Clinical Microbiology, 2026

How did I get here?

Adenovirus-host interactions for vector development

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I can't think of anything to say except
laughing is nice

Pink Floyd - Brain Damage

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Abstract

Adenoviruses (AdVs) have been developed as vectors for gene therapy, vaccines, and oncolytic applications, owing to their transduction efficiency, broad tropism, and well-established production platforms. Still, clinical translation is limited due to insufficient targeting and complex interactions with host factors. A detailed understanding of AdV-host interactions is needed for the rational design of next-generation vectors. This thesis explores AdV-host interactions in the context of vector development.

Human adenoviruses (HAdVs) are genetically divided into seven species (A-G), where species D is the largest and most diverse. The low seroprevalence of species D HAdVs has made them interesting as gene delivery platforms. In the first study, we identified a new chimeric HAdV-D virus, HAdV-20-42-42. HAdV-20-42-42 was vectorised and we demonstrated that it used the coxsackievirus and adenovirus receptor (CAR) and CD46 for attachment. We also demonstrated the vector's ability to induce a T cell response in mouse splenocytes. The ability to use dual receptors and activate adaptive immunity highlights the potential of species D HAdV as versatile gene delivery platforms.

In the second study, we generated four distinct A549 knockout models by targeting known HAdV receptors, resulting in cells deficient in CAR, CD46, DSG2, or sialic acid. Upon infection with HAdVs, we identified CD46 as the primary entry receptor for a majority of species D HAdVs. We used SPR analysis to demonstrate that binding to CD46 was mediated primarily by the HAdV hexon protein, suggesting the possibility for an interaction with high avidity. Together, these findings provide mechanistic insight into the molecular basis underlying the broad cellular tropism of species D HAdVs.

Given the central role of skeletal muscle as a target tissue for vaccine vectors, we wanted to explore whether HAdV transduction of these cells could be improved. Previously, the endogenous peptide lactoferricin (Lfcin) has been recognised to enhance HAdV-C5 infection in epithelial cells. In the third study, we investigated whether Lfcin could also enhance HAdV-C5 infection in human skeletal muscle cells. We found that Lfcin enhanced infection in a dose-dependent manner, but at very high concentrations it promoted viral particle clustering, which correlated with reduced enhancement. Addition of Lfcin during the early stages of infection markedly improved viral entry and transduction in both proliferating

myoblasts and terminally differentiated myotubes. In addition, Lfcin reduced serum-mediated neutralisation of HAdV-C5. These findings demonstrate how endogenous host factors can modulate HAdV infectivity, influence biodistribution, and counter neutralising antibodies.

These studies provide additional insights into HAdV-host interactions by exploring determinants of HAdV tropism, and can be used for the development of safer, more targeted, and more effective AdV-based vectors.

Enkel sammanfattning på svenska

Det uppskattas finnas närmare 10^{31} viruspartiklar på jorden [1]. Trots denna enorma mängd finns det många som är relativt okända utanför forskarvärlden, däribland de virus jag forskat på: adenovirus. Adenovirus är en stor familj med många olika virus. De som kan infektera människor är kategoriserade i sju grupper (A-G), men historiskt har dessa främst förknippats med lindriga symptom såsom en lätt förkylning. Till utseendet ser alla adenovirus ut som en mikroskopisk 20-sidig tärning där det från var och ett av de tolv hörnen sticker ut armar som kallas fibrer. Ungefär tiotusen virus får plats på längden av en millimeter.

Så varför studera virus som historiskt bara ansetts ge lindriga symptom? Jo, medicinskt kan adenovirus användas som *vektorer*; det vill säga virus som modifierats för att fungera som leveransbud för till exempel vacciner eller terapeutiska gener. Risken för framtida pandemier gör det viktigt att vi utvecklar nya plattformar för vaccinutveckling och en del genetiska sjukdomar saknar idag effektiv behandling. För att en vektor ska fungera optimalt måste man se till att den hamnar på rätt plats i kroppen och att den överlever kroppens immunförsvar länge nog för att leverera sin last.

Denna avhandling handlar om adenovirus och hur vi kan använda dem som vektorer för vaccination och andra tillämpningar.

I första delen av detta arbete upptäckte vi ett nytt adenovirus, som visade sig tillhöra den största guppen adenovirus, grupp D. Vi modifierade det till vektor för att utvärdera dess potential som leveransplattform för vacciner eller genterapi och fann att det hade många egenskaper som lämpade sig för detta. Genom att använda en biobank av mänskliga blodprover kunde vi se att de flesta inte hade antikroppar mot detta virus, vilket är bra om man vill använda det som vektor. Vi undersökte sedan hur viruset tar sig in i celler och fann att det kan använda sig av två olika proteiner på cellytan, vilket gör att det kan användas på flera ställen i kroppen. Vi undersökte också hur virusvektorn påverkade immunsvaret hos möss och fann att den stimulerar utvecklingen av immunitet mot den vaccin-antigen som vektorn bär på. Denna information påverkar när och hur vi kan använda adenovirus som vektorer.

I den andra delen av detta arbete ville vi förstå hur adenovirus från grupp D sprids i kroppen. För spridning måste virus hitta en väg in i våra celler och

vi upptäckte att grupp D adenovirus använder ett protein som heter CD46 för detta. Vi kunde visa att många adenovirus från grupp D binder till CD46 på ett sätt som gör interaktionen extra stabil. Genom att viruset har många bindningspunkter på sin yta kan det interagera med flera CD46-proteiner samtidigt. CD46 finns för övrigt på nästan alla celler i vår kropp, vilket kan förklara varför vissa adenovirus från grupp D kan infektera många olika celler och vävnader i kroppen. Detta har både för och nackdelar när man vill använda dem som vektorer och ger en inblick i hur deras väg in i celler kan styra var de infekterar.

Skelettmuskler är en viktig målvävnad för vektorbaserade vacciner, men adenovirus från grupp C har svårt att ta sig in i muskelceller. Problemet är att dessa celler inte har det viktiga ytproteinet CAR (som står för Coxsackie och Adenovirus-Receptorn). Utan CAR på cellytan har grupp C adenovirus ingen effektiv väg in. Tidigare studier har visat att ett kroppseget proteinfragment, lactoferricin, ökar upptag av adenovirus i lungceller och vi ville därför undersöka om lactoferricin även kan öka upptag av adenovirus i skelettmuskelceller. Vi fann att tillsats av lactoferricin tidigt under infektion kraftigt förbättrade virusupptaget i skelettmuskelceller. Dessutom kunde lactoferricin delvis skydda adenoviruset mot neutraliserande antikroppar, vilka annars hämmar vektorer från att fungera optimalt. Dessa resultat ger en inblick i hur vi kan använda olika molekyler för att förbättra upptag av adenovirus-baserade vektorer.

Sammanfattningsvis ger dessa arbeten en ökad förståelse för hur adenovirus interagerar med värdceller och identifierar faktorer som styr var och hur olika adenovirus kan infektera. Resultaten öppnar dörren för att utveckla säkrare, mer effektiva adenovirus-baserade vektorer.

List of publications

Publications included in thesis

The included publications in the thesis are referred to by their roman numeral (I-III) and are published under a Creative Commons Attribution 4.0 International (CC BY 4.0) License and the content, including text and figures, is reused in this thesis with permission from the original publisher.

