaction. The co-localization is more clearly demonstrated as yellow dots in the cropped image (right panel). This confirms the findings of Heikkilä et al. (2010), i.e. CV-A9 and β2M are closely associated at the early stage of infection in A549 cell line. Similar co-localization occurs with HPeV-1 (Figure 12, lower panels). These data together with the results of siRNA screen and antibody blocking experiment, suggest that β2M is essential for successful CV-A9 and HPeV-1 infection in both SW480 and A549 cell lines.

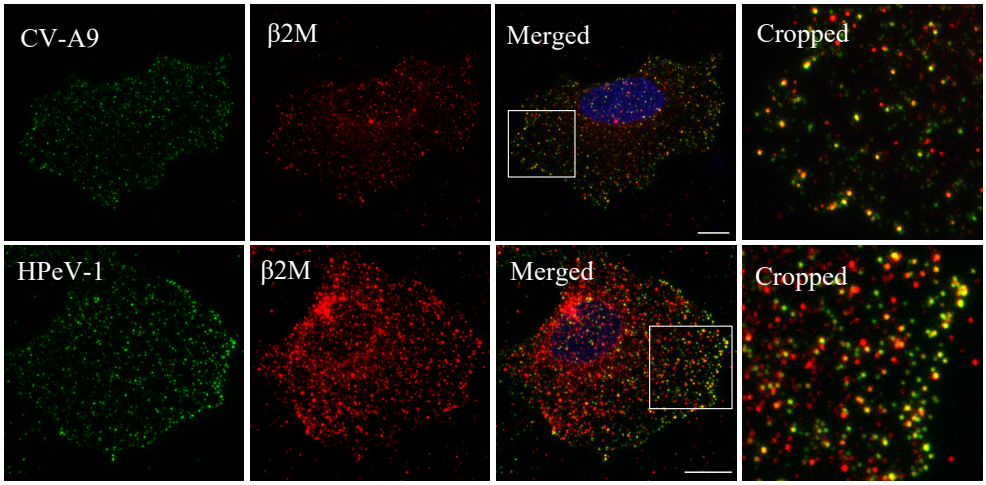

Figure 12: Co-localization of β2M with CV-A9 and HPeV-1 in A549 cells at 0 min time point. *A549 cells were inoculated with CV-A9 and HPeV-1, fixed, stained and imaged with confocal microscope. β2M (red) co-localizes with CV-A9 (upper panel, green) and HPeV-1 (lower panel, green), shown as yellow, in the early stage of infection. Non-permeabilized cells. Bar 10 µm.*

5.1.3 HSPA5 in CV-A9 and HPeV-1 infection (I)

Phage peptide library screening was used to look for the non-integrin receptor of CV-A9. One of the binding peptides, ESPLSLVA, partially aligned with the N-terminal sequence (LSLVA) of the HSPA5 protein. Previous studies have demonstrated interaction between CV-A9 and HSPA5 (Triantafilou et al. 2000b, Triantafilou et al. 2002, Triantafilou and Triantafilou 2003), in contrast, the role of HSPA5 in HPeV-1 infection is unknown. Using high concentration of ESPLSLVA peptide in blocking assay, CV-A9 infectivity decreased significantly in both SW480 and A549 cells, while control peptide did not affect infectivity (**I / Fig. 4 A and Fig. S2**). Using similar blocking assay (**Figure 13**) HPeV-1 infectivity in SW480 cells decreased by 40% and 25% in A549 cells. Although the p-values (SW480 0.08 and A549 0.32) were not statistically significant, these preliminary results suggest that HSPA5 may contribute to HPeV-1 infection. Further investigations are needed to elucidate the role of HSPA5 in HPeV-1 infection.

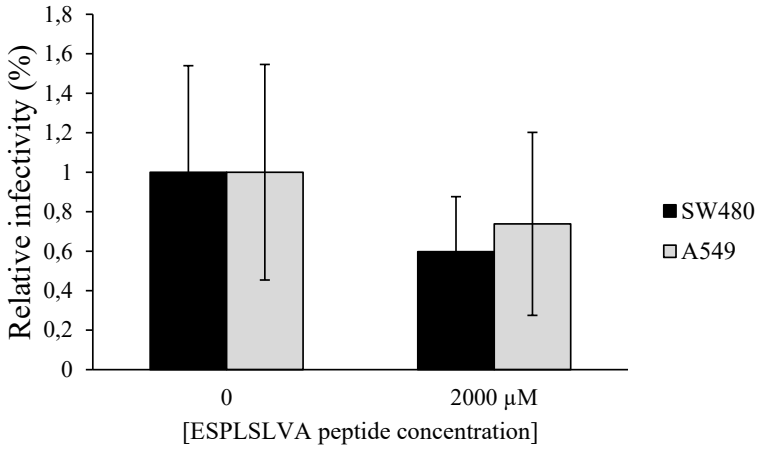

Figure 13: Effect of HSPA5 blocking in HPeV-1 infection in SW480 and A549 cells. *The cells were incubated with or without ESPLSLVA peptide before HPeV-1 infection. Infection efficiency was analyzed with fluorescence microscopy after staining, and relative infectivities were calculated. Blocking of HSPA5 with peptide decreased HPeV-1 infection, but not statistically significantly.*

To further verify the role of HSPA5 in CV-A9 infection, experiments using immunofluorescence confocal microscopy were performed. First, the localization of HSPA5 on the surface of SW480 cells was verified by immunostaining in the absence of virus in both permeabilized and non-permeabilized cells (**I / Fig. 4 B**). Next, confocal images show that HSPA5 co-localizes with CV-A9 during the attachment (at 0 min) and at the early stage of internalization (at 5 min) (**I / Fig. 4 C**). The results indicate that HSPA5 would act in both the attachment and early stages of CV-A9 infection in SW480 cells.

5.2 Role of integrins in CV-A9 and HPeV-1 infection (I, II)

5.2.1 Utilization of αVβ3 and αVβ6 integrins in HPeV-1 infection (II)

Three different cell lines, A549, SW480 and HeLa, were infected with HPeV-1 (**II / Fig. 1 A**). All cell lines were susceptible to HPeV-1 infection. To determine the receptor profile on the surface of the different cell lines, flow cytometry analysis was performed using antibodies against αV, β1, αVβ3, αVβ6 and α5β1 integrin (**I / Fig. 1 A and 2 C**, **II / Fig. 1 B**). Flow cytometry analysis demonstrated that all these cell lines express high amounts of αV, β1 and α5β1 integrins. However, neither HeLa nor SW480 cells expressed αVβ3 or αVβ6 integrins. The results obtained with these two cell lines suggest that neither αVβ3 nor αVβ6 can act as the primary receptor of HPeV-1.

5.2.2 Role of α5β1 integrin in CV-A9 and HPeV-1 infection (I, II)

To determine the role of α5β1 integrin in HPeV-1 and CV-A9 infection, antibody-blocking assays were performed with SW480 cells. Function-blocking antibody against α5β1 did not affect the infectivity of CV-A9 (**I / Fig. 2 A**) or HPeV-1 (**II / Fig. 2 A and B**). In addition, binding of α5β1 integrin to CV-A9 or HPeV-1 *in vitro* was not detected (**I / Fig. 2 D** / **Figure 14**). Together the results suggest that α5β1 integrin does not contribute to CV-A9 and HPeV-1 infection, which is in line with the previous study by Pulli et al. (1997).

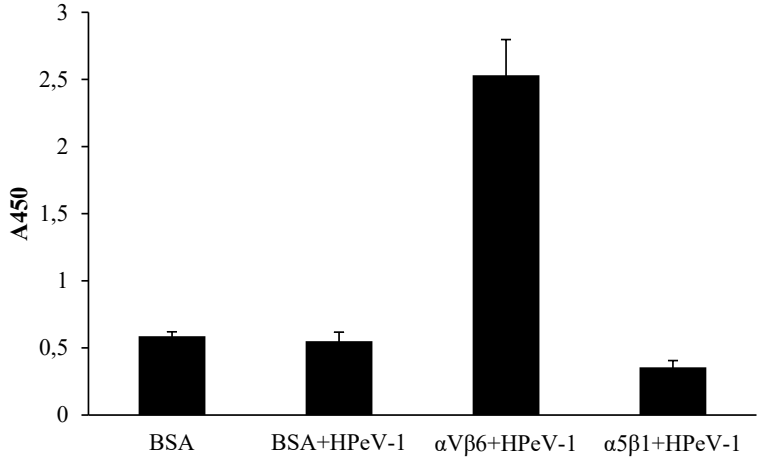

***Figure 14: Binding of HPeV-1 to α5β1 integrin in vitro**. In vitro integrin binding assay was performed using BSA as a negative control. αVβ6 integrin was used as a positive control, because Seitsonen et al. (2010) showed that HPeV-1 binds to αVβ6 in vitro. HPeV-1 does not bind to α5β1 integrin. Instead, HPeV-1 binds in vitro to αVβ6 integrin as expected.*

5.2.3 Function of αVβ1 integrin in HPeV-1 infection (II)

To analyze the involvement of αVβ1 integrin in HPeV-1 infection, antibody blocking assay on SW480 cells was performed by using αV and β1 integrin function-blocking antibodies separately and in combination (**II / Fig. 2 A and B**). It was shown that a combination of αV and β1 integrin antibodies most efficiently blocked HPeV-1 infection. This supported αVβ1 being the principal receptor in infectious entry of HPeV-1 in SW480 cell line.

The role of β1 integrin was further investigated utilizing a β1 integrin knock-out cell line (mouse epithelial cell line, GE11-KO) (Gimond et al. 1999). GE11-KO and its derivative, β1 integrin transfected GE11-β1, were infected with HPeV-1 and confocal microscopy

was performed at 6 h post infection (**II / Fig. 3 A**). Immunofluorescence staining (blue staining in **II / Fig. 3 A**) and flow cytometry analysis (**II / Fig 3 A**, right panel) were used to verify the β1 integrin expression profiles of both cell lines. It was shown that HPeV-1 enters the β1 integrin transfected but not the β1 integrin knock-out cells.

5.2.4 Co-localization of HPeV-1 with β1 integrin (II)

It was clearly shown with GE11 cells that β1 integrin is essential for HPeV-1 infection. To analyze the function of β1 integrin with HPeV-1, a co-localization assay was performed (**II / Fig. 5**). HPeV-1 co-localize significantly with β1 integrin in GE11-β1 cell line at different time points. This demonstrates that HPeV-1 is co-endocytosed with β1 integrin. The co-localization analysis was performed with BioimageXD, which further indicated that almost every virion interacts with β1 integrin between 0 to 30 min post infection, indicating that β1 integrin is required for HPeV-1 infection.

5.2.5 CV-A9 and clinical isolates of HPeV-1 in GE11 cells (II)

The prototype HPeV-1 (Harris strain) might not possess the characteristics of the recent HPeV-1 isolates, since it is cell culture-adapted. To rule out this possibility, three clinical HPeV-1 isolates were included into the receptor studies with GE11 cells (**II / Fig. 3 B**). The results show that clinical HPeV-1 isolates do not enter β1-deficient cell line, but the β1-expressing cell line is permissive for all HPeV-1 isolates. This indicates that both the HPeV-1 prototype and recent clinical HPeV-1 isolates utilize β1 integrin for their entry.

According to literature β1 integrin does not interact with CV-A9 (Triantafilou et al. 2000c) but it still may have a role in CV-A9 infection (Pulli et al. 1997). The GE11-KO cells serve an unambiguous tool to study the role of β1 integrin in virus entry, providing clear-cut results. The results show that CV-A9 can enter, and apparently, replicate in GE11-KO cells (**II / Fig. 3 C**) confirming that the entry of CV-A9 is β1 integrin independent.

5.2.6 Integrin activation in HPeV-1 infection (II)

Integrin activation is a controlled procedure by which the cell regulates ligand binding. Only few examples exist on the role of integrin activation in virus infection, these include E-1 (Jokinen et al. 2010) and adenovirus 5 (Ad5) (Davison et al. 1997). β1 integrin activating antibody, TS2/16, was used to investigate the role of integrin activation in HPeV-1 infection in SW480 cells (**II / Fig. 4 A and B**). As shown, the activation of β1 integrins

significantly increase (up to 2.5-fold) HPeV-1 infectivity. Another compound affecting integrin activation is 12-*O*-Tetradecanoylphorbol-13-acetate (TPA, also known as PMA). Cells were incubated with TPA, which increased HPeV-1 infectivity as compared to the mock-treated control cells (**Figure 15**). This result, together with the results of the previous experiments, strongly suggest that HPeV-1 favors activated integrins for infectious entry.