(I) **Human AdV-20-42-42, a Promising Novel Adenoviral Vector for Gene Therapy and Vaccine Product Development**
M. Z. Ballmann, S. Raus, R. Engelhart, G. L. Kajan, A. Beqqali, P.W.F. Hadoke, C. van der Zalm, T. Papp, L. John, S. Khan, S. Boedhoe, K. Danskog, L. Frangsmyr, J. Custers, W.A.M. Bakker, H.M. van der Schaar, N. Arnberg, A.A.C. Lemckert, M. Havenga and A.H. Baker (2021). J Virol 95(22): e0038721.

(II) **Lactoferricin enables adenovirus infection of human skeletal muscle cells**
K. Danskog, N. Mistry, C. Ardahl, M. Durbeej, M.N.E. Forsell, A. Lenman and N. Arnberg (2025). Npj Viruses 3(1): 62.

(III) **CD46 is a cellular receptor for species D human adenovirus**
K. Danskog, F. Petersen, L. Frangsmyr, G. Gonzalez, M. Becker, A. Lenman and N. Arnberg (2025). mBio 16(11): e0158725.

Publications not included in thesis

i. **Structure-guided design of trivalent 5-N-acetylneuraminic acid inhibitors to improve potency and explore range against human adenovirus infections**
M. Strebl, E. Johansson, P. Kumar, R. Caraballo, B.D. Persson, P. Bachmann, K. Danskog, F. Petersen, M.A. Liaci, N. Arnberg, M. Elofsson, T. Stehle (2026). (Manuscript)

ii. **Bile acids accumulate norovirus-like particles and enhance binding to and entry into human enteric epithelial cells**
E. Hahlin*, K. Danskog*, S. Nord, M. Becker, S.M.A. Willekens, C. Wibom, H. Tanner, D. Öhlund, L. Sandblad, A. Lenman, N. Arnberg (2026) J Virol. doi:10.1128/jvi.00342-26 (*authors contributed equally)

iii. **Adenovirus cell entry. Chapter 2, Adenoviral Vectors for Gene Therapy (Third Edition)**
K. Danskog, E. Hahlin and N. Arnberg (2025). D. T. Curiel and A. L. Parker. San Diego, Academic Press: 45-73.

iv. **Structural insights into the interaction between adenovirus C5 hexon and human lactoferrin**
A. Dhillon, B.D. Persson, A. N. Volkov, H. Sulzen, A. Kadek, P. Pompach, S. Kereiche, M. Lepsik, K. Danskog, C. Uetrecht, N. Arnberg and S. Zoll (2024). J Virol 98(3): e0157623.

v. **BAF45b Is Required for Efficient Zika Virus Infection of HAP1 Cells**
B. D. Persson, S. Nord, R. Lindqvist, K. Danskog, A. K. Overby, A. Kohl, H. J. Willison, A. Lenman and N. Arnberg (2021). Viruses 13(10).

Abbreviations

AdV	Adenovirus
ARD	Acute respiratory disease
AVP	Adenovirus Protease
CAR	Coxsackie and Adenovirus Receptor
ChAd	Chimpanzee adenovirus
CD46/MCP	Cluster of differentiation 46/Membrane cofactor protein
cDNA	Complementary DNA
CMAS	Cytidine monophosphate N-acetylneuraminic acid synthetase
CMV	Cytomegalovirus
CS	Chondroitin sulfate
CsCl	Caesium Chloride
CTL	Cytotoxic T cell
DBP	DNA-binding protein
dsDNA	Double-stranded DNA
DSG2	Desmoglein 2
E	Early gene
EDTA	Ethylenediaminetetraacetic acid
EM	Electron Microscopy
EKC	Epidemic Keratoconjunctivitis
FFA	Focus Forming Assay
FFU	Focus Forming Unit
FIX	Coagulation Factor IX
FVII	Coagulation factor VII
FX	Coagulation Factor X
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
Gla	Glutamic acid
gRNA	Guide RNA
HAdV	Human Adenovirus
HD-5	Human alpha-defensin 5
HepIII	Heparinase III
HIV	Human Immunodeficiency Virus
HNP-1	Human alpha-defensin 1 derivative
HPLC	High performance liquid chromatography
HSPG	Heparan sulfate proteoglycans
HVR	Hypervariable region
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
ITR	Inverted terminal repeat
kDa	Kilo Dalton
K_D	Equilibrium dissociation constant
L	Late gene
LF	Lactoferrin
Lfcin	Lactoferricin
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NAb	Neutralising antibody
PEG	Polyethylene glycol
PLL	Poly-L-lysine
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RGD	Arginine-Glycine-Asparagine
RU	Response unit
SAdV	Simian adenovirus
SEM	Standard error of the mean
SCR	Short consensus repeat
SD	Standard deviation
SPR	Surface Plasmon Resonance
SR	Scavenger Receptor
STP region	Serine, threonine, proline rich region
TLR	Toll like receptor
TP	Terminal Protein
vp	Virus particle

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or how did I get here?
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Musical references, in order of appearance: Talking Heads – Once in a Lifetime; Magdalena Bay – The Beginning; Beastie Boys – The Sound of Science; Talking Heads – Pulled Up; Tina Turner – The Best; Jack Johnson – Better Together; Shania Twain – Man! I Feel Like a Woman!; Dire Straits – Walk of Life; Jessica Andersson – Party Voice; Anna Book – Samba Sambero; Chely Wright – The Bumper of My S.U.V.; Celine Dion – That's the Way it is; Pink Floyd – Shine on You Crazy Diamond; Helen Sjöholm – I gott bevar; FELICIA – My System; Charli xcx – Rewind; Carly Rae Jepsen – First Time; Billy Joel – Scenes from an Italian Restaurant; Nine Inch Nails – Meet Your Master; Queen – Bohemian Rhapsody; DJ Sabrina The Teenage DJ – Next To Me; AC/DC – It's a Long Way To The Top (If You Wanna Rock 'N' Roll); Talking Heads – Uh-Oh, Love Comes to Town; HOOJA – GAMMAL I GEMET; Helene Fischer – Herzbeben; Björk – Undo; Monty Python – Always Look On The Bright Side Of Life; Pusha T – If You Know You Know; Euskefeurat – Det Är Hit Man Kommer När Man Kommer Hem; Sindey Gish – Impostor Syndrome; Fricky – Aqua Aura; Tommy Körberg - Som en bro över mörka vatten; Electric Banana Band – Banankontakt; Gustaf Fröding – Strövtåg i hembygden; Ted Gärdestad – Sol, vind och vatten; Uje Brandelius – Ramlar mot varandra; Molly Sandén - Det bästa kanske inte hänt än

Introduction

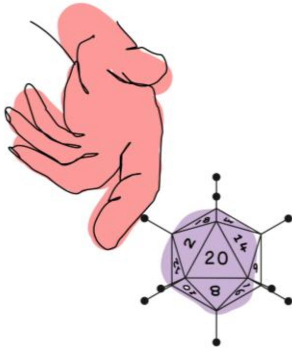

Figure 1. *An adenovirus looks like a 20-sided die used in role playing games like dungeons and dragons.*

Statistically, there is a greater than 50% probability that I have encountered at least one adenovirus at some point in my life [2], (a likelihood that has increased after years of working with them in the lab). As a player of dungeons and dragons I also find myself carrying the virus during every session, metaphorically speaking, as the 20-sided die (d20 for short) happens to be an almost perfect replica of the adenovirus capsid (**Figure 1**). Viruses have evolved to replicate efficiently with minimal resources, and constructing a capsid using triangular lattices of repeating proteins is thermodynamically very efficient [3]. In fact, the d20 structure (called icosahedral) of adenoviruses is one of the more prevalent geometric shapes in nature. It maximises storage capacity by being the closest you can get to a sphere without actually being one, and triangles are a strong geometrical shape, providing the virus with a rigid shell that can endure the conditions inside and outside the body.