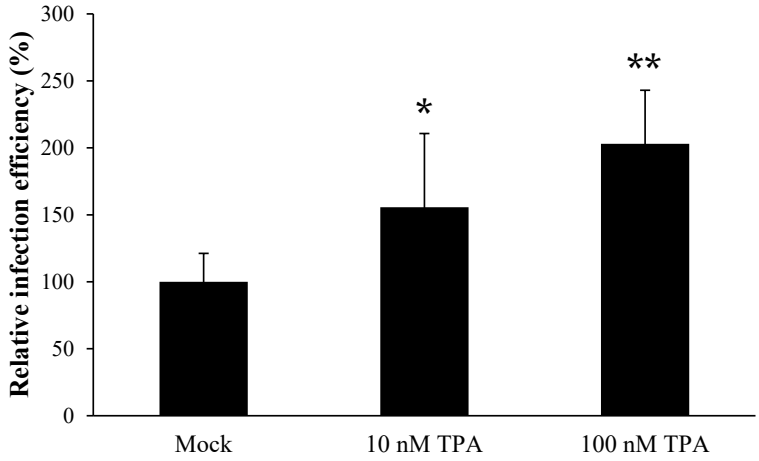

Figure 15: Effect of TPA in HPeV-1 infection in SW480 cells. *The cells were preincubated with different concentrations of TPA prior to HPeV-1 infection. Infection efficiencies were analyzed with fluorescence microscopy after staining, and relative infectivities were calculated. Incubation of the cells with TPA prior to HPeV-1 infection increased HPeV-1 infection significantly. * indicates p<0,01 and ** indicates p<0,001.*

5.2.7 Integrin clustering (II)

As with integrin activation, no studies exist on integrin clustering during HPeV-1 infection. Clustering assay in SW480 cells show that HPeV-1 does not induce clustering of β1 integrins (**II / Fig. 4 C**). Although macroclusters, which were described in e.g. E-1 infection (Jokinen et al. 2010, Upla et al. 2004), were not observed by HPeV-1, the presence of microclusters, which are not possible to visualize with the equipment used, cannot be excluded.

5.3 Utilization of heparan sulfate proteoglycans by CV-A9 and HPeV-1 (III)

5.3.1 Prevalence of putative HS-binding site in CV-A9 isolates (III)

McLeish et al. (2012) proposed the amino acid 132 of CV-A9 VP1 to mediate HS binding. They suggested that virus containing a threonine at this site (T132) does not bind heparin, but viruses with positively charged residue (T132R or T132K) interact with the negatively charged heparin. They also suggested that CV-A9 prototype strain (Griggs), which contains T at position 132, does not bind to heparin. However, sequence analysis of 54 clinical CV-A9 isolates revealed that the incidence of T132 mutation is rare in clinical isolates (**III / Fig. 3**). Only a single isolate contained T132R substitution and nine isolates presented T132K substitution, which questions the importance of this putative HS-binding site in the natural infection.

5.3.2 siRNA silencing of exostosin-1 in CV-A9 infection (III)

To investigate the role of HS in CV-A9 infection, A549 cells were used. Exostosin-1, which participates in the chain elongation step of HS biosynthesis, was silenced with siRNA (**III / Fig. 1 A and B**) resulting in almost 70% decrease in the CV-A9 infectivity. This suggests that interference with HS's biosynthetic pathway hampers CV-A9 infection.

5.3.3 Effect of treatments against HSPG in CV-A9 and HPeV-1 infections (III)

To investigate more closely the significance of HSPGs in CV-A9 and HPeV-1 infections, experiments with different strategies were performed in A549 cells.

5.3.3.1 Sodium chlorate and heparinase I with CV-A9 and HPeV-1 prototypes

siRNA silencing of exostosin-1 interferes with HS biosynthesis similarly to sodium chlorate ($NaClO_3$) (Humphries and Silbert 1988). Heparinase I enzymatically digests glycosidic linkages presented in the HSPG core. $NaClO_3$ and Heparinase I treated cells were infected with CV-A9 and HPeV-1 and visualized by immunofluorescence staining (**III / Fig. 2 A and B**). Both treatments affected CV-A9 infectivity substantially, and $NaClO_3$ decreased HPeV-1 infectivity substantially. However, heparinase I had a weaker effect

on HPeV-1 infectivity but still it inhibited significantly HPeV-1 infection at high concentrations.

5.3.3.2 Effect of heparin on infection by CV-A9 and HPeV-1 prototypes and clinical isolates

The finding that $NaClO_3$ and heparinase I treatments inhibited CV-A9 and HPeV-1 infection suggested that, in contrast to previous studies (Boonyakiat et al. 2001, McLeish et al. 2012), they interact with HSPGs. To further study the role of HSPGs in CV-A9 and HPeV-1 infection, their ability to bind heparin was examined. In addition to the prototype viruses (CV-A9 Griggs and HPeV-1 Harris), several clinical isolates were analyzed, including four CV-A9-T132, four CV-A9-T132K/R isolates, and six HPeV-1 isolates. Viruses were incubated with heparin prior to inoculation, and recorded the results using immunofluorescence microscopy (**III / Fig. 4**). CV-A9 Griggs infectivity was reduced up to 25%, but heparin treatment did not reduce the infectivity of other T132 isolates. Instead, the infection efficiency was increased (**III / Fig. 4 A and B**). However, heparin almost completely blocked the infectivity of CV-A9-T132K/R isolates, which is in line with results of McLeish et al. (2012), providing further support on T132K/R being the heparin-binding site.

The effect of heparin on HPeV-1 infection varied (**III / Fig. 4 C and D**). Infectivity of most HPeV-1 isolates, including Harris strain, decreased after heparin treatment. In fact, the infectivity of only isolate, 550163, remained unaltered. In contrast to CV-A9, the treatment did not increase the infectivity. These data suggest that HPeV-1 capsid protein may contain a specific heparin-binding site similarly to CV-A9.

5.3.3.3 Role of protamine in infection by CV-A9 and HPeV-1 prototypes and clinical isolates

Protamine sulfate (PS) is a drug that reverses the anticoagulant effects of heparin by binding to it, and it antagonizes protein interactions with heparin and HS. A549 cells were preincubated with protamine sulfate prior to virus infection, and nine CV-A9 isolates and seven HPeV-1 isolates were used as in the heparin assay. At 2 mg/ml concentration, protamine sulfate decreased the infectivity of all 16 viruses irrespective of T132 site (**III / Fig. 5**). This suggests that HSPGs have a role in both CV-A9 and HPeV-1 infections, but their action is CV-A9-VP1-T132 independent.

6 DISCUSSION

The aims of this thesis were to analyze the receptor tropism of two RGD-containing picornaviruses, CV-A9 and HPeV-1. Besides αVβ3 and αVβ6 integrins, previously suggested act as CV-A9 receptors, the virus can infect integrin-independently. CV-A9 utilizes HSPA5 and β2M and interacts with HS. In contrast, HPeV-1 infection is dependent of αVβ1 integrin, but β2M and HS also possess a role in HPeV-1 infection.

6.1 Integrin-independent infection mechanisms of CV-A9 and HPeV-1

Previous studies concerning picornaviruses containing the RGD motif have focused on binding to integrins. CV-A9 and HPeV-1 both contain the RGD motif, but their receptor tropism is rather different. CV-A9 can infect some cell lines RGD-independently (Roivainen et al. 1991, Hughes et al. 1995, Roivainen et al. 1996, Triantafilou et al. 2000c), and CV-A9 utilizes other receptors in cell binding and internalization. Although CV-A9 can infect cells independent of the RGD motif (Hughes et al. 1995), the motif is conserved among isolates and no RGDdel mutants exist in the nature (Santti et al. 2000). This suggests that integrin(s) have a role at least in some stages of infection. A recent structural study suggested that αVβ6 integrin would not induce uncoating and release of CV-A9 RNA during entry (Shakeel et al. 2013), suggesting that other molecules would mediate CV-A9 internalization and entry. HSPA5, β2M, Arf6 and HS are proposed to participate internalization (Triantafilou et al. 1999, Triantafilou et al. 2000b, Triantafilou et al. 2002, Triantafilou and Triantafilou 2003, Heikkila et al. 2010, McLeish et al. 2012), but all of them have been studied mainly in combination with integrins. In all, the evidence favors CV-A9 infection to be αVβ6 integrin dependent (Williams et al. 2004). HPeV-1 infection, instead, is RGD-dependent (Stanway et al. 1994, Boonyakiat et al. 2001), but non-RGD HPeV-1 strains do exist (Benschop et al. 2008, Westerhuis 2014). Primary receptor candidates for HPeV-1 include only several RGD-binding integrins (Pulli et al. 1997, Triantafilou et al. 2000a, Joki-Korpela et al. 2001, Seitsonen et al. 2010).

This study reveals that CV-A9 can infect human colon adenocarcinoma cell line (SW480) independently of RGD and αV integrins. SW480 does not express αVβ3 and αVβ6 integrins, but CV-A9 and CV-A9 RGDdel viruses were able to infect the cells efficiently. Blocking the αV did not affect CV-A9 infectivity in SW480 cells, supporting the independency of infection on αV integrins. To find other potential receptors, siRNA blocking and peptide phage library screening were performed. These revealed two possible receptor candidates, β2M and HSPA5. Triantafilou et al. (1999) and Heikkilä et al. (2010) showed that β2M plays a role in the CV-A9 infection, in both CHO and A549 cells. Triantafilou et al. (2000b, 2002 and 2003) studied the role of HSPA5 in CV-A9 infection in

GMK cell line. However, all these previous results of β2M and HSPA5 have been linked to function of integrins as the primary receptor. Based on the above results, antibody and peptide blocking assays were conducted. Blocking of β2M in SW480 and A549 cells resulted in a dose-dependent reduction of CV-A9 infectivity. This shows that β2M has a significant role in CV-A9 infection in many cells, while integrins function as receptors only in some cells lines. Peptide blocking assay with peptide binder (ESPLSLVA), which aligned with the N-terminus of HSPA5, decreased CV-A9 infectivity in both SW480 and A549 cells. Furthermore, HSPA5 co-localized with CV-A9 in SW480 during the attachment and at the early stages of infection, supporting the findings of Triantafilou et al. (2002). They concluded that HSPA5 acts as a co-receptor for CV-A9 together with integrins, but these results show that HSPA5 can act independent of integrins to mediate CV-A9 infection in the cell lines tested. However, the role of HSPA5 as natural CV-A9 receptor may be shadowed by the fact that HSPA5 is present on the surface of cancer cells but not in normal cells *in vivo* (Ni et al. 2011).

Boonyakiat et al. (2001) and Joki-Korpela et al. (2001) have questioned the role of β2M in HPeV-1 infection in RD and A549 cells, respectively. However, blocking of β2M by antibodies in SW480 cells efficiently decreased HPeV-1 infectivity. HPeV-1 also co-localizes with β2M in A549 cells, the same cells that Joki-Korpela et al. (2001) used, with a similar pattern as seen with CV-A9. These results suggest that in contrast to previous findings, β2M does have a role in HPeV-1 infection. β2M may act as a co-receptor or accessory molecule supporting the infection, possibly via interaction with integrins and acting in the post-attachment stage. The role of the other non-integrin receptor molecule, HSPA5, was studied in HPeV-1 infection with a peptide-blocking assay. No previous studies on HSPA5 and HPeV-1 exist, but our preliminary results suggest that HSPA5 plays a role in HPeV-1 infection.

6.2 β1 integrin in CV-A9 and HPeV-1 infection

The literature of CV-A9 and β1 integrin is relatively limited, and somewhat contradictory. Pulli et al. (1997) deduced that αVβ1 may have a role in CV-A9 infection, whereas Triantafilou et al. (2000c) stated that αVβ1 integrin does not participate in CV-A9 infection. α5β1 integrin has been shown not to affect CV-A9 or HPeV-1 infection (Pulli et al. 1997). HPeV-1 interacts with αVβ1 integrin in cell entry (Pulli et al. 1997, Joki-Korpela et al. 2001), but it has been proposed to act as a secondary receptor used only in the absence of αVβ3 integrin (Triantafilou et al. 2000a). In addition to αVβ3 integrin, also αVβ6 integrin has been suggested to act as HPeV-1 receptor (Seitsonen et al. 2010). With the exception of the paper by Seitsonen et al. (2010), all studies concerning HPeV-1 receptors are over 15 years old. An update on HPeV-1 receptor interactions/usage was therefore needed.

Three different cell lines, SW480, A549, and HeLa, were infected with HPeV-1 and results show, that all cell lines were susceptible to HPeV-1 infection. Flow cytometry analysis revealed that in addition to SW480, also HeLa cells were devoid of αVβ3 or αVβ6 integrins confirming that these molecules are not the sole receptors for HPeV-1. To determine the role of α5β1 integrin in CV-A9 and HPeV-1 infection, an antibody blocking and *in vitro* binding assays were performed. Function-blocking antibody against α5β1 integrin did not have an effect on CV-A9 or HPeV-1 infections, and CV-A9 and HPeV-1 did not bind to α5β1 integrin *in vitro*. These results support the view by Pulli et al. (1997) that α5β1 integrin does not participate in CV-A9 and HPeV-1 infections.

After excluding αVβ3, αVβ6, α5β1 in HPeV-1 infection, the role of αVβ1 integrin was further examined. αV and β1 integrin blocking antibodies separately and together blocked HPeV-1 infection efficiently in SW480 cells, suggesting αVβ1 to have a role in HPeV-1 infection. β1 integrin knock-out, mouse epithelial cell line GE11-KO, as well as its derivative, a β1-transfected GE11 cell line, GE11-β1 (Gimond et al. 1999) were used to verify the role of β1 integrin in HPeV-1 infection. The cells were inoculated with HPeV-1 and imaged with confocal microscope at 6 h post infection. HPeV-1 internalized into GE11-β1 but not into GE11-KO cell cells. Cell culture adapted HPeV-1 prototype and more recent clinical isolates produced the same result. Prototype viruses may be cell culture adapted, and thus not reflect the infections observed in ”real life”. The use of clinical low-passage isolates thus provided the natural aspect to the study, and the results may thus be generalized. In conclusion, all these results together confirmed that αVβ1 integrin is essential for HPeV-1 entry.