1. Adenoviruses

Adenoviruses (AdVs) were first isolated in 1953 from adenoid tissue [4], and initially termed an adenoid degeneration agent. Further studies of the isolated virus recognised it as a causative agent of acute respiratory disease (ARD). Through AdVs we have learned much about virus and human biology. In 1962 a human AdV (HAdV) was shown to induce tumour growth in hamster, providing the first evidence of oncogenic activity by a human virus [5]. In 1968, the HAdV hexon was the first viral protein to be crystallised [6]. In 1977 RNA splicing was discovered in HAdVs [7]; a discovery which in 1993 was awarded the Nobel Prize. Today, HAdVs are known as ubiquitous human pathogens, frequently associated with self-limiting respiratory illness, such as the common cold. Serological studies demonstrate that by adulthood, most people have been exposed to at least one HAdV type, as evidenced by circulating neutralising antibodies (NAbs) [2]. This widespread prevalence and well-characterised biology have

positioned AdVs as important models in biology, and more recently, as platforms for gene therapy, vaccination, and oncolytic applications.

Within the *Adenoviridae* family there are six known genera of AdVs, of which one, *Mastadenoviruses* contain the HAdV types that infect humans [8]. Today, more than 100 distinct HAdVs have been identified [9]. Based on genetic variations they are divided into seven species, HAdV-A to -G. Prior to the 21st century, serology was the predominant method for identification of new types, while today, sequencing-based methods are being used. This has sky-rocketed the number of new types discovered (**Figure 2**) [10]. By a large margin, species D HAdVs contain the most members, presumably because of recombination [11, 12]. Within species D, the genome is mostly conserved. The GC content is above 50%, but it decreases sharply in regions flanking structural proteins, facilitating recombination of hexon, penton and fiber between species D members during co-infections [13].

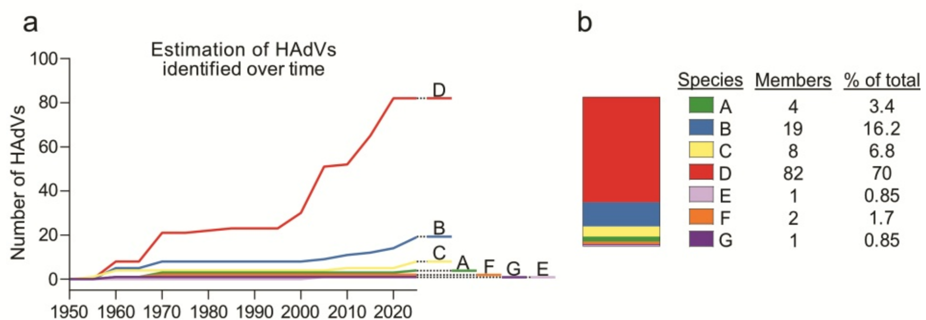

Figure 2. *There are seven species of HAdVs, where species D is the largest.* ***a.*** *Estimation of HAdVs identified over time, divided by species. HAdV typing shifted from serology to sequencing-based methods in the late 1990s.* ***b.*** *HAdV members per species, presented as absolute numbers and as percent of total HAdVs identified. HAdV-E and G contain only one member each, and HAdV-F two. HAdV-C were the first to be isolated, and 70% of HAdVs are species D. Information is from Human Adenovirus Working Group [9].*

Members of different HAdV species present with varied but also overlapping tropism, infecting respiratory, enteric, renal, ocular, and lymphoid tissues. Species C HAdVs are mainly associated with respiratory tropism, but can cause persistent infections in adenoids and tonsils, where T cells are major targets [14, 15], which is enigmatic as they lack the textbook primary attachment factor, the Coxsackie and adenovirus receptor (CAR). Species F (HAdV-F40, and -F41) are a leading cause of diarrhoea-associated mortality in children under the age of five [16]. Some species D HAdVs can cause epidemic keratoconjunctivitis (EKC), including HAdV-D8,

-D37, -D53, -D54, -D56, and -D64 [17-19], where HAdV-D8 and -D37 are also associated with genital infections [20], and HAdV-D56 was isolated from a case of fatal neonatal pneumonia [13]. Species D HAdVs are associated with gastroenteritis, laryngitis and bronchiolitis. Also, hepatitis and urethritis has been observed in immunocompromised individuals infected by HAdVs.

2. Structure

Although HAdVs were discovered in 1953, a high-resolution structure was available first in 2010 [21]. From this, the organisation of the individual capsid proteins could be determined. HAdVs are non-enveloped, double-stranded (ds)DNA viruses with an icosahedral capsid built by three major exposed capsomers (hexon/II penton base/III, and fiber/IV), along with four minor capsid proteins (IIIa, IX, VI, and VIII), and six core proteins (µ, V, VII, IVa2, terminal protein (TP), and the AdV protease (AVP)) (**Figure 3**). The weight and copy numbers of the structural protein is given in **Table 1.**

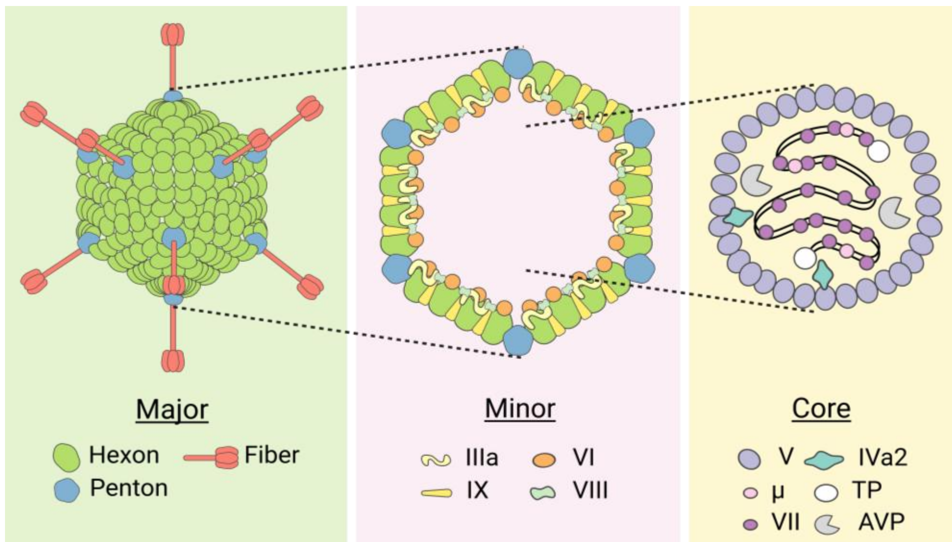

Figure 3. *AdV structural proteins. HAdVs consist of three major capsomers forming the icosahedral capsid: the hexon (green), penton (blue), fiber (coral). There are four minor proteins important for retaining the structure of the capsid: protein IIIa, VIII and VI are located inside the capsid underneath the hexon, while minor protein IX forms a triskeletal structure on the outside of the viral capsid, around the hexon trimers [22, 23]. The core proteins are multifunctional and help with condensation and protection of the viral genome, regulation of gene expression, and coordination during assembly and uncoating [24, 25]. Image created with BioRender.com (2026).*

Table 1. *Protein name, molecular weight, and the copy numbers of each HAdV protein reported in the references in the last column.*

	Protein	Size (kDa)	Copy nr.	References
Major	Hexon (II)	108	720	[26]
	Penton base (III)	63	60	[26]
	Fiber (IV)	61	36	[26]
Minor	IIIa	65	60	[27, 28]
	VI	27	360	[27, 28]
	VIII	25	120	[27, 28]
	IX	14.3	600	[28]
Core	V	41.5	150	[28]
	VII	22	500-800	[27, 28]
	μ	8.8	100-290	[27, 28]
	IVa2	50.8	5	[28]
	TP	76.5	2	[28]
	AVP	23	7	[28]

The fiber protein is considered as primary mediator of receptor interactions. The molecular structure of the fiber is shown in **Figure 4**. It is a trimeric protein of 319 to 587 amino acid in length (depending on HAdV type) with three domains: a tail, a shaft, and a knob. The tail anchors the fiber to the penton base, and the protruding shaft ends with an N-terminal knob domain that has different properties depending on the species and genotype. The shaft domain of the fiber is built up by repeats of two β-strands, where the number of repeats determines length of the shaft. The fiber shaft also contains flexible repeats which affect its ability to bend and find a receptor. For example, the HAdV-C5 shaft has 22 repeats with several flexible parts, whereas HAdV-D37 has 8 repeats and is more rigid [29, 30]. In the case of HAdV-D37 this interferes with the ability to engage with CAR [29].

The fiber knob has a trimeric propeller shape, where each monomer is an eight-stranded antiparallel β-sandwich consisting of two separate β-sheets, where one sheet is built by strands named G, H, I, and D, and the other built

by strands named A, B, C, and J. Loops are formed between the β-strands, and are named according to the strands they connect, for example, between the D and the G strand spans the DG loop. The loops are important in binding, where for example the AB loop is the main source of contact with CAR [31]. Most *Mastadenoviruses* contain only one gene for the fiber protein, but species F (HAdV-F40, and -41) and G (HAdV-G52) HAdVs has two fibers: a long and a short. Simian AdVs (SAdVs) from species G have three different fiber genes [32], and the only member of the genus *Ichtadenovirus*, the white sturgeon, AdV-1 was found to contain four fiber-like genes in genomic analysis, although it is unclear whether all code for functional proteins [33].

The hexon covers a majority of the capsid surface and contributes to around 60 % of the virion mass [34]. The molecular structure of the hexon is shown in **Figure 4**. The hexon is a trimer where each facet of the virus capsid contains 12 trimers, and in total each virus is built by 240 hexon trimers. In total there are 720 hexon monomers, each made up of 919 to 968 amino acids depending on the type [35]. The base of the hexon is folded into two eight-stranded β-barrels separated by a smaller β-sheet, and from the base, loops extend to form the tower region [34]. The overall structure and sequence identity of the hexon is highly conserved within the *Adenoviridae* family, but the hexon tower loops vary in length, composition, and form the so-called hexon hypervariable regions (HVRs). In total, there are seven serotype-specific HVRs per hexon, named HVR1-7, with a length between 2–38 residues, located on two loops which together form the epsilon determinant: serotype-specific B-cell epitopes, recognised by antibodies [36, 37]. The HVRs are also important for interactions *in vivo*. For example, HVR1 of HAdV-C5 has a strong negative charge [38], which enables its interactions with defensins and lactoferrin [39-42]. Additionally, HVR5 and HVR7 are important in mediating interaction with blood coagulation factors [43].

At each vertex of the HAdV capsid is a penton base that anchors the fiber to the capsid, and as its name suggests, it is a pentameric protein. The molecular structure of the penton base is shown in **Figure 4**. The penton base is 470-570 (depending on HAdV type) residues with highly conserved homology between species. The differences in length can be pinpointed to the two exposed penton hypervariable loops, of which one contains the integrin-binding RGD-motif, present in all HAdV species but F [44]. The second penton hypervariable loop is shorter, varying between 12 and 23 residues, and its role is less defined, but its high sequence variability

implies it as an immune epitope for NAbs [44, 45]. The integrin-binding properties of HAdVs is considered essential for infectious uptake in many cell types. Early on it was discovered that HAdV-B3 penton bases self-assembles into dodecameric forms during infection [46, 47]. These forms sometimes also contain fiber proteins and can enter cells the same way as complete virus particles [48]. The HAdV-B3 penton dodecamers have been shown to loosen epithelial cell junctions by binding to desmoglein-2 (DSG2) [49], a similar mechanism to how HAdV-C2 fibers loosen CAR-CAR interactions between cells to facilitate apical escape [50].

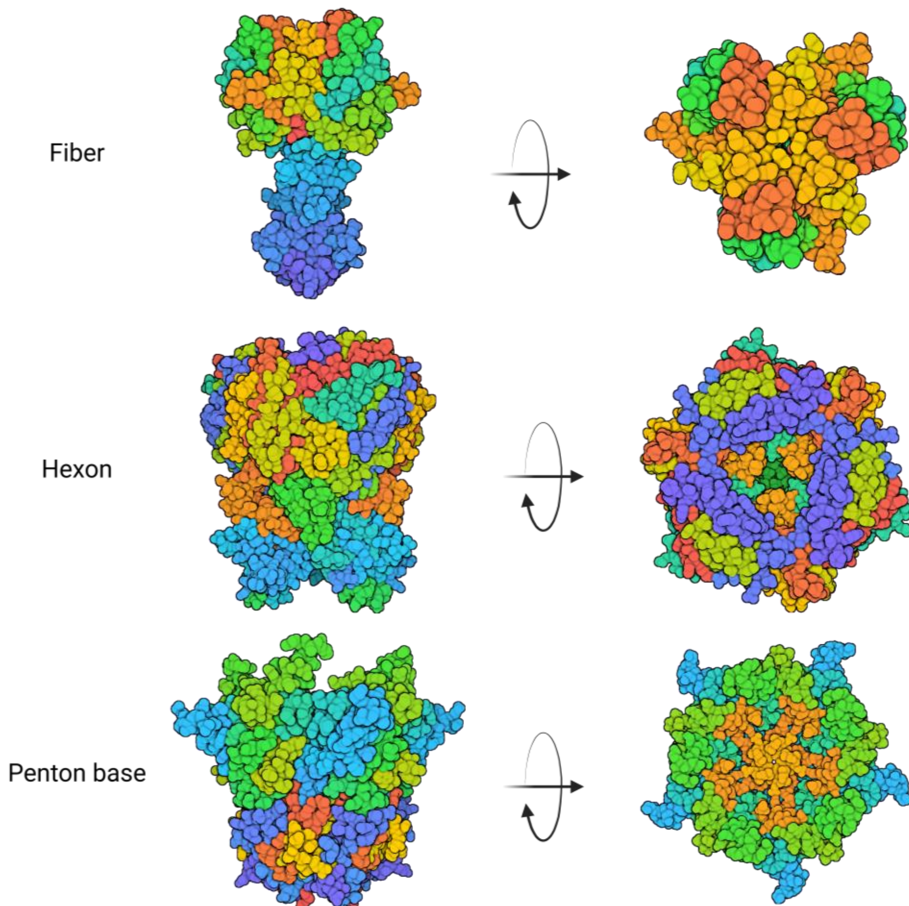

Figure 4. *Space-filling models of the fiber knob trimer (top), hexon trimer (middle), and penton base pentamer (bottom). Lateral view (left), and top-down perspective (right). Colours represent individual chains of the proteins. Image created with BioRender.com (2026), using PDB structures of the HAdV-C2 fiber: 1QIU; HAdV-F41 penton base: 6Z7Q; and HAdV-C5 hexon: 3TG7.*

HAdVs also contain four minor structural proteins: IIIa, VI, VIII, and IX. Although they are called minor, they provide important functions in that they help cementing, and structuring the capsid, as well as arranging the genome. Proteins IIIa, VI, and VIII are located inside the capsid, and IX twists around the hexon proteins, partially exposed on the outside. Protein IIIa stabilises the capsid by connecting the penton bases to the hexons [34], but the full extent of its functions is unknown. Protein VI is involved in transport of hexons via its nuclear localisation signals and nuclear export signals [51]. Protein VIII function as a reinforcement between hexons at angles to increase stability [34]. Protein IX is the only exposed minor capsid protein and unique to the *Mastadenovirus* genus [34], providing a potentially interesting target for capsid modifications.