To verify that β1 integrin has no functional role in CV-A9 infection, GE11 cells were infected with CV-A9. The virus internalized, and was able to replicate in both cell lines confirming independency of β1 integrin. CV-A9 is able to replicate in mouse cells (Lerner et al. 1962, Harvala et al. 2003), however, HPeV-1 does not replicate in GE11-β1 cells, which may indicate that HPeV-1 cannot infect mice. This could further indicate that experimental infection in mice is not possible with HPeV-1.

Previously Joki-Korpela et al. (2001) studied HPeV-1 and β1 integrin interaction using confocal microscopy, but did not detect significant co-localization. In contrast, clear co-localization between HPeV-1 and β1 was obtained in the early stages of internalization in GE11-β1 cells. The use of a modern microscope and antibodies that are more specific could explain the discrepancy, as well as utilization of different cell lines. The data suggest that the virus is internalized together with β1 integrin from the plasma membrane into the cytoplasm. This is the first time when HPeV-1 receptor interactions are detected visually.

Virus attachment to inactive or active conformation of β1 integrins has been studied earlier with E-1 (Jokinen et al. 2010) and Ad5 (Davison et al. 1997). Ad5 attaches to the active conformation of α5β1 integrin, but E-1 attaches to α2β1 integrin in its inactive

conformation (Davison et al. 1997, Jokinen et al. 2010). Davison et al. (1997) used β1 integrin activating antibody, TS2/16, to demonstrate the effect of β1 integrin activation to infection efficiency, the same antibody was used in this study. Incubation of SW480 cells with TS2/16 prior to infection increased HPeV-1 infectivity to 2.5-fold with higher antibody concentrations. In addition, another integrin activating compound, TPA, which increased HPeV-1 infection in SW480 cells significantly, was used. TPA is usually linked to protein kinase C (PKC) activation, which in turn induces ligand-independent clustering of integrins, as well as an activating conformational change in integrins (Connors et al. 2007, Jokinen et al. 2010). To exclude the possible inducing effect of integrin clustering in HPeV-1 infection, a clustering assay was performed. Integrin clustering assay showed that attachment of HPeV-1 to β1 integrin in SW480 cells did not induce formation of visible clusters. This suggests that the inducing effect of HPeV-1 infection caused by TPA treatment was not the result of TPA-induced integrin clustering but integrin activation, and integrin clustering is not essential for HPeV-1 endocytosis. Based on these results, it is likely that HPeV-1 co-internalizes into the cell with active αVβ1 integrin using the normal recycling route of β1 integrin. Active β1 integrins are recycled from the plasma membrane in a clathrin-dependent manner and transported to early endosomes (Arjonen et al. 2012, Alanko 2016), which is compatible with the previous studies of HPeV-1 endocytosis (Joki-Korpela et al. 2001).

In conclusion, HPeV-1 internalizes via αVβ1 integrin, which is a typical RGD-binding integrin widely expressed on the cell surface. HPeV-1 is strictly dependent on its RGD-motif because cultivation of HPeV-1-RGDdel mutated viruses has not been successful. Interestingly, few HPeV-1-RGDdel mutants have been found in the nature (Westerhuis 2014). HPeV-1 binds αVβ3 and αVβ6 integrins *in vitro* (Seitsonen et al. 2010). However, in the articles included in this thesis it was shown that HPeV-1 utilizes active form of αVβ1 integrin. Furthermore, HPeV-1 likely enters the cells via normal β1 integrin recycling route instead of integrin clustering-induced internalization. While HPeV-1 binding to αVβ3 or αVβ6 integrins does not trigger the internalization, the endocytosis of the virus occurs only when HPeV-1 binds to active αVβ1 integrin, and the integrin-HPeV-1 complex is then recycled into the cell via regular β1 integrin recycling mechanism. *In vitro* binding assays with αVβ1 integrin cannot be performed in the absence of this heterodimer protein, and there are no commercial αVβ1 integrin antibodies available, which complicates αVβ1 integrin related studies.

6.3 HS in CV-A9 and HPeV-1 infection

The initial step in virus infection is the attachment to the cell surface and this often occurs via HS. Binding of viruses to carbohydrate moieties, such as HS, acts as a key step that

induces conformational changes in the viral structure (Hussein et al. 2015). These conformational changes are critical for interactions with receptors that promote viral entry, and often these receptors are integrins. Blocking of HS function thus impairs viral entry via integrins. HSPGs are heparan sulfate glycosaminoglycan molecules (HSGAGs), which are covalently attached to syndecan core proteins on the cell surface. HSPGs mediate their functions mainly via HS side chains (Sasisekharan et al. 2002). Heparin, which is commonly used in HS-related studies, differs from HS mainly with its origin of secretion, because heparin is only produced by mast cells, whereas HS is produced by all cell types. Boonyakiat et al. (2001) could not demonstrate interaction with HPeV-1 and HS. A relatively recent paper by McLeish et al. (2012) proposed that CV-A9 contain a HS-binding site in its VP1 region, more precisely position 132, which in most isolates harbors a threonine (T132). They suggested that only those viruses containing a mutation in T132 could interact with heparin. The importance of this putative HS-binding site is, however, questionable, because sequence analysis performed in this study (**III**) revealed that the prevalence of this mutation is less than 20%. Thus, its role in natural infection is unlikely significant.

Heparin incubation with CV-A9 before infecting A549 cells revealed, indeed, that CV-A9 isolates that contain the specific mutation, T132R/K, were binding to heparin. However, silencing of HS biosynthesis with EXT-1 siRNA and sodium chlorate, heparinase I and protamine treatments showed that CV-A9 is susceptible to HS despite possible mutation in T132. All treatments decreased the infection of CV-A9 prototype strain Griggs. The results with CV-A9 Griggs with sodium chlorate were contradictory to the results of McLeish et al. (2012). This may be the consequence of different cell line used, or cell culture adaptation of the prototype strain. To exclude the relevance of cell culture adaptation, several low passage clinical isolates were used in protamine assay, and with the clinical isolates the role of HS in CV-A9 infection was confirmed.

In contrast to CV-A9, HPeV-1 had not been shown to interact with HS (Boonyakiat et al. 2001). Sodium chlorate, heparinase I and protamine treatments decreased HPeV-1 infection in A549 cell line. However, similarly to CV-A9, heparin treatment caused variable effect on HPeV-1 infection. Heparin treatment affected the prototype strain, Harris, and several clinical isolates similarly to CV-A9 isolates containing T132 mutation, but the effect of heparin treatment was relatively small in a few clinical isolates. This suggests that HPeV-1, similarly to CV-A9, may contain a heparin-binding site. However, the role of the putative heparin-binding site in the natural HPeV-1 infection might be irrelevant, because all the other treatments against HS affected HPeV-1 infection.

7 CONCLUSIONS

In conclusion, the cell surface interactions of CV-A9 and HPeV-1 are complex. More studies exist on the receptor usage of CV-A9, and both RGD-independent and RGD-dependent entry are proposed. HPeV-1 entry is RGD-dependent and thus its receptor interactions might be less complex (**Figure 16)**.

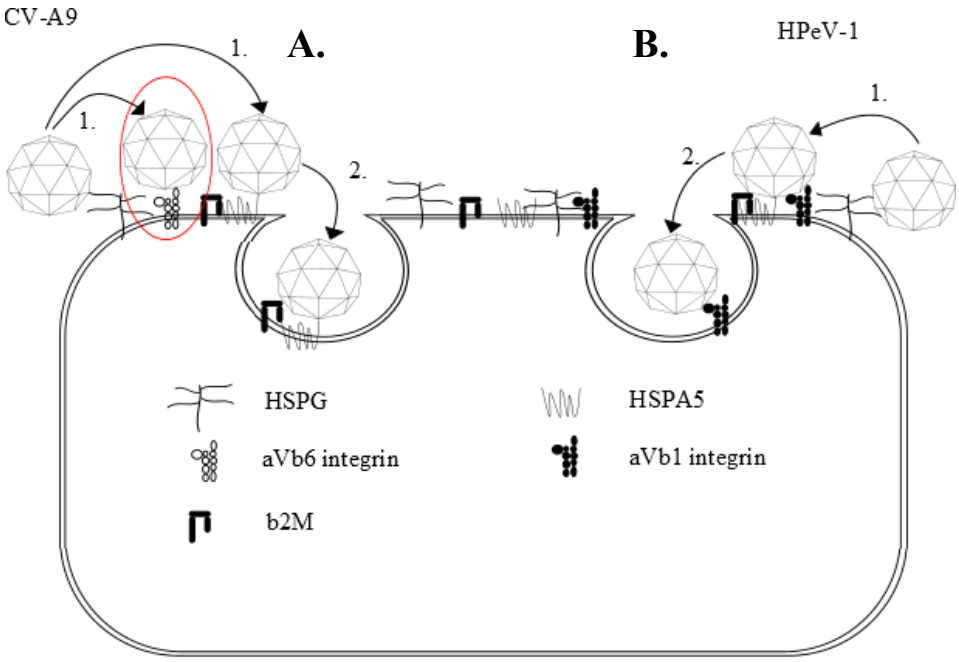

Figure 16: Cell surface interactions of CV-A9 and HPeV-1. A) *CV-A9 binds to cell surface either via integrins (indicated by a red circle) or integrin-independently. First CV-A9 attaches to HSPG, which acts as "sticky surface". Next CV-A9 binds to its specific receptors, either αVβ6 integrin or HSPA5, and β2M is involved in both pathways. This thesis showed that both HSPA5 and β2M interact with CV-A9 in the early stages of infection independently of integrins.* ***B)*** *HPeV-1, similarly to CV-A9, first binds HSPG, after which the virus binds to its primary receptor, αVβ1 integrin. β2M acts as a co-receptor, and HSPA5 may also contribute to HPeV-1 infection. αVβ1 integrin is internalized with HPeV-1.*

HSPG, including HS, act as the first attachment sites for both CV-A9 and HPeV-1. HSPGs are widely expressed on the cell surfaces, which facilitates the initial virus-cell interaction. Thereafter the virus can bind its primary receptor, which initiates the viral endocytosis. In a cell line without αVβ6 integrins, CV-A9 utilizes HSPA5 and β2M for infectious entry. HPeV-1, instead, is dependent on RGD-binding integrins, specifically αVβ1 integrin. HPeV-1 also utilizes β2M as a co-receptor. Interestingly, we found that HPeV-1 prefers the active form of αVβ1 integrin, which would suggest that the virus exploits normal β1 integrin recycling system for internalization.

To alleviate any (further) receptor studies one should carefully consider, which cell line to use for the experimental work. Many authors suggest using primary cell lines for *in vitro* studies; however, no consensus exists on which cell line to use. Targeted genome editing via CRISPR/Cas9 technology will allow for example generation of receptor knock-out cell lines, which will enable more thorough receptor tropism and entry mechanism studies.

The detailed knowledge on CV-A9 and HPeV-1 cell surface interactions offers the opportunity to develop antivirals against picornaviruses. HSPGs participate to infection of several picornaviruses, and there are commercially available drugs affecting their function, such as protamine. For instance HS function-blocking drugs might be used in the future to treat hospitalized children diagnosed with entero- or parechovirus infection. There are also therapeutic reagents against HSPA5, such as compound OSU03012+sildenafil, which decrease expression of HSPA5 (Booth et al. 2015). Integrins are a target of novel therapeutic reagents, mainly due to their role in cancer development, but these reagents could also help in the battle against virus infections. The knowledge of specific receptors and/or entry mediators will allow targeted choice or design of drug molecules against (picorna)viral diseases.

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REFERENCES

Abed Y, Boivin G. 2006. Human parechovirus types 1, 2 and 3 infections in Canada. Emerg.Infect.Dis. **12:**969-75.

Abraham G, Colonno RJ. 1984. Many rhinovirus serotypes share the same cellular receptor. J.Virol. **51:**340-5.

Adams MJ, Lefkowitz EJ, King AM, Harrach B, Harrison RL, Knowles NJ, Kropinski AM, Krupovic M, Kuhn JH, Mushegian AR, Nibert M, Sabanadzovic S, Sanfacon H, Siddell SG, Simmonds P, Varsani A, Zerbini FM, Gorbalenya AE, Davison AJ. 2016. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2016). Arch.Virol.

Agrez MV, Shafren DR, Gu X, Cox K, Sheppard D, Barry RD. 1997. Integrin alpha v beta 6 enhances coxsackievirus B1 lytic infection of human colon cancer cells. Virology **239:**71-7.

Alanko J. 2016. Integrin traffic and signalling - from plasma membrane to endosomes.

Alexander DA, Dimock K. 2002. Sialic acid functions in enterovirus 70 binding and infection. J.Virol. **76:**11265-72.

Alexander JP,Jr, Baden L, Pallansch MA, Anderson LJ. 1994. Enterovirus 71 infections and neurologic disease--United States, 1977-1991. J.Infect.Dis. **169:**905-8.

Aliabadi N, Messacar K, Pastula DM, Robinson CC, Leshem E, Sejvar JJ, Nix WA, Oberste MS, Feikin DR, Dominguez SR. 2016. Enterovirus D68 Infection in Children with Acute Flaccid Myelitis, Colorado, USA, 2014. Emerg.Infect.Dis. **22:**1387-94.

Ambros V, Baltimore D. 1980. Purification and properties of a HeLa cell enzyme able to remove the 5'-terminal protein from poliovirus RNA. J.Biol.Chem. **255:**6739-44.

Arjonen A, Alanko J, Veltel S, Ivaska J. 2012. Distinct recycling of active and inactive beta1 integrins. Traffic **13:**610-25.

Arnold E, Luo M, Vriend G, Rossmann MG, Palmenberg AC, Parks GD, Nicklin MJ, Wimmer E. 1987. Implications of the picornavirus capsid structure for polyprotein processing. Proc.Natl.Acad.Sci.U.S.A. **84:**21-5.

Baranowski E, Ruiz-Jarabo CM, Sevilla N, Andreu D, Beck E, Domingo E. 2000. Cell recognition by foot-and-mouth disease virus that lacks the RGD integrin-binding motif: flexibility in aphthovirus receptor usage. J.Virol. **74:**1641-7.

Barczyk M, Carracedo S, Gullberg D. 2010. Integrins. Cell Tissue Res. **339:**269-80.

Bauer K. 1997. Foot- and-mouth disease as zoonosis. Arch.Virol.Suppl. **13:**95-7.

Belnap DM, McDermott BM,Jr, Filman DJ, Cheng N, Trus BL, Zuccola HJ, Racaniello VR, Hogle JM, Steven AC. 2000. Three-dimensional structure of poliovirus receptor bound to poliovirus. Proc.Natl.Acad.Sci.U.S.A. **97:**73-8.

Benschop K, Thomas X, Serpenti C, Molenkamp R, Wolthers K. 2008. High prevalence of human Parechovirus (HPeV) genotypes in the Amsterdam region and identification of specific HPeV variants by direct genotyping of stool samples. J.Clin.Microbiol. **46:**3965-70.

Benschop KS, Schinkel J, Luken ME, van den Broek PJ, Beersma MF, Menelik N, van Eijk HW, Zaaijer HL, VandenBroucke-Grauls CM, Beld MG, Wolthers KC. 2006. Fourth human parechovirus serotype. Emerg.Infect.Dis. **12:**1572-5.

Bergelson JM. 2010. Receptors.The Picornaviruses73-86.

Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. 1997a. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science **275:**1320-3.

Bergelson JM, Modlin JF, Wieland-Alter W, Cunningham JA, Crowell RL, Finberg RW. 1997b. Clinical coxsackievirus B isolates differ from laboratory strains in their interaction with two cell surface receptors. J.Infect.Dis. **175:**697-700.

Bergelson JM, Mohanty JG, Crowell RL, St John NF, Lublin DM, Finberg RW. 1995. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). J.Virol. **69:**1903-6.

Bergelson JM, Chan M, Solomon KR, St John NF, Lin H, Finberg RW. 1994a. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc.Natl.Acad.Sci.U.S.A. **91:**6245-8.

Bergelson JM, St John NF, Kawaguchi S, Pasqualini R, Berdichevsky F, Hemler ME, Finberg RW. 1994b. The I domain is essential for echovirus 1 interaction with VLA-2. Cell Adhes.Commun. **2:**455-64.

Bergelson JM, Shepley MP, Chan BM, Hemler ME, Finberg RW. 1992. Identification of the integrin VLA-2 as a receptor for echovirus 1. Science **255:**1718-20.

Berinstein A, Roivainen M, Hovi T, Mason PW, Baxt B. 1995. Antibodies to the vitronectin receptor

(integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J.Virol. **69:**2664-6.

Berkovich S, Pangan J. 1968. Recoveries of virus from premature infants during outbreaks of respiratory disease: the relation of ECHO virus type 22 to disease of the upper and lower respiratory tract in the premature infant. Bull.N.Y.Acad.Med. **44:**377-87.

Berryman S, Clark S, Monaghan P, Jackson T. 2005. Early events in integrin alphavbeta6-mediated cell entry of foot-and-mouth disease virus. J.Virol. **79:**8519-34.

Birenbaum E, Handsher R, Kuint J, Dagan R, Raichman B, Mendelson E, Linder N. 1997. Echovirus type 22 outbreak associated with gastrointestinal disease in a neonatal intensive care unit. Am.J.Perinatol. **14:**469-73.

Bissel SJ, Auer RN, Chiang CH, Kofler J, Murdoch GH, Nix WA, Painter M, Richer M, Sartelet H, Wang G, Wiley CA. 2015. Human Parechovirus 3 Meningitis and Fatal Leukoencephalopathy. J.Neuropathol.Exp.Neurol. **74:**767-77.

Bizzintino J, Lee WM, Laing IA, Vang F, Pappas T, Zhang G, Martin AC, Khoo SK, Cox DW, Geelhoed GC, McMinn PC, Goldblatt J, Gern JE, Le Souef PN. 2011. Association between human rhinovirus C and severity of acute asthma in children. Eur.Respir.J. **37:**1037-42.

Blomqvist S, Paananen A, Savolainen-Kopra C, Hovi T, Roivainen M. 2008. Eight years of experience with molecular identification of human enteroviruses. J.Clin.Microbiol. **46:**2410-3.

Blomqvist S, Roivainen M, Puhakka T, Kleemola M, Hovi T. 2002. Virological and serological analysis of rhinovirus infections during the first two years of life in a cohort of children. J.Med.Virol. **66:**263-8.

Bochkov YA, Watters K, Ashraf S, Griggs TF, Devries MK, Jackson DJ, Palmenberg AC, Gern JE. 2015. Cadherin-related family member 3, a childhood asthma susceptibility gene product, mediates rhinovirus C binding and replication. Proc.Natl.Acad.Sci.U.S.A. **112:**5485-90.

Boivin G, Abed Y, Boucher FD. 2005. Human parechovirus 3 and neonatal infections. Emerg.Infect.Dis. **11:**103-5.

Boonyakiat Y, Hughes PJ, Ghazi F, Stanway G. 2001. Arginine-glycine-aspartic acid motif is critical for human parechovirus 1 entry. J.Virol. **75:**10000-4.

Booth L, Roberts JL, Cash DR, Tavallai S, Jean S, Fidanza A, Cruz-Luna T, Siembiba P, Cycon KA, Cornelissen CN, Dent P. 2015. GRP78/BiP/HSPA5/Dna K is a universal therapeutic target for human disease. J.Cell.Physiol. **230:**1661-76.

Brabec M, Baravalle G, Blaas D, Fuchs R. 2003. Conformational changes, plasma membrane penetration, and infection by human rhinovirus type 2: role of receptors and low pH. J.Virol. **77:**5370-7.

Bridgewater RE, Norman JC, Caswell PT. 2012. Integrin trafficking at a glance. J.Cell.Sci. **125:**3695-701.

Britton PN, Dale RC, Nissen MD, Crawford N, Elliott E, Macartney K, Khandaker G, Booy R, Jones CA, PAEDS-ACE Investigators. 2016. Parechovirus Encephalitis and Neurodevelopmental Outcomes. Pediatrics **137:**e20152848,2848. Epub 2016 Jan 20.

Burke MJ. 2016. Oncolytic Seneca Valley Virus: past perspectives and future directions. Oncolytic Virother **5:**81-9.

Calvo D, Dopazo J, Vega MA. 1995. The CD36, CLA-1 (CD36L1), and LIMPII (CD36L2) gene family: cellular distribution, chromosomal location, and genetic evolution. Genomics **25:**100-6.

Caswell PT, Vadrevu S, Norman JC. 2009. Integrins: masters and slaves of endocytic transport. Nat.Rev.Mol.Cell Biol. **10:**843-53.

Centers for Disease Control and Prevention (CDC). 2011. Clusters of acute respiratory illness associated with human enterovirus 68--Asia, Europe, and United States, 2008-2010. MMWR Morb.Mortal.Wkly.Rep. **60:**1301-4.

Chang KH, Day C, Walker J, Hyypia T, Stanway G. 1992. The nucleotide sequences of wild-type coxsackievirus A9 strains imply that an RGD motif in VP1 is functionally significant. J.Gen.Virol. **73 (Pt 3):**621-6.

Chang KH, Auvinen P, Hyypia T, Stanway G. 1989. The nucleotide sequence of coxsackievirus A9; implications for receptor binding and enterovirus classification. J.Gen.Virol. **70 (Pt 12):**3269-80.

Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM. 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proc.Natl.Acad.Sci.U.S.A. **98:**15191-6.

Colonno RJ, Condra JH, Mizutani S, Callahan PL, Davies ME, Murcko MA. 1988. Evidence for the direct involvement of the rhinovirus canyon in receptor binding. Proc.Natl.Acad.Sci.U.S.A. **85:**5449-53.

Colonno RJ. 1986. Cell surface receptors for picornaviruses. Bioessays **5:**270-4.

Colonno RJ, Callahan PL, Long WJ. 1986. Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinoviruses. J.Virol. **57:**7-12.

Connors WL, Jokinen J, White DJ, Puranen JS, Kankaanpaa P, Upla P, Tulla M, Johnson MS,

Heino J. 2007. Two synergistic activation mechanisms of alpha2beta1 integrin-mediated collagen binding. J.Biol.Chem. **282:**14675-83.

Coyne CB, Shen L, Turner JR, Bergelson JM. 2007. Coxsackievirus entry across epithelial tight junctions requires occludin and the small GTPases Rab34 and Rab5. Cell.Host Microbe **2:**181-92.

Coyne CB, Bergelson JM. 2006. Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. Cell **124:**119-31.

Cui A, Yu D, Zhu Z, Meng L, Li H, Liu J, Liu G, Mao N, Xu W. 2010. An outbreak of aseptic meningitis caused by coxsackievirus A9 in Gansu, the People's Republic of China. Virol.J. **7:**72.

Curry S, Fry E, Blakemore W, Abu-Ghazaleh R, Jackson T, King A, Lea S, Newman J, Stuart D. 1997. Dissecting the roles of VP0 cleavage and RNA packaging in picornavirus capsid stabilization: the structure of empty capsids of foot-and-mouth disease virus. J.Virol. **71:**9743-52.

Davison E, Diaz RM, Hart IR, Santis G, Marshall JF. 1997. Integrin alpha5beta1-mediated adenovirus infection is enhanced by the integrin-activating antibody TS2/16. J.Virol. **71:**6204-7.

de Crom SC, Rossen JW, van Furth AM, Obihara CC. 2016. Enterovirus and parechovirus infection in children: a brief overview. Eur.J.Pediatr. **175:**1023-9.

Dicara D, Burman A, Clark S, Berryman S, Howard MJ, Hart IR, Marshall JF, Jackson T. 2008. Foot-and-mouth disease virus forms a highly stable, EDTA-resistant complex with its principal receptor, integrin alphavbeta6: implications for infectiousness. J.Virol. **82:**1537-46.

Dickinson CD, Veerapandian B, Dai XP, Hamlin RC, Xuong NH, Ruoslahti E, Ely KR. 1994. Crystal structure of the tenth type III cell adhesion module of human fibronectin. J.Mol.Biol. **236:**1079-92.

Ehrenfeld E, Domingo E, Roos RP. 2010. The picornaviruses.

Ehrnst A, Eriksson M. 1993. Epidemiological features of type 22 echovirus infection. Scand.J.Infect.Dis. **25:**275-81.

Eisenhut M, Algawi B, Wreghitt T, Foweraker J, McKee T, Miles R, Challener J. 2000. Fatal Coxsackie A9 virus infection during an outbreak in a neonatal unit. J.Infect. **40:**297-8.

Esposito S, Rahamat-Langendoen J, Ascolese B, Senatore L, Castellazzi L, Niesters HG. 2014. Pediatric parechovirus infections. J.Clin.Virol. **60:**84-9.

Evans DJ, Almond JW. 1998. Cell receptors for picornaviruses as determinants of cell tropism and pathogenesis. Trends Microbiol. **6:**198-202.

Feigelstock D, Thompson P, Mattoo P, Zhang Y, Kaplan GG. 1998. The human homolog of HAVcr-1 codes for a hepatitis A virus cellular receptor. J.Virol. **72:**6621-8.

Feng Z, Lemon SM. 2010. Hepatitis A Virus.The Picornaviruses383-96.

Figueroa JP, Ashley D, King D, Hull B. 1989. An outbreak of acute flaccid paralysis in Jamaica associated with echovirus type 22. J.Med.Virol. **29:**315-9.

Fischer TK, Midgley S, Dalgaard C, Nielsen AY. 2014. Human parechovirus infection, Denmark. Emerg.Infect.Dis. **20:**83-7.

Flanegan JB, Petterson RF, Ambros V, Hewlett NJ, Baltimore D. 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5'-terminus of virion and replicative intermediate RNAs of poliovirus. Proc.Natl.Acad.Sci.U.S.A. **74:**961-5.

Forss S, Schaller H. 1982. A tandem repeat gene in a picornavirus. Nucleic Acids Res. **10:**6441-50.