The HAdV genome codes for six core proteins: V, VII, μ, TP, IVa2, and AVP. Proteins V, VII, and μ all carry a net positive charge, enabling interaction with the negatively charged genome [28, 52]. Protein V is essential for efficient assembly [53]. Protein VII is important in transporting the viral genome into the nucleus early in infection, and in condensing the genome during assembly [25]. The μ protein has been shown to condense dsDNA in solution together with protein VII [54]. The genome is covalently bound to a TP on each of its 5' ends, that serves as primers for DNA replication and protects the genome from nucleases [55]. The IVa2 protein is multifunctional, and present during transcription, where it regulates the expression of structural proteins. Studies have also shown that it is found at a single vertex within the capsid, where it provides the energy required to package DNA into the newly formed capsids [56]. The AVP is a cysteine endopeptidase essential for successful infection. It is active early, involved in virus uncoating at the nuclear membrane [57], and during assembly, where it cleaves several HAdV precursor proteins. It is a unique viral protease in that its activity is enhanced by DNA, essentially using it as a cofactor [27].

The HAdV genome contains additional genes, coding for non-structural proteins. The functions of these are less well described, but many are associated with regulatory functions. For example, the L4-100 kDa non-structural protein is essential late in infection by virtue of it functioning as a driver of late gene expression and acting as chaperone for hexon assembly [58]. Another example is the viral DNA-binding protein (DBP), which protects viral DNA from nuclease activity and helps unwinding it during replication [27].

3. The infectious cycle

The HAdV infectious cycle is initiated by primary attachment via the fiber knob followed by a lower affinity binding between penton and cellular integrins, which triggers endocytosis [59, 60]. The HAdV infectious cycle is summarised in **Figure 5**.

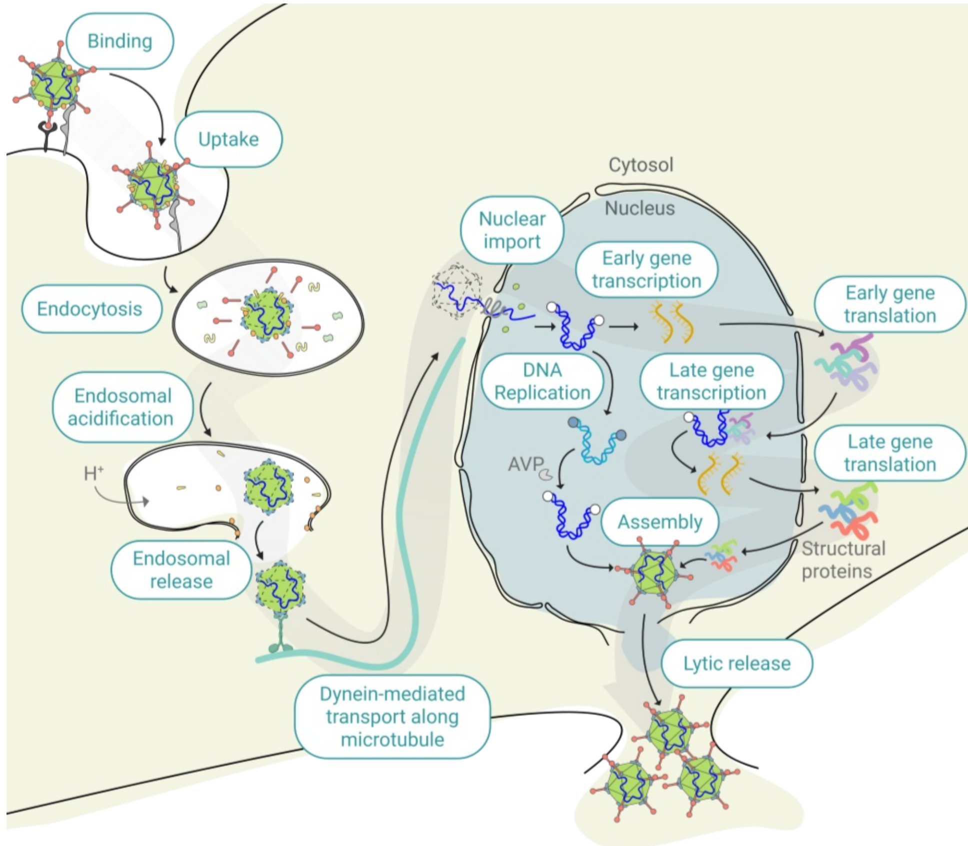

Figure 5. *The HAdV infectious cycle. After binding to a primary receptor, HAdV particles are internalised via receptor-mediated endocytosis. Endosomal acidification induces conformational changes and partial capsid disassembly, allowing viral release into the cytoplasm. The partially uncoated capsid is transported along microtubules toward the nucleus, where viral DNA is imported through the nuclear pore complex and replication begins. Early gene expression drives the production of regulatory and non-structural proteins that prime the cellular environment to support viral replication. In the late phase, structural proteins are synthesised and assembled into mature virions, which are packaged with newly replicated genomes and released upon host cell lysis. Image created with BioRender.com (2026).*

3.1. Attachment

Much like Bear Grylls, viruses are survivors, and live by *improvise, adapt, and overcome* to ensure successful infection. However, even the most elite infiltrator requires a way through the front door. The ability of HAdVs to enter cells is largely dependent on the molecules at the cellular surface, which is why the identification and characterisation of these have become an important topic within HAdV research. Several host proteins and glycans interact with the HAdV capsid, and although the fiber is mediating many of these interactions, the penton and hexon are equally important considering the whole life cycle. Some of the most important interaction partners of the HAdV capsid proteins are summarised in **Figure 6**.

Different surface molecules support different functions during attachment and entry. To clarify the roles of each interaction, the following definitions are used: **Receptors** bind the virus and actively promote entry; **attachment factors** bind the virus particle but are not required for uptake; **entry receptors** are defined as molecules capable of binding virions and are essential for infection; **bridging factors** bind the virus capsid and can mediate attachment to cell surface molecules.