Fry E, Stuart DI. 2010. Virion Structure.The Picornaviruses59-71.

Fry EE, Lea SM, Jackson T, Newman JW, Ellard FM, Blakemore WE, Abu-Ghazaleh R, Samuel A, King AM, Stuart DI. 1999. The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. EMBO J. **18:**543-54.

Fujita H, Takata Y, Kono A, Tanaka Y, Takahashi T, Himeno M, Kato K. 1992. Isolation and sequencing of a cDNA clone encoding the 85 kDa human lysosomal sialoglycoprotein (hLGP85) in human metastatic pancreas islet tumor cells. Biochem.Biophys.Res.Commun. **184:**604-11.

Gern JE, Busse WW. 1999. Association of rhinovirus infections with asthma. Clin.Microbiol.Rev. **12:**9-18.

Gimond C, van Der Flier A, van Delft S, Brakebusch C, Kuikman I, Collard JG, Fassler R, Sonnenberg A. 1999. Induction of cell scattering by expression of beta1 integrins in beta1-deficient epithelial cells requires activation of members of the rho family of GTPases and downregulation of cadherin and catenin function. J.Cell Biol. **147:**1325-40.

Goodfellow IG, Powell RM, Ward T, Spiller OB, Almond JW, Evans DJ. 2000. Echovirus infection of rhabdomyosarcoma cells is inhibited by antiserum to the complement control protein CD59. J.Gen.Virol. **81:**1393-401.

Gouy M, Guindon S, Gascuel O. 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol.Biol.Evol. **27:**221-4.

Grant RA, Hiremath CN, Filman DJ, Syed R, Andries K, Hogle JM. 1994. Structures of poliovirus complexes with anti-viral drugs: implications for viral stability and drug design. Curr.Biol. **4:**784-97.

Greve JM, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, Kamarck ME, McClelland A.

1989. The major human rhinovirus receptor is ICAM-1. Cell **56:**839-47.

Grist NR, Bell EJ, Assaad F. 1978. Enteroviruses in human disease. Prog.Med.Virol. **24:**114-57.

Grunert HP, Wolf KU, Langner KD, Sawitzky D, Habermehl KO, Zeichhardt H. 1997. Internalization of human rhinovirus 14 into HeLa and ICAM-1-transfected BHK cells. Med.Microbiol.Immunol. **186:**1-9.

Hafenstein S, Bowman VD, Chipman PR, Bator Kelly CM, Lin F, Medof ME, Rossmann MG. 2007. Interaction of decay-accelerating factor with coxsackievirus B3. J.Virol. **81:**12927-35.

Harvala H, Wolthers KC, Simmonds P. 2010. Parechoviruses in children: understanding a new infection. Curr.Opin.Infect.Dis. **23:**224-30.

Harvala H, Robertson I, Chieochansin T, McWilliam Leitch EC, Templeton K, Simmonds P. 2009. Specific association of human parechovirus type 3 with sepsis and fever in young infants, as identified by direct typing of cerebrospinal fluid samples. J.Infect.Dis. **199:**1753-60.

Harvala H, Simmonds P. 2009. Human parechoviruses: biology, epidemiology and clinical significance. J.Clin.Virol. **45:**1-9.

Harvala H, Kalimo H, Stanway G, Hyypia T. 2003. Pathogenesis of coxsackievirus A9 in mice: role of the viral arginine-glycine-aspartic acid motif. J.Gen.Virol. **84:**2375-9.

Hayden FG, Herrington DT, Coats TL, Kim K, Cooper EC, Villano SA, Liu S, Hudson S, Pevear DC, Collett M, McKinlay M, Pleconaril Respiratory Infection Study Group. 2003. Efficacy and safety of oral pleconaril for treatment of colds due to picornaviruses in adults: results of 2 double-blind, randomized, placebo-controlled trials. Clin.Infect.Dis. **36:**1523-32.

He Y, Chipman PR, Howitt J, Bator CM, Whitt MA, Baker TS, Kuhn RJ, Anderson CW, Freimuth P, Rossmann MG. 2001. Interaction of coxsackievirus B3 with the full length coxsackievirus-adenovirus receptor. Nat.Struct.Biol. **8:**874-8.

He Y, Bowman VD, Mueller S, Bator CM, Bella J, Peng X, Baker TS, Wimmer E, Kuhn RJ, Rossmann MG. 2000. Interaction of the poliovirus receptor with poliovirus. Proc.Natl.Acad.Sci.U.S.A. **97:**79-84.

Heikkila O, Susi P, Tevaluoto T, Harma H, Marjomaki V, Hyypia T, Kiljunen S. 2010. Internalization of coxsackievirus A9 is mediated by {beta}2-microglobulin, dynamin, and Arf6 but not by caveolin-1 or clathrin. J.Virol. **84:**3666-81.

Heikkila O, Susi P, Stanway G, Hyypia T. 2009. Integrin alphaVbeta6 is a high-affinity receptor for coxsackievirus A9. J.Gen.Virol. **90:**197-204.

Hendry E, Hatanaka H, Fry E, Smyth M, Tate J, Stanway G, Santti J, Maaronen M, Hyypia T, Stuart D. 1999. The crystal structure of coxsackievirus A9: new insights into the uncoating mechanisms of enteroviruses. Structure **7:**1527-38.

Hershenson MB. 2010. Rhinovirus and Respiratory Disease.The Picornaviruses369-81.

Hertzler S, Luo M, Lipton HL. 2000. Mutation of predicted virion pit residues alters binding of Theiler's murine encephalomyelitis virus to BHK-21 cells. J.Virol. **74:**1994-2004.

Hewat EA, Neumann E, Conway JF, Moser R, Ronacher B, Marlovits TC, Blaas D. 2000. The cellular receptor to human rhinovirus 2 binds around the 5-fold axis and not in the canyon: a structural view. EMBO J. **19:**6317-25.

Hindiyeh M, Li QH, Basavappa R, Hogle JM, Chow M. 1999. Poliovirus mutants at histidine 195 of VP2 do not cleave VP0 into VP2 and VP4. J.Virol. **73:**9072-9.

Hofer F, Gruenberger M, Kowalski H, Machat H, Huettinger M, Kuechler E, Blaas D. 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. Proc.Natl.Acad.Sci.U.S.A. **91:**1839-42.

Hogle JM, Chow M, Filman DJ. 1985. Three-dimensional structure of poliovirus at 2.9 A resolution. Science **229:**1358-65.

Holland JJ, Kiehn ED. 1968. Specific cleavage of viral proteins as steps in the synthesis and maturation of enteroviruses. Proc.Natl.Acad.Sci.U.S.A. **60:**1015-22.

Holtz LR, Finkbeiner SR, Zhao G, Kirkwood CD, Girones R, Pipas JM, Wang D. 2009. Klassevirus 1, a previously undescribed member of the family Picornaviridae, is globally widespread. Virol.J. **6:**86,422X-6-86.

Holtz LR, Finkbeiner SR, Kirkwood CD, Wang D. 2008. Identification of a novel picornavirus related to cosaviruses in a child with acute diarrhea. Virol.J. **5:**159,422X-5-159.

Howes MT, Kirkham M, Riches J, Cortese K, Walser PJ, Simpson F, Hill MM, Jones A, Lundmark R, Lindsay MR, Hernandez-Deviez DJ, Hadzic G, McCluskey A, Bashir R, Liu L, Pilch P, McMahon H, Robinson PJ, Hancock JF, Mayor S, Parton RG. 2010. Clathrin-independent carriers form a high capacity endocytic sorting system at the leading edge of migrating cells. J.Cell Biol. **190:**675-91.

Huber SA. 1994. VCAM-1 is a receptor for encephalomyocarditis virus on murine vascular endothelial cells. J.Virol. **68:**3453-8.

Hughes PJ, Horsnell C, Hyypia T, Stanway G. 1995. The coxsackievirus A9 RGD motif is not essential for virus viability. J.Virol. **69:**8035-40.

Humphries DE, Silbert JE. 1988. Chlorate: a reversible inhibitor of proteoglycan sulfation. Biochem.Biophys.Res.Commun. **154:**365-71.

Humphries JD, Byron A, Humphries MJ. 2006. Integrin ligands at a glance. J.Cell.Sci. **119:**3901-3.

Hussein HA, Walker LR, Abdel-Raouf UM, Desouky SA, Montasser AK, Akula SM. 2015. Beyond RGD: virus interactions with integrins. Arch.Virol. **160:**2669-81.

Hynes RO. 2002. Integrins: bidirectional, allosteric signaling machines. Cell **110:**673-87.

Hyoty H. 2016. Viruses in type 1 diabetes. Pediatr.Diabetes **17 Suppl 22:**56-64.

Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, Koskela P, Roivainen M, Leinikki P, Hovi T. 1995. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. Diabetes **44:**652-7.

Hyypia T, Hovi T, Knowles NJ, Stanway G. 1997. Classification of enteroviruses based on molecular and biological properties. J.Gen.Virol. **78 (Pt 1):**1-11.

Hyypia T, Horsnell C, Maaronen M, Khan M, Kalkkinen N, Auvinen P, Kinnunen L, Stanway G. 1992. A distinct picornavirus group identified by sequence analysis. Proc.Natl.Acad.Sci.U.S.A. **89:**8847-51.

Jaaskelainen AJ, Kolehmainen P, Kallio-Kokko H, Nieminen T, Koskiniemi M, Tauriainen S, Lappalainen M. 2013. First two cases of neonatal human parechovirus 4 infection with manifestation of suspected sepsis, Finland. J.Clin.Virol. **58:**328-30.

Jackson T, Clark S, Berryman S, Burman A, Cambier S, Mu D, Nishimura S, King AM. 2004. Integrin alphavbeta8 functions as a receptor for foot-and-mouth disease virus: role of the beta-chain cytodomain in integrin-mediated infection. J.Virol. **78:**4533-40.

Jackson T, Mould AP, Sheppard D, King AM. 2002. Integrin alphavbeta1 is a receptor for foot-and-mouth disease virus. J.Virol. **76:**935-41.

Jackson T, Blakemore W, Newman JW, Knowles NJ, Mould AP, Humphries MJ, King AM. 2000a. Foot-and-mouth disease virus is a ligand for the high-affinity binding conformation of integrin alpha5beta1: influence of the leucine residue within the RGDL motif on selectivity of integrin binding. J.Gen.Virol. **81:**1383-91.

Jackson T, Sheppard D, Denyer M, Blakemore W, King AM. 2000b. The epithelial integrin alphavbeta6 is a receptor for foot-and-mouth disease virus. J.Virol. **74:**4949-56.

Jackson T, Ellard FM, Ghazaleh RA, Brookes SM, Blakemore WE, Corteyn AH, Stuart DI, Newman JW, King AM. 1996. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. J.Virol. **70:**5282-7.

Jacobs SE, Lamson DM, St George K, Walsh TJ. 2013. Human rhinoviruses. Clin.Microbiol.Rev. **26:**135-62.

Jacobson MF, Baltimore D. 1968. Polypeptide cleavages in the formation of poliovirus proteins. Proc.Natl.Acad.Sci.U.S.A. **61:**77-84.

Jadrnickova-Volakova, Schnellerova M, Lokajova M, Jandasek L. 1967. Outbreak of respiratory infection in the newborn infant department, induced by Coxsackie virus A-9. Cesk.Pediatr. **22:**809-13.

Jheng JR, Wang SC, Jheng CR, Horng JT. 2016. Enterovirus 71 induces dsRNA/PKR-dependent cytoplasmic redistribution of GRP78/BiP to promote viral replication. Emerg.Microbes Infect. **5:**e23.

Joffret ML, Bouchier C, Grandadam M, Zeller H, Maufrais C, Bourhy H, Despres P, Delpeyroux F, Dacheux L. 2013. Genomic characterization of Sebokele virus 1 (SEBV1) reveals a new candidate species among the genus Parechovirus. J.Gen.Virol. **94:**1547-53.

Joki-Korpela P, Marjomaki V, Krogerus C, Heino J, Hyypia T. 2001. Entry of human parechovirus 1. J.Virol. **75:**1958-67.

Joki-Korpela P, Hyypia T. 1998. Diagnosis and epidemiology of echovirus 22 infections. Clin.Infect.Dis. **27:**129-36.

Jokinen J, White DJ, Salmela M, Huhtala M, Kapyla J, Sipila K, Puranen JS, Nissinen L, Kankaanpaa P, Marjomaki V, Hyypia T, Johnson MS, Heino J. 2010. Molecular mechanism of alpha2beta1 integrin interaction with human echovirus 1. EMBO J. **29:**196-208.

Jones MS, Lukashov VV, Ganac RD, Schnurr DP. 2007. Discovery of a novel human picornavirus in a stool sample from a pediatric patient presenting with fever of unknown origin. J.Clin.Microbiol. **45:**2144-50.

Junttila N, Leveque N, Kabue JP, Cartet G, Mushiya F, Muyembe-Tamfum JJ, Trompette A, Lina B, Magnius LO, Chomel JJ, Norder H. 2007. New enteroviruses, EV-93 and EV-94, associated with acute flaccid paralysis in the Democratic Republic of the Congo. J.Med.Virol. **79:**393-400.