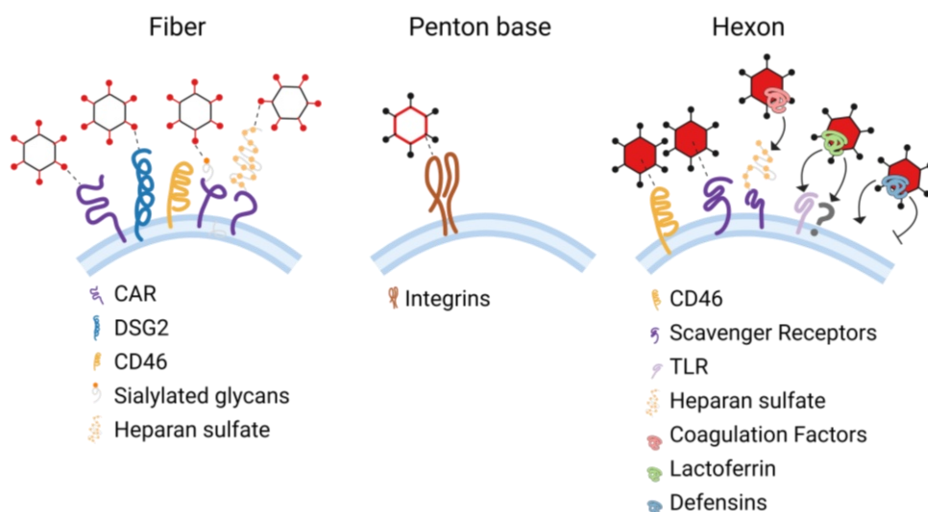

Figure 6**. Human adenovirus-host interactions. HAdV interactions with host proteins and receptors divided by which viral capsid protein mediates the interaction. Fiber: CAR [61, 62],** *DSG2 [63], CD46 [64-66], Heparan sulfate proteoglycans (HSPGs) [67, 68], sialic acid [69-72]. Penton: integrins [73-78]. Hexon: CD46 [79], Scavenger receptors [80, 81], Coagulation factors [82-85], Defensins [86, 87], Lactoferrin [40, 41, 88, 89]. Image created with BioRender.com* (2026).

3.1.1. Fiber-mediated interactions

The fiber projects outward from the HAdV capsid, scanning the cellular surface for binding partners. By coordinating this initial contact, it is not surprising that it is considered the primary mediator of viral attachment.

The main attachment factor for many HAdV species *in vitro* is CAR. All HAdV species except B have been shown to bind CAR either *in vivo*, *in vitro,* or biochemically, via the fiber knob [61, 62], and the HAdV-CAR interactions have been studied in detail where the structure and residues mediating binding have been identified [31, 90, 91]. CAR itself is composed of two extracellular domains (D1 and D2), a transmembrane domain, and a C-terminal cytoplasmic tail. The D1 domain provides the majority of the surface contact with the HAdV fiber knob, primarily to the AB loop, but also the CD loop and the FG loop. The nanomolar affinity between HAdV-C5 fiber knob and CAR [92], enables attachment, and lead to receptor-mediated entry via integrins, in otherwise non-permissive tissues. For instance, expression of human CAR in mice (which are naturally refractory to HAdVs) enable more efficient transduction of the virus [93, 94]. Despite the strong binding of fiber knob to CAR, the interaction is a weak determinant of HAdV tropism. CAR is not expressed apically, but rather basally and in tight junctions on polarised epithelial cells. Therefore, it cannot function as main attachment factor during primary infection at the apical surface. Yet, studies done on polarised epithelial cells show that progeny HAdV-C2 released basolaterally can exploit excess fiber proteins, which disrupt CAR-CAR interactions and loosen the tight junctions between cells. This facilitates viral spread to the apical side where a new round of infection can proceed [50, 90]. Species D HAdV fiber knobs can interact with CAR biochemically at micro- to nanomolar affinities, yet the interaction appears non-functional *in vivo*, as the DG loop provides steric hinderance for engagement with entry receptors [29, 61, 95].

The long fibers of HAdV-F40, -F41, and -G52 are most similar to species C HAdVs and can engage CAR. The short fiber of HAdV-G52 can use sialic acid-containing glycans as attachment factors [62], whereas the short fiber of HAdV-F40, and -F41 bind HSPGs [67]. HSPGs are found on the surface of most cells and have further been identified as a decoy receptor for HAdV-D37 [96]. The exceptionally high positive charge of the HAdV-D37 fiber knob also enables direct interactions with sialic acid-containing glycans and the ganglioside GD1a glycan [69, 70]. HAdV-D15, -D64 -D24, -D26, -D29 and -D53 fiber knobs have been shown to bind sialic acid, albeit to a varying degree

depending on the cell line [71, 72, 97], indicating that a combination of attachment factors might be at play during species D HAdV binding.

Species B HAdVs have members that primarily binds either CD46 or desmoglein2 (DSG2) via the fiber knob [63-66] as attachment factors. HAdV-B3, -B7, -B11, and -B35 have all been shown to utilise CD46 for infection. In HAdV-B11, the HI, DG and IJ loop are involved in binding to CD46 [98]. CD46 is expressed on nearly all nucleated cells and plays a role in immune homeostasis via its regulatory effect on complement activation, and during infections as several viruses can use it as a receptor, including measles virus vaccine strains, and human herpesvirus 6 [99]. CD46 is a glycoprotein that consists of four short consensus repeats (SCR1-4) and a serine, threonine, proline rich region (STP region). It is anchored to the cell by a transmembrane domain followed by a short cytoplasmic tail. Human CD46 exists in multiple isoforms, where the most common variants, BC1, BC2, C1, and C2 (**Figure 7**), differ in the constitution of the STP region, which is also a major site for *O*-glycosylation. The BC1 and BC2 isoforms feature a longer STP region, and the C1 and C2 isoforms have a shorter STP region and consequently fewer *O*-glycosylation sites [100]. The SCRs are responsible for the interaction with HAdVs from species B, specifically the SCR1 and SCR2.

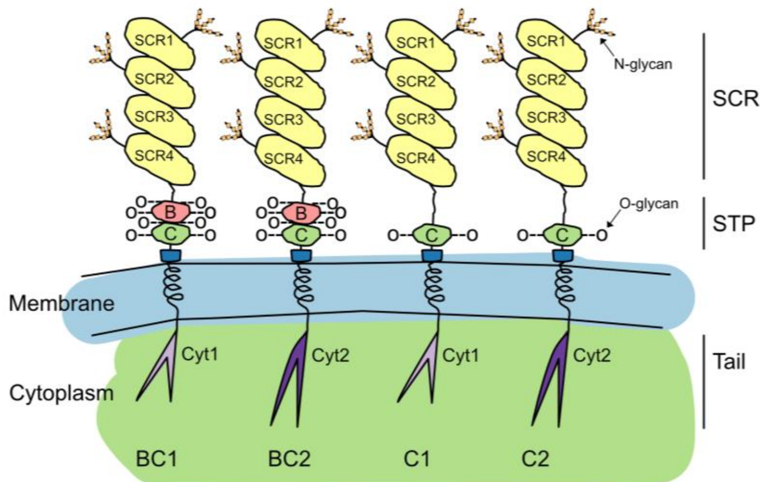

Figure 7. *Human CD46 exist in several isoforms, where the most common are called BC1, BC2, C1, and C2. The isoforms share the same structure in the short consensus repeat regions (SCRs), which consist of four repeats called SCR1-4, and contain three sites for N-glycosylation. The isoforms differ in the serine, threonine, proline-rich (STP) region (which additionally harbours several O-glycosylation sites), and in the cytoplasmic tail region.*

DSG2 is a cell adhesion molecule that together with desmocollin form junctions between epithelial cells [101]. HAdV-B3, -B7, -B11, -B14, and -B14p1 have been shown to use DSG2 as attachment factor on epithelial cells, and can use this interaction to open cellular junctions, providing greater access to other attachment factors [63, 102]. The EF loop of the fiber knob mediate DSG2 binding and may be involved in stabilising the interaction [102]. HAdVs that bind DSG2, such as HAdV-B3 and -B7, can still use CD46 if it is present in high density through increased avidity [103], and the HAdV-B3 fiber knob interacts with HSPGs, although in a low-affinity manner [68].