Kalynych S, Palkova L, Plevka P. 2015. The Structure of Human Parechovirus 1 Reveals an Association of the RNA Genome with the Capsid. J.Virol. **90:**1377-86.

Kankaanpaa P, Paavolainen L, Tiitta S, Karjalainen M, Paivarinne J, Nieminen J, Marjomaki V, Heino J, White DJ. 2012. BioImageXD: an open, general-purpose and high-throughput image-processing platform. Nat.Methods **9:**683-9.

Kaplan G, Totsuka A, Thompson P, Akatsuka T, Moritsugu Y, Feinstone SM. 1996. Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. EMBO J. **15:**4282-96.

Karjalainen M, Kakkonen E, Upla P, Paloranta H, Kankaanpaa P, Liberali P, Renkema GH, Hyypia T, Heino J, Marjomaki V. 2008. A Raft-derived, Pak1-regulated entry participates in alpha2beta1 integrin-dependent sorting to caveosomes. Mol.Biol.Cell **19:**2857-69.

Karnauchow TM, Tolson DL, Harrison BA, Altman E, Lublin DM, Dimock K. 1996. The HeLa cell receptor for enterovirus 70 is decay-accelerating factor (CD55). J.Virol. **70:**5143-52.

Khan AG, Pickl-Herk A, Gajdzik L, Marlovits TC, Fuchs R, Blaas D. 2010. Human rhinovirus 14 enters rhabdomyosarcoma cells expressing icam-1 by a clathrin-, caveolin-, and flotillin-independent pathway. J.Virol. **84:**3984-92.

Khan AG, Pichler J, Rosemann A, Blaas D. 2007. Human rhinovirus type 54 infection via heparan sulfate is less efficient and strictly dependent on low endosomal pH. J.Virol. **81:**4625-32.

Khatami A, McMullan BJ, Webber M, Stewart P, Francis S, Timmers KJ, Rodas E, Druce J, Mehta B, Sloggett NA, Cumming G, Papadakis G, Kesson AM. 2015. Sepsis-like disease in infants due to human parechovirus type 3 during an outbreak in Australia. Clin.Infect.Dis. **60:**228-36.

Kim MC, Kwon YK, Joh SJ, Lindberg AM, Kwon JH, Kim JH, Kim SJ. 2006. Molecular analysis of duck hepatitis virus type 1 reveals a novel lineage close to the genus Parechovirus in the family Picornaviridae. J.Gen.Virol. **87:**3307-16.

Kitamura N, Semler BL, Rothberg PG, Larsen GR, Adler CJ, Dorner AJ, Emini EA, Hanecak R, Lee JJ, van der Werf S, Anderson CW, Wimmer E. 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature **291:**547-53.

Knowles NJ, Hovi T, King AMQ, Stanway G. 2010. Overview of Taxonomy.The Picornaviruses19-32.

Koivunen E, Arap W, Rajotte D, Lahdenranta J, Pasqualini R. 1999a. Identification of receptor ligands with phage display peptide libraries. J.Nucl.Med. **40:**883-8.

Koivunen E, Restel BH, Rajotte D, Lahdenranta J, Hagedorn M, Arap W, Pasqualini R. 1999b. Integrin-binding peptides derived from phage display libraries. Methods Mol.Biol. **129:**3-17.

Kolatkar PR, Bella J, Olson NH, Bator CM, Baker TS, Rossmann MG. 1999. Structural studies of two rhinovirus serotypes complexed with fragments of their cellular receptor. EMBO J. **18:**6249-59.

Kolehmainen P, Jaaskelainen A, Blomqvist S, Kallio-Kokko H, Nuolivirta K, Helminen M, Roivainen M, Lappalainen M, Tauriainen S. 2014. Human parechovirus type 3 and 4 associated with severe infections in young children. Pediatr.Infect.Dis.J. **33:**1109-13.

Koskiniemi M, Paetau R, Linnavuori K. 1989. Severe encephalitis associated with disseminated echovirus 22 infection. Scand.J.Infect.Dis. **21:**463-6.

Kotaniemi-Syrjanen A, Vainionpaa R, Reijonen TM, Waris M, Korhonen K, Korppi M. 2003. Rhinovirus-induced wheezing in infancy--the first sign of childhood asthma? J.Allergy Clin.Immunol. **111:**66-71.

Kreuter JD, Barnes A, McCarthy JE, Schwartzman JD, Oberste MS, Rhodes CH, Modlin JF, Wright PF. 2011. A fatal central nervous system enterovirus 68 infection. Arch.Pathol.Lab.Med. **135:**793-6.

Kvist S, Levy F. 1993. Early events in the assembly of MHC class I antigens. Semin.Immunol. **5:**105-16.

Laitinen OH, Honkanen H, Pakkanen O, Oikarinen S, Hankaniemi MM, Huhtala H, Ruokoranta T, Lecouturier V, Andre P, Harju R, Virtanen SM, Lehtonen J, Almond JW, Simell T, Simell O, Ilonen J, Veijola R, Knip M, Hyoty H. 2014. Coxsackievirus B1 is associated with induction of beta-cell autoimmunity that portends type 1 diabetes. Diabetes **63:**446-55.

Lau SK, Yip CC, Woo PC, Yuen KY. 2010. Human rhinovirus C: a newly discovered human rhinovirus species. Emerg.Health.Threats J. **3:**e2.

Lau SK, Yip CC, Tsoi HW, Lee RA, So LY, Lau YL, Chan KH, Woo PC, Yuen KY. 2007. Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. J.Clin.Microbiol. **45:**3655-64.

Lea S. 2002. Interactions of CD55 with non-complement ligands. Biochem.Soc.Trans. **30:**1014-9.

Lee YF, Nomoto A, Detjen BM, Wimmer E. 1977. A protein covalently linked to poliovirus genome RNA. Proc.Natl.Acad.Sci.U.S.A. **74:**59-63.

Legay V, Chomel JJ, Fernandez E, Lina B, Aymard M, Khalfan S. 2002. Encephalomyelitis due to human parechovirus type 1. J.Clin.Virol. **25:**193-5.

Leino T, Leinikki P, Hyypia T, Ristola M, Suni J, Sutinen J, Holopainen A, Haikala O, Valle M, Rostila T. 1997. Hepatitis A outbreak amongst intravenous amphetamine abusers in Finland. Scand.J.Infect.Dis. **29:**213-6.

Lemon SM. 1985. Type A viral hepatitis. New developments in an old disease. N.Engl.J.Med. **313:**1059-67.

Lerner AM, Levin HS, Finland M. 1962. Age and susceptibility of mice to Coxsackie A viruses. J.Exp.Med. **115:**745-62.

Levy H, Bostina M, Filman DJ, Hogle JM. 2010. Cell Entry: a Biochemical and Structural Perspective.The Picornaviruses87-104.

Li JP, Spillmann D. 2012. Heparan sulfate proteoglycans as multifunctional cell regulators: cell surface receptors. Methods Mol.Biol. **836:**239-55.

Li L, Victoria J, Kapoor A, Naeem A, Shaukat S, Sharif S, Alam MM, Angez M, Zaidi SZ, Delwart E. 2009. Genomic characterization of novel human parechovirus type. Emerg.Infect.Dis. **15:**288-91.

Liberali P, Kakkonen E, Turacchio G, Valente C, Spaar A, Perinetti G, Bockmann RA, Corda D, Colanzi A, Marjomaki V, Luini A. 2008. The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS. EMBO J. **27:**970-81.

Lublin DM, Atkinson JP. 1989. Decay-accelerating factor: biochemistry, molecular biology, and function. Annu.Rev.Immunol. **7:**35-58.

Luo M, Vriend G, Kamer G, Minor I, Arnold E, Rossmann MG, Boege U, Scraba DG, Duke GM, Palmenberg AC. 1987. The atomic structure of Mengo virus at 3.0 A resolution. Science **235:**182-91.

Maier MK, Seth S, Czeloth N, Qiu Q, Ravens I, Kremmer E, Ebel M, Muller W, Pabst O, Forster R, Bernhardt G. 2007. The adhesion receptor CD155 determines the magnitude of humoral immune responses against orally ingested antigens. Eur.J.Immunol. **37:**2214-25.

Makela MJ, Puhakka T, Ruuskanen O, Leinonen M, Saikku P, Kimpimaki M, Blomqvist S, Hyypia T, Arstila P. 1998. Viruses and bacteria in the etiology of the common cold. J.Clin.Microbiol. **36:**539-42.

Maller HM, Powars DF, Horowitz RE, Portnoy B. 1967. Fatal myocarditis associated with ECHO virus, type 22, infection in a child with apparent immunological deficiency. J.Pediatr. **71:**204-10.

Mao Q, Wang Y, Bian L, Xu M, Liang Z. 2016. EV-A71 vaccine licensure: a first step for multivalent enterovirus vaccine to control HFMD and other severe diseases. Emerg.Microbes Infect. **5:**e75.

Marjomaki V, Pietiainen V, Matilainen H, Upla P, Ivaska J, Nissinen L, Reunanen H, Huttunen P, Hyypia T, Heino J. 2002. Internalization of echovirus 1 in caveolae. J.Virol. **76:**1856-65.

Marlovits TC, Zechmeister T, Schwihla H, Ronacher B, Blaas D. 1998. Recombinant soluble low-density lipoprotein receptor fragment inhibits common cold infection. J.Mol.Recognit. **11:**49-51.

Martin EK, Kuypers J, Chu HY, Lacombe K, Qin X, Strelitz B, Bradford M, Jones C, Klein EJ, Englund JA. 2015. Molecular epidemiology of human rhinovirus infections in the pediatric emergency department. J.Clin.Virol. **62:**25-31.

Martinez MA, Verdaguer N, Mateu MG, Domingo E. 1997. Evolution subverting essentiality: dispensability of the cell attachment Arg-Gly-Asp motif in multiply passaged foot-and-mouth disease virus. Proc.Natl.Acad.Sci.U.S.A. **94:**6798-802.

Matheny SC, Kingery JE. 2012. Hepatitis A. Am.Fam.Physician **86:**1027,34; quiz 1010-2.

McLeish NJ, Williams CH, Kaloudas D, Roivainen MM, Stanway G. 2012. Symmetry-related clustering of positive charges is a common mechanism for heparan sulfate binding in enteroviruses. J.Virol. **86:**11163-70.

McMinn PC. 2002. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol.Rev. **26:**91-107.

Mendelsohn CL, Wimmer E, Racaniello VR. 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56:**855-65.

Mercer J, Greber UF. 2013. Virus interactions with endocytic pathways in macrophages and dendritic cells. Trends Microbiol. **21:**380-8.

Mercer J, Schelhaas M, Helenius A. 2010. Virus entry by endocytosis. Annu.Rev.Biochem. **79:**803-33.

Merilahti P, Koskinen S, Heikkila O, Karelehto E, Susi P. 2012. Endocytosis of integrin-binding human picornaviruses. Adv.Virol. **2012:**547530.

Midgley CM, Jackson MA, Selvarangan R, Turabelidze G, Obringer E, Johnson D, Giles BL, Patel A, Echols F, Oberste MS, Nix WA, Watson JT, Gerber SI. 2014. Severe respiratory illness associated with enterovirus D68 - Missouri and Illinois, 2014. MMWR Morb.Mortal.Wkly.Rep. **63:**798-9.

Milstone AM, Petrella J, Sanchez MD, Mahmud M, Whitbeck JC, Bergelson JM. 2005. Interaction with coxsackievirus and adenovirus receptor, but not with decay-accelerating factor (DAF), induces A-particle formation in a DAF-binding coxsackievirus B3 isolate. J.Virol. **79:**655-60.

Mirkovic RR, Kono R, Yin-Murphy M, Sohier R, Schmidt NJ, Melnick JL. 1973. Enterovirus type 70: the etiologic agent of pandemic acute haemorrhagic conjunctivitis. Bull.World Health Organ. **49:**341-6.

Mistry N, Inoue H, Jamshidi F, Storm RJ, Oberste MS, Arnberg N. 2011. Coxsackievirus A24 variant uses sialic acid-containing O-linked glycoconjugates as cellular receptors on human ocular cells. J.Virol. **85:**11283-90.

Modlin JF. 2007. Enterovirus deja vu. N.Engl.J.Med. **356:**1204-5.

Monaghan P, Gold S, Simpson J, Zhang Z, Weinreb PH, Violette SM, Alexandersen S, Jackson T. 2005. The alpha(v)beta6 integrin receptor for Foot-and-mouth disease virus is expressed constitutively on the epithelial cells targeted in cattle. J.Gen.Virol. **86:**2769-80.

Muller L, Mack I, Tapparel C, Kaiser L, Alves MP, Kieninger E, Frey U, Regamey N, Latzin P. 2015. Human Rhinovirus Types and Association with

Respiratory Symptoms During the First Year of Life. Pediatr.Infect.Dis.J. **34:**907-9.

Nelsen-Salz B, Eggers HJ, Zimmermann H. 1999. Integrin alpha(v)beta3 (vitronectin receptor) is a candidate receptor for the virulent echovirus 9 strain Barty. J.Gen.Virol. **80 (Pt 9):**2311-3.

Ng T, Shima D, Squire A, Bastiaens PI, Gschmeissner S, Humphries MJ, Parker PJ. 1999. PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic. EMBO J. **18:**3909-23.

Ni M, Zhang Y, Lee AS. 2011. Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. Biochem.J. **434:**181-8.

Nicholas KB, Nicholas HBJ, Deerfield DW. 1997. GeneDoc: Analysis and Visualization of Genetic Variation. Embnew.news **4:**14.

Nicodemou A, Petsch M, Konecsni T, Kremser L, Kenndler E, Casasnovas JM, Blaas D. 2005. Rhinovirus-stabilizing activity of artificial VLDL-receptor variants defines a new mechanism for virus neutralization by soluble receptors. FEBS Lett. **579:**5507-11.

Niklasson B, Kinnunen L, Hornfeldt B, Horling J, Benemar C, Hedlund KO, Matskova L, Hyypia T, Winberg G. 1999. A new picornavirus isolated from bank voles (Clethrionomys glareolus). Virology **255:**86-93.

Nilsson EC, Jamshidi F, Johansson SM, Oberste MS, Arnberg N. 2008. Sialic acid is a cellular receptor for coxsackievirus A24 variant, an emerging virus with pandemic potential. J.Virol. **82:**3061-8.

Nishimura Y, Shimizu H. 2012. Cellular receptors for human enterovirus species a. Front.Microbiol. **3:**105.

Nishimura Y, Shimojima M, Tano Y, Miyamura T, Wakita T, Shimizu H. 2009. Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nat.Med. **15:**794-7.

Nugent CI, Kirkegaard K. 1995. RNA binding properties of poliovirus subviral particles. J.Virol. **69:**13-22.

O'Donnell V, LaRocco M, Duque H, Baxt B. 2005. Analysis of foot-and-mouth disease virus internalization events in cultured cells. J.Virol. **79:**8506-18.

O'Regan S, Robitaille P, Mongeau JG, McLaughlin B. 1980. The hemolytic uremic syndrome associated with ECHO 22 infection. Clin.Pediatr.(Phila) **19:**125-7.

Oberste MS, Maher K, Schnurr D, Flemister MR, Lovchik JC, Peters H, Sessions W, Kirk C, Chatterjee N, Fuller S, Hanauer JM, Pallansch MA. 2004. Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. J.Gen.Virol. **85:**2577-84.

Olson NH, Kolatkar PR, Oliveira MA, Cheng RH, Greve JM, McClelland A, Baker TS, Rossmann MG. 1993. Structure of a human rhinovirus complexed with its receptor molecule. Proc.Natl.Acad.Sci.U.S.A. **90:**507-11.

Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. 2010. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. **9:**1097-105.

Osterback R, Vuorinen T, Linna M, Susi P, Hyypia T, Waris M. 2009. Coxsackievirus A6 and hand, foot, and mouth disease, Finland. Emerg.Infect.Dis. **15:**1485-8.

Pallansch MA, Oberste MS, Whitton JL. 2013. Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses.Fields Virology490-530.

Palmenberg AC, Neubauer D, Skern T. 2010. Genome Organization and Encoded Proteins.The Picornaviruses3-17.

Papadopoulos NG, Moustaki M, Tsolia M, Bossios A, Astra E, Prezerakou A, Gourgiotis D, Kafetzis D. 2002. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. Am.J.Respir.Crit.Care Med. **165:**1285-9.

Pariani E, Pellegrinelli L, Pugni L, Bini P, Perniciaro S, Bubba L, Primache V, Amendola A, Barbarini M, Mosca F, Binda S. 2014. Two cases of neonatal human parechovirus 3 encephalitis. Pediatr.Infect.Dis.J. **33:**1191-3.

Paul AV, van Boom JH, Filippov D, Wimmer E. 1998. Protein-primed RNA synthesis by purified poliovirus RNA polymerase. Nature **393:**280-4.

Pearson WR, Lipman DJ. 1988. Improved tools for biological sequence comparison. Proc.Natl.Acad.Sci.U.S.A. **85:**2444-8.

Pellinen T, Ivaska J. 2006. Integrin traffic. J.Cell.Sci. **119:**3723-31.

Pietiainen V, Marjomaki V, Upla P, Pelkmans L, Helenius A, Hyypia T. 2004. Echovirus 1 endocytosis into caveosomes requires lipid rafts, dynamin II, and signaling events. Mol.Biol.Cell **15:**4911-25.

Plevka P, Perera R, Cardosa J, Kuhn RJ, Rossmann MG. 2012. Crystal structure of human enterovirus 71. Science **336:**1274.

Powelka AM, Sun J, Li J, Gao M, Shaw LM, Sonnenberg A, Hsu VW. 2004. Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic **5:**20-36.

Powell RM, Ward T, Goodfellow I, Almond JW, Evans DJ. 1999. Mapping the binding domains on decay accelerating factor (DAF) for haemagglutinating enteroviruses: implications for the evolution of a DAF-binding phenotype. J.Gen.Virol. **80 (Pt 12):**3145-52.

Prchla E, Kuechler E, Blaas D, Fuchs R. 1994. Uncoating of human rhinovirus serotype 2 from late endosomes. J.Virol. **68:**3713-23.

Prempeh H, Smith R, Muller B. 2001. Foot and mouth disease: the human consequences. The health consequences are slight, the economic ones huge. BMJ **322:**565-6.

Pulli T, Roivainen M, Hovi T, Hyypia T. 1998. Induction of neutralizing antibodies by synthetic peptides representing the C terminus of coxsackievirus A9 capsid protein VP1. J.Gen.Virol. **79 (Pt 9):**2249-53.

Pulli T, Koivunen E, Hyypia T. 1997. Cell-surface interactions of echovirus 22. J.Biol.Chem. **272:**21176-80.

Pulli T, Koskimies P, Hyypia T. 1995. Molecular comparison of coxsackie A virus serotypes. Virology **212:**30-8.

Racaniello VR. 2013. *Picornaviridae*: The Viruses and Their Replication.Fields Virology453-89.

Racaniello VR, Baltimore D. 1981a. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc.Natl.Acad.Sci.U.S.A. **78:**4887-91.

Racaniello VR, Baltimore D. 1981b. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214:**916-9.

Rahamat-Langendoen J, Riezebos-Brilman A, Borger R, van der Heide R, Brandenburg A, Scholvinck E, Niesters HG. 2011. Upsurge of human enterovirus 68 infections in patients with severe respiratory tract infections. J.Clin.Virol. **52:**103-6.

Rankl C, Kienberger F, Wildling L, Wruss J, Gruber HJ, Blaas D, Hinterdorfer P. 2008. Multiple receptors involved in human rhinovirus attachment to live cells. Proc.Natl.Acad.Sci.U.S.A. **105:**17778-83.

Repass GL, Palmer WC, Stancampiano FF. 2014. Hand, foot, and mouth disease: identifying and managing an acute viral syndrome. Cleve.Clin.J.Med. **81:**537-43.

Rintanen N, Karjalainen M, Alanko J, Paavolainen L, Maki A, Nissinen L, Lehkonen M, Kallio K, Cheng RH, Upla P, Ivaska J, Marjomaki V. 2012. Calpains promote alpha2beta1 integrin turnover in nonrecycling integrin pathway. Mol.Biol.Cell **23:**448-63.

Rivera VM, Welsh JD, Maizel JV,Jr. 1988. Comparative sequence analysis of the 5' noncoding region of the enteroviruses and rhinoviruses. Virology **165:**42-50.

Roivainen M, Klingel K. 2009. Role of enteroviruses in the pathogenesis of type 1 diabetes. Diabetologia **52:**995-6.

Roivainen M, Alfthan G, Jousilahti P, Kimpimaki M, Hovi T, Tuomilehto J. 1998. Enterovirus infections as a possible risk factor for myocardial infarction. Circulation **98:**2534-7.

Roivainen M, Piirainen L, Hovi T. 1996. Efficient RGD-independent entry process of coxsackievirus A9. Arch.Virol. **141:**1909-19.

Roivainen M, Piirainen L, Hovi T, Virtanen I, Riikonen T, Heino J, Hyypia T. 1994. Entry of coxsackievirus A9 into host cells: specific interactions with alpha v beta 3 integrin, the vitronectin receptor. Virology **203:**357-65.

Roivainen M, Hyypia T, Piirainen L, Kalkkinen N, Stanway G, Hovi T. 1991. RGD-dependent entry of coxsackievirus A9 into host cells and its bypass after cleavage of VP1 protein by intestinal proteases. J.Virol. **65:**4735-40.

Rossmann MG, He Y, Kuhn RJ. 2002. Picornavirus-receptor interactions. Trends Microbiol. **10:**324-31.

Rossmann MG, Arnold E, Erickson JW, Frankenberger EA, Griffith JP, Hecht HJ, Johnson JE, Kamer G, Luo M, Mosser AG. 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature **317:**145-53.

Rueckert RR, Wimmer E. 1984. Systematic nomenclature of picornavirus proteins. J.Virol. **50:**957-9.

Ruoslahti E, Pierschbacher MD. 1987. New perspectives in cell adhesion: RGD and integrins. Science **238:**491-7.

Russell SJ, Bell EJ. 1970. Echoviruses and carditis. Lancet **1:**784-5.

Sa-Carvalho D, Rieder E, Baxt B, Rodarte R, Tanuri A, Mason PW. 1997. Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. J.Virol. **71:**5115-23.

Sainato R, Flanagan R, Mahlen S, Fairchok M, Braun L. 2011. Severe human parechovirus sepsis beyond the neonatal period. J.Clin.Virol. **51:**73-4.

Santti J, Harvala H, Kinnunen L, Hyypia T. 2000. Molecular epidemiology and evolution of coxsackievirus A9. J.Gen.Virol. **81:**1361-72.

Sasisekharan R, Raman R, Prabhakar V. 2006. Glycomics approach to structure-function relationships of glycosaminoglycans. Annu.Rev.Biomed.Eng. **8:**181-231.

Sasisekharan R, Shriver Z, Venkataraman G, Narayanasami U. 2002. Roles of heparan-sulphate glycosaminoglycans in cancer. Nat.Rev.Cancer. **2:**521-8.

Schaffer FL, Schwerdt CE. 1955. Crystallization of Purified Mef-1 Poliomyelitis Virus Particles. Proc.Natl.Acad.Sci.U.S.A. **41:**1020-3.

Schnurr D, Dondero M, Holland D, Connor J. 1996. Characterization of echovirus 22 variants. Arch.Virol. **141:**1749-58.

Schuffenecker I, Javouhey E, Gillet Y, Kugener B, Billaud G, Floret D, Lina B, Morfin F. 2012. Human parechovirus infections, Lyon, France, 2008-10: evidence for severe cases. J.Clin.Virol. **54:**337-41.

Sedmak G, Nix WA, Jentzen J, Haupt TE, Davis JP, Bhattacharyya S, Pallansch MA, Oberste MS. 2010. Infant deaths associated with human parechovirus infection in Wisconsin. Clin.Infect.Dis. **50:**357-61.

Seitsonen J, Susi P, Heikkila O, Sinkovits RS, Laurinmaki P, Hyypia T, Butcher SJ. 2010. Interaction of alphaVbeta3 and alphaVbeta6 integrins with human parechovirus 1. J.Virol. **84:**8509-19.

Selvarangan R, Nzabi M, Selvaraju SB, Ketter P, Carpenter C, Harrison CJ. 2011. Human parechovirus 3 causing sepsis-like illness in children from midwestern United States. Pediatr.Infect.Dis.J. **30:**238-42.

Shafren DR, Dorahy DJ, Ingham RA, Burns GF, Barry RD. 1997. Coxsackievirus A21 binds to decay-accelerating factor but requires intercellular adhesion molecule 1 for cell entry. J.Virol. **71:**4736-43.

Shafren DR, Bates RC, Agrez MV, Herd RL, Burns GF, Barry RD. 1995. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. J.Virol. **69:**3873-7.

Shakeel S, Seitsonen JJ, Kajander T, Laurinmaki P, Hyypia T, Susi P, Butcher SJ. 2013. Structural and functional analysis of coxsackievirus A9 integrin alphavbeta6 binding and uncoating. J.Virol. **87:**3943-51.

Silberstein E, Xing L, van de Beek W, Lu J, Cheng H, Kaplan GG. 2003. Alteration of hepatitis A virus (HAV) particles by a soluble form of HAV cellular receptor 1 containing the immunoglobin-and mucin-like regions. J.Virol. **77:**8765-74.

Skinner MA, Racaniello VR, Dunn G, Cooper J, Minor PD, Almond JW. 1989. New model for the secondary structure of the 5' non-coding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J.Mol.Biol. **207:**379-92.

Smits SL, Raj VS, Oduber MD, Schapendonk CM, Bodewes R, Provacia L, Stittelaar KJ, Osterhaus AD, Haagmans BL. 2013. Metagenomic analysis of the ferret fecal viral flora. PLoS One **8:**e71595.

Smyth MS, Martin JH. 2002. Picornavirus uncoating. Mol.Pathol. **55:**214-9.

Snyers L, Zwickl H, Blaas D. 2003. Human rhinovirus type 2 is internalized by clathrin-mediated endocytosis. J.Virol. **77:**5360-9.

Sorkin A, von Zastrow M. 2009. Endocytosis and signalling: intertwining molecular networks. Nat.Rev.Mol.Cell Biol. **10:**609-22.