3.1.2. Hexon-mediated interactions

The hexons play an important role *in vivo* and mediate several interactions. It is a major immunological target, and it was recently discovered that several species D HAdVs can also engage with CD46 via the hexon [79], although it is not clear exactly which residues are mediating this interaction.

The hexon can via coagulation factors bridge binding to cellular heparan sulfate [82, 83]. Coagulation factors are proteolytic enzymes (zymogens) which circulate in the plasma as inactive agents. They have an N-terminal γ-carboxyl glutamic acid (Gla) domain, and a C-terminal serine protease domain. Carboxylation of the Gla domain enables binding of divalent cations such as calcium and this primes the coagulation factors for clotting [104]. The vitamin K-dependent factors; coagulation factor XI (FXI), coagulation factor X (FX), protein C, and coagulation factor VII (FVII) have all been shown to interact with the HAdV capsid and act as bridging factors [82-85]. FX in particular can interact with HVR7 and HVR5 on the hexon of some HAdV species A (HAdV-A12, -A18, and A31), and C (HAdV-C5, -C2) via the Gla domain [43, 105]. In HAdV-C5, a stretch of six residues in HVR7 and a glutamic acid residue in HVR5 are the primary mediators of the interaction to FX [106], and the interaction can be abolished by mutating HVR5 or HVR7 [43]. No additional HAdV species are known to interact with FX, but species A HAdV-A31 can bind coagulation factor IX (FIX) [84, 85].

Scavenger receptors (SRs) can also bind the HAdV-C5 hexon via HVRs, as PEGylation of HVR1, 2, 3, 5, and 7, and removing HVR1 prevents HAdV-C5 binding to murine alveolar macrophage-like MPI cells [80, 107]. In this regard, SRs also function as entry receptors, as preincubating HAdV-C5 with SR-A ligands reduces uptake in J774 mouse macrophages, and

knockdown of SR-A6 (also known as MARCO) in MPI cells prevents binding and uptake of HAdV-C5 [80, 81].

In vivo, coagulation factors and SRs can redirect HAdVs to the liver, and intravenous delivery lead to virus elimination by resident liver macrophages known as Kupffer cells. Coagulation factors work as a bridging factor to heparan sulfate, which is present in abundance on hepatocytes, and SRs are macrophage receptors that HAdVs can bind to. Indeed, Kupffer cell-mediated clearance of HAdV-C5 is dependent on SR-A, as polyinosinic acid (which act as a ligand for SR-A), prevents Kupffer cells from clearing the viral particles [108, 109]. *In vivo* interactions are complex, as although FX promotes viral clearance by redirecting the virus to liver macrophages, it has also been shown to block hexon-targeting NAbs from binding to the virus capsid [110].

Defensins are small cationic amphipathic peptides that are part of the innate immune system. In humans two types of defensins are expressed, α- and β-defensins, where α-defensins are, in turn, classified as myeloid or enteric depending on tissue expression. Human α-defensin 5 (HD-5) and human α-defensin 1 derivative (HNP-1) have been shown to block HAdV-C5 by binding directly to the capsid, preventing endosomal escape and uncoating [42, 111]. HAdV species A, B, C, and E are sensitive to HD-5, and species A, B, and C are to varying degree inhibited by HNP-1 [42], On the other hand, HD-5 has been shown to enhance HAdV-D64 infection in A549 cells, also when the primary attachment factor sialic acid is blocked [87]. HD-5-mediated neutralisation has been linked to hexon HVR-1, HVR-7, but also to negatively charged regions in the fiber, and a conserved region within the penton base [39, 42, 87].

The hexon of HAdV species C as well as HAdV-D26 and -B35 interact with lactoferrin (LF) and its N-terminal peptide lactoferricin (Lfcin), and through this interaction infect cells in a CAR-independent manner [40, 41, 88, 89]. Structure of LF and amino acid composition of Lfcin are shown in **Figure 8**. Lactoferrin is a globular iron-binding glycoprotein of approximately 80 kDa. As its name suggests, it plays an important role in iron metabolism. Although it is present in multiple body fluids including blood serum, saliva and tear fluid, it is named after having been discovered in breast milk and is in fact continuously secreted by mammary glands [112]. Lactoferrin is also secreted locally by neutrophils at sites of inflammation, and by epithelial cells into body fluids [113]. LF has innate antimicrobial action and

exerts anticancer properties [114-117]. Due to its non-toxic nature in humans, LF has been used in clinical trials to treat for example anaemia [118, 119], gastrointestinal and respiratory symptoms in infants [120], and reduce mortality in preterm infants [121, 122]. Importantly, LF displays antiviral properties against many RNA- and DNA-viruses including SARS-CoV-2 [123, 124], respiratory syncytial virus [125] and herpes simplex virus 1 [126]. However, for species C HAdVs, LF is pro-viral [40]. It interacts with the HAdV hexon and is a bridging factor to Toll-like receptor 4 (TLR4) on dendritic cells [88], and to an unknown receptor on other cells. LF in the mucosa is cleaved by proteases into a 49 amino acid cationic peptide called lactoferricin (Lfcin), which also displays immunomodulatory effects [127, 128], and is pro-viral for HAdV-C5 [41]. At neutral pH Lfcin is positively charged and there are a number of residues in Lfcin implied in the interaction with HVR1 of the HAdV-C5 hexon, these being three Arginine residues R24, R27 and R30 as well as five lysine residues K28, K277, K280, K282 and K285 [89].

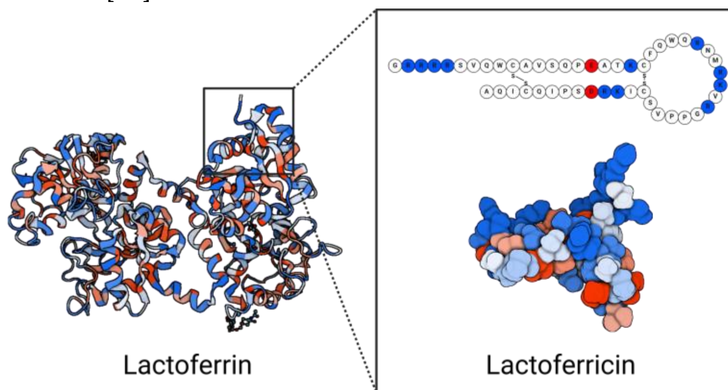

Figure 8. *Lactoferrin and lactoferricin. Lactoferrin is an approximately 80 kDa iron-binding glycoprotein that is cleaved by proteases, and from the N-terminal part a 49 amino acid cationic peptide called lactoferricin is produced. Image created with BioRender.com (2026), using PDB structures for lactoferrin: 1LFG; and lactoferricin (bottom): 1Z6V, coloured by hydrophobicity. In lactoferricin (top), residues marked in blue are positively charged and residues in red are negatively charged.*

3.1.3. Penton-mediated interactions

Although primary attachment factors differ between species, all HAdV can bind integrins via the penton base. The penton base anchors the fiber to the capsid, and acts as a hinge at the vertex of the viral shell. Integrins are generally not considered mediating primary interaction between virus and cell, but they are essential for infectious uptake in several cell types by conferring actin reorganisation and facilitating endocytosis [129]. This

makes them function as entry receptors. The fiber length affects the role of integrins, as long fiber length require more flexibility to allow for penton-integrin interactions, whereas shorter fiber length allows for better access to integrins [29, 95]. Integrins are heterodimeric proteins with an α and a β subunit that form a structure able to assume a bent, inactive, conformation, and an extended, active, conformation [130]. The partial obstruction of the penton base by the fiber makes conformational flexibility of integrins important, and structural studies have shown that the penton base can engage integrins in multiple binding modes [131]. Most HAdVs contain a highly conserved RGD motif within the penton base that mediates binding to RGD-recognising integrins [73-77], except species F (HAdV-F40 and -F41), which instead are able to bind laminin-binding integrins [78], and HAdV-D60, which does not have an RGD motif, but instead a Tyr-Gly-Asp (YGD) motif [132]. Different types of RGD-binding integrins are engaged by HAdVs: for example HAdV-C5 binds αvβ1, αvβ3, αvβ5, α5β1 and α3β1 integrins [75], HAdV-D26 use αvβ3 integrins for uptake in epithelial cells [77], HAdV-D37 can use αvβ1 and α3β1 integrins to enter human corneal cells [74] and HAdV-D56 transduction is improved in CHO cells expressing the laminin-binding integrin α7, which is a muscle cell marker [133].