Stanway G, Joki-Korpela P, Hyypia T. 2000. Human parechoviruses--biology and clinical significance. Rev.Med.Virol. **10:**57-69.

Stanway G, Hyypia T. 1999. Parechoviruses. J.Virol. **73:**5249-54.

Stanway G, Kalkkinen N, Roivainen M, Ghazi F, Khan M, Smyth M, Meurman O, Hyypia T. 1994. Molecular and biological characteristics of echovirus 22, a representative of a new picornavirus group. J.Virol. **68:**8232-8.

Staunton DE, Merluzzi VJ, Rothlein R, Barton R, Marlin SD, Springer TA. 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56:**849-53.

Steinke JW, Borish L. 2016. Immune Responses in Rhinovirus-Induced Asthma Exacerbations. Curr.Allergy Asthma Rep. **16:**78.

Summers DF, Maizel JV,Jr. 1968. Evidence for large precursor proteins in poliovirus synthesis. Proc.Natl.Acad.Sci.U.S.A. **59:**966-71.

Tami C, Silberstein E, Manangeeswaran M, Freeman GJ, Umetsu SE, DeKruyff RH, Umetsu DT, Kaplan GG. 2007. Immunoglobulin A (IgA) is a natural ligand of hepatitis A virus cellular receptor 1 (HAVCR1), and the association of IgA with HAVCR1 enhances virus-receptor interactions. J.Virol. **81:**3437-46.

Tauriainen S, Oikarinen S, Oikarinen M, Hyoty H. 2011. Enteroviruses in the pathogenesis of type 1 diabetes. Semin.Immunopathol. **33:**45-55.

Tauriainen S, Oikarinen S, Taimen K, Laranne J, Sipila M, Lonnrot M, Ilonen J, Simell O, Knip M, Hyoty H. 2008. Temporal relationship between human parechovirus 1 infection and otitis media in young children. J.Infect.Dis. **198:**35-40.

Tauriainen S, Martiskainen M, Oikarinen S, Lonnrot M, Viskari H, Ilonen J, Simell O, Knip M, Hyoty H. 2007. Human parechovirus 1 infections in young children--no association with type 1 diabetes. J.Med.Virol. **79:**457-62.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673-80.

Thompson P, Lu J, Kaplan GG. 1998. The Cys-rich region of hepatitis A virus cellular receptor 1 is required for binding of hepatitis A virus and protective monoclonal antibody 190/4. J.Virol. **72:**3751-61.

Toivonen L, Schuez-Havupalo L, Karppinen S, Teros-Jaakkola T, Rulli M, Mertsola J, Waris M, Peltola V. 2016. Rhinovirus Infections in the First 2 Years of Life. Pediatrics **138:**10.1542/peds.2016-1309.

Tomassini JE, Graham D, DeWitt CM, Lineberger DW, Rodkey JA, Colonno RJ. 1989. cDNA cloning reveals that the major group rhinovirus receptor on HeLa cells is intercellular adhesion molecule 1. Proc.Natl.Acad.Sci.U.S.A. **86:**4907-11.

Toth KS, Zhang CX, Lipton HL, Luo M. 1993. Crystallization and preliminary X-ray diffraction studies of Theiler's virus (GDVII strain). J.Mol.Biol. **231:**1126-9.

Triantafilou K, Triantafilou M. 2003. Lipid raft microdomains: key sites for Coxsackievirus A9 infectious cycle. Virology **317:**128-35.

Triantafilou K, Fradelizi D, Wilson K, Triantafilou M. 2002. GRP78, a coreceptor for coxsackievirus A9, interacts with major histocompatibility complex class I molecules which mediate virus internalization. J.Virol. **76:**633-43.

Triantafilou K, Triantafilou M. 2001. A biochemical approach reveals cell-surface molecules utilised by Picornaviridae: Human Parechovirus 1 and Echovirus 1. J.Cell.Biochem. **80:**373-81.

Triantafilou K, Triantafilou M, Takada Y, Fernandez N. 2000a. Human parechovirus 1 utilizes integrins alphavbeta3 and alphavbeta1 as receptors. J.Virol. **74:**5856-62.

Triantafilou M, Triantafilou K, Wilson KM. 2000b. A 70 kDa MHC class I associated protein (MAP-70) identified as a receptor molecule for Coxsackievirus A9 cell attachment. Hum.Immunol. **61:**867-78.

Triantafilou M, Triantafilou K, Wilson KM, Takada Y, Fernandez N. 2000c. High affinity interactions of Coxsackievirus A9 with integrin alphavbeta3 (CD51/61) require the CYDMKTTC sequence of beta3, but do not require the RGD sequence of the CAV-9 VP1 protein. Hum.Immunol. **61:**453-9.

Triantafilou M, Triantafilou K, Wilson KM, Takada Y, Fernandez N, Stanway G. 1999. Involvement of beta2-microglobulin and integrin alphavbeta3 molecules in the coxsackievirus A9 infectious cycle. J.Gen.Virol. **80 (Pt 10):**2591-600.

Tsang SK, McDermott BM, Racaniello VR, Hogle JM. 2001. Kinetic analysis of the effect of poliovirus receptor on viral uncoating: the receptor as a catalyst. J.Virol. **75:**4984-9.

Turnbull J, Powell A, Guimond S. 2001. Heparan sulfate: decoding a dynamic multifunctional cell regulator. Trends Cell Biol. **11:**75-82.

Tuthill TJ, Groppelli E, Hogle JM, Rowlands DJ. 2010. Picornaviruses. Curr.Top.Microbiol.Immunol. **343:**43-89.

Upla P, Marjomaki V, Kankaanpaa P, Ivaska J, Hyypia T, Van Der Goot FG, Heino J. 2004. Clustering induces a lateral redistribution of alpha 2 beta 1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. Mol.Biol.Cell **15:**625-36.

van der Linden L, Wolthers KC, van Kuppeveld FJ. 2015. Replication and Inhibitors of Enteroviruses and Parechoviruses. Viruses **7:**4529-62.

van der Sanden S, de Bruin E, Vennema H, Swanink C, Koopmans M, van der Avoort H. 2008. Prevalence of human parechovirus in the Netherlands in 2000 to 2007. J.Clin.Microbiol. **46:**2884-9.

van Zwol AL, Lequin M, Aarts-Tesselaar C, van der Eijk AA, Driessen GA, de Hoog M, Govaert P. 2009. Fatal neonatal parechovirus encephalitis. BMJ Case Rep. **2009:**10.1136/bcr.05.2009.1883. Epub 2009 Dec 14.

Varki A, Cummings R, Esko J. 2009. Essentials of glycobiology.

Varki NM, Varki A. 2007. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab.Invest. **87:**851-7.

Verboon-Maciolek MA, Groenendaal F, Hahn CD, Hellmann J, van Loon AM, Boivin G, de Vries LS. 2008. Human parechovirus causes encephalitis with white matter injury in neonates. Ann.Neurol. **64:**266-73.

Vlasak M, Goesler I, Blaas D. 2005a. Human rhinovirus type 89 variants use heparan sulfate proteoglycan for cell attachment. J.Virol. **79:**5963-70.

Vlasak M, Roivainen M, Reithmayer M, Goesler I, Laine P, Snyers L, Hovi T, Blaas D. 2005b. The minor receptor group of human rhinovirus (HRV) includes HRV23 and HRV25, but the presence of a lysine in the VP1 HI loop is not sufficient for receptor binding. J.Virol. **79:**7389-95.

Walters B, Penaranda S, Nix WA, Oberste MS, Todd KM, Katz BZ, Zheng X. 2011. Detection of human parechovirus (HPeV)-3 in spinal fluid specimens from pediatric patients in the Chicago area. J.Clin.Virol. **52:**187-91.

Ward T, Powell RM, Pipkin PA, Evans DJ, Minor PD, Almond JW. 1998. Role for beta2-microglobulin in echovirus infection of rhabdomyosarcoma cells. J.Virol. **72:**5360-5.

Ward T, Pipkin PA, Clarkson NA, Stone DM, Minor PD, Almond JW. 1994. Decay-accelerating factor CD55 is identified as the receptor for echovirus 7 using CELICS, a rapid immuno-focal cloning method. EMBO J. **13:**5070-4.

Watanabe K, Oie M, Higuchi M, Nishikawa M, Fujii M. 2007. Isolation and characterization of novel human parechovirus from clinical samples. Emerg.Infect.Dis. **13:**889-95.

Weinacker A, Chen A, Agrez M, Cone RI, Nishimura S, Wayner E, Pytela R, Sheppard D. 1994. Role of the integrin alpha v beta 6 in cell attachment to fibronectin. Heterologous expression of intact and secreted forms of the receptor. J.Biol.Chem. **269:**6940-8.

Westerhuis B. 2014. The 3-dimensional play of human parechovirus infection; Cell, virus and antibody.

Westerhuis BM, Jonker SC, Mattao S, Benschop KS, Wolthers KC. 2013. Growth characteristics of human parechovirus 1 to 6 on different cell lines and cross- neutralization of human parechovirus antibodies: a comparison of the cytopathic effect and real time PCR. Virol.J. **10:**146,422X-10-146.

Whitton JL, Cornell CT, Feuer R. 2005. Host and virus determinants of picornavirus pathogenesis and tropism. Nat.Rev.Microbiol. **3:**765-76.

Wigand R, Sabin AB. 1961. Properties of ECHO types 22, 23 and 24 viruses. Arch.Gesamte Virusforsch. **11:**224-47.

Wildenbeest JG, Wolthers KC, Straver B, Pajkrt D. 2013. Successful IVIG treatment of human parechovirus-associated dilated cardiomyopathy in an infant. Pediatrics **132:**e243-7.

Wildenbeest JG, Harvala H, Pajkrt D, Wolthers KC. 2010. The need for treatment against human parechoviruses: how, why and when? Expert Rev.Anti Infect.Ther. **8:**1417-29.

Williams CH, Kajander T, Hyypia T, Jackson T, Sheppard D, Stanway G. 2004. Integrin alpha v beta 6 is an RGD-dependent receptor for coxsackievirus A9. J.Virol. **78:**6967-73.

Wolthers KC, Benschop KS, Schinkel J, Molenkamp R, Bergevoet RM, Spijkerman IJ, Kraakman HC, Pajkrt D. 2008. Human parechoviruses as an important viral cause of sepsislike illness and meningitis in young children. Clin.Infect.Dis. **47:**358-63.

Xing L, Tjarnlund K, Lindqvist B, Kaplan GG, Feigelstock D, Cheng RH, Casasnovas JM. 2000. Distinct cellular receptor interactions in poliovirus and rhinoviruses. EMBO J. **19:**1207-16.

Yamashita T, Sakae K, Tsuzuki H, Suzuki Y, Ishikawa N, Takeda N, Miyamura T, Yamazaki S. 1998. Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the Picornaviridae associated with acute gastroenteritis in humans. J.Virol. **72:**8408-12.

Yamashita T, Kobayashi S, Sakae K, Nakata S, Chiba S, Ishihara Y, Isomura S. 1991. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. J.Infect.Dis. **164:**954-7.

Yamayoshi S, Iizuka S, Yamashita T, Minagawa H, Mizuta K, Okamoto M, Nishimura H, Sanjoh K, Katsushima N, Itagaki T, Nagai Y, Fujii K, Koike S. 2012. Human SCARB2-dependent infection by coxsackievirus A7, A14, and A16 and enterovirus 71. J.Virol. **86:**5686-96.

Yamayoshi S, Yamashita Y, Li J, Hanagata N, Minowa T, Takemura T, Koike S. 2009. Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nat.Med. **15:**798-801.

Yang B, Chuang H, Yang KD. 2009a. Sialylated glycans as receptor and inhibitor of enterovirus 71 infection to DLD-1 intestinal cells. Virol.J. **6:**141.

Yang B, Chuang H, Yang KD. 2009b. Sialylated glycans as receptor and inhibitor of enterovirus 71 infection to DLD-1 intestinal cells. Virol.J. **6:**141,422X-6-141.

Yang SL, Chou YT, Wu CN, Ho MS. 2011. Annexin II binds to capsid protein VP1 of enterovirus 71 and enhances viral infectivity. J.Virol. **85:**11809-20.

Ylipaasto P, Eskelinen M, Salmela K, Hovi T, Roivainen M. 2010. Vitronectin receptors, alpha v integrins, are recognized by several non-RGD-containing echoviruses in a continuous laboratory cell line and also in primary human Langerhans' islets and endothelial cells. J.Gen.Virol. **91:**155-65.

Zhang P, Mueller S, Morais MC, Bator CM, Bowman VD, Hafenstein S, Wimmer E, Rossmann MG. 2008. Crystal structure of CD155 and electron microscopic studies of its complexes with polioviruses. Proc.Natl.Acad.Sci.U.S.A. **105:**18284-9.

Zhao X, Shi Y, Xia Y. 2016. Genome analysis revealed novel genotypes and recombination of the human parechoviruses prevalent in children in Eastern China. Gut Pathog. **8:**52.

Zimmermann H, Eggers HJ, Zimmermann A, Kraus W, Nelsen-Salz B. 1995. Complete nucleotide sequence and biological properties of an infectious clone of prototype echovirus 9. Virus Res. **39:**311-9.

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