3.2. Entry

HAdVs enter cells via receptor-mediated endocytosis, however, the exact mechanisms seem to differ between species. Inhibitors of the micropinocytosis pathway effectively blocks HAdV-B35 entry in epithelial cells [134], caveolin and dynamin appears to play a role in trafficking of HAdV-D26 and -D37 [135, 136] and HAdV-F40 and -F41 show a dependency on clathrin and dynamin during entry into intestinal cells [137]. Colocalisation has been observed between HAdV-C5 and lipid rafts in U266 plasmocytes, implying a role for caveolin in entry [138].

Early studies on HAdV-C2 entry revealed that the virus is internalised 5 minutes after binding, escapes the endosome after 15 minutes, and reaches the nuclear envelope after 35-45 minutes [139]. Within the endosomes, the capsid sheds the fibers [139]. Protein VI mediates partial viral disassembly, and infiltration of ions into the endosome reduce the pH, resulting in virus release into the cytoplasm [140]. The low pH treatment causes minor structural changes to the hexons, which prime them for interaction with dynein and transport along microtubules to the nuclear pore [141-144]. The genome is bound to core protein VII that protects it from the cellular DNA damage response [145], and the package is imported into the nucleus.

3.3. Replication

The AdV genome consists of linear dsDNA, and the size varies between genera. The HAdV genome is approximately 36 kb with regions of inverted repeats, and both ends are bound covalently by TP that enhance DNA replication [55]. Replication is divided into an early and a late phase. Early (E) transcriptional units consist of proteins E1A, E1B, E2, E3, E4 that regulate transcription and suppress the host immune response. The early phase triggers the cell to enter S phase whereby it can increase production of viral genes and downregulate production of cellular genes. The E1A protein binds to promoter regions to either promote or repress transcription of cellular genes, and E1B inhibits cellular genes associated with antiviral defence. The E2 gene unit codes for the HAdV DNA-binding protein (DBP), TP, and DNA polymerase [146]. The E3 protein dampens the host immune response, and the E4 promote cell survival, blocks apoptosis, and stimulate translation [146]. There is an intermediate transcriptional unit that codes for proteins IX and IVa2 [147]. The early genes allow for continuous amplification of the viral genome that serves as template for the late (L) transcriptional units (L1-L5), which code for the major structural protein penton, hexon, and fiber [147]. The L4-100 kDa protein induces selective translation of the other late viral proteins. In the final stages of replication, pre-TP is cleaved by a viral protease to TP, resulting in progeny DNA, which is packaged in the newly formed capsids [148]. After approximately 40 h, an infected cell can produce one million copies of viral DNA.

3.4. Assembly and release

The HAdV capsid proteins are translated and assembled in the cytoplasm. The chaperone protein L4-100 kDa helps with folding of the hexon monomers and assembly of the trimeric hexons [149]. The pentons, formed by the penton bases and fibers, are also assembled and are together with the hexons transported back into the nucleus. In the nucleus they associate with the other minor structural proteins to form empty capsids. Protein IVa2, L1-52/55K, L4-22K and IIIa are required for genome packaging [150]. The AVP cleaves proteins VI, VII, VIII, μ, and TP, completing the assembly to form infectious particles [150]. Virus release is facilitated by the adenovirus death protein (ADP), which is expressed late in infection. It has been suggested that the level of ADP expression determines whether the infection becomes lytic or persistent [151].

4. Immunological aspects

Natural HAdV infections often cause mild, transient diseases in healthy individuals, but there have been cases of outbreaks with high mortality. In the 1960s, ARD caused by HAdV-E4 and -B7 affected US military recruits resulting in hospitalisation rates approaching 50% [152]. This prompted development of live HAdV-E4 and -B7 vaccines, which reduced disease incidence. Immunogenicity of AdVs also needs consideration in vector development, as adverse effects can result in clinical complications. An early HAdV vector developed in the late 90s inadvertently led to systemic inflammation and fatality in a young boy [153]. Later, a HAdV-C5-based HIV vaccine gave higher risk of acquiring HIV after immunisation [154]. The underlying mechanism remains a subject of debate, but theories include proliferation of HAdV-C5-specific CD4+ T cells, or that pre-existing HAdV-C5 immunity dampens the vaccine-induced response [155, 156]. The recent use of the Astra Zeneca chimpanzee AdV (ChAdV)-based vaccine ChAdOx1 (Oxford/AstraZeneca) and the HAdV-D26-based Ad26.COV2.S (Johnson & Johnson/Janssen) led to heightened incidence of vaccine-induced immune thrombocytopenia and thrombosis [157, 158]. This was recently shown to be caused by antibodies against viral core protein VII showing cross-reactivity against platelet factor 4 [159].

The innate immune response is driven in large part by pathogen recognition receptors (PRRs), which include Toll-like receptors (TLRs). TLRs, for example TLR9, sense conserved nucleic acid motifs and initiate inflammatory signalling through the release of type I interferons (IFNs) and other cytokines [160]. Excessive innate immune activation and strong type-I IFN responses can lead to decreased transgene expression, toxicity, chronic inflammation, and impair the antigen-specific antibody and CD8+ T-cell responses. Yet some level of activation is beneficial for priming the adaptive immune response for lasting immunity. Different HAdV species give different immunological responses, which means that care must be taken when choosing the backbone for viral vectors. In non-human primate models, HAdV-C5–based vectors trigger a weaker innate immune response than vectors derived from HAdV-B35, -D26, and -D48, as indicated by lower levels of inflammatory markers IL-1RA and IL-6 [161]. Notably, swapping the HAdV-C5 fiber and penton for those of HAdV-B35 heightens cytokine production in human peripheral blood mononuclear cells, revealing an important role for capsid proteins in immune stimulation [161].

The adaptive immune response is initiated through cytokine signalling and antigen presentation by professional antigen-presenting cells like dendritic cells, monocytes, macrophages, and B cells. This leads to the activation of T and B lymphocytes in secondary lymphoid organs. A simplified overview of the T and B lymphocyte function is presented in **Figure 9**. CD4+ T cells act as helpers that aid in the expansion of CTLs and activation of antibody-producing plasma B cells. CD4+ T cells directed against HAdVs often display cross-reactivity, consistent with the presence of conserved CD4^{+} T cell epitopes within the hexon protein [162]. Although adaptive immunity confers protection, it also represents a significant obstacle for vector re-administration and sustained gene expression, as it will additionally confer protection against the vector itself. HAdVs captured by IgG, IgM, and IgA antibodies is an important way for the body to handle pathogens. Antibodies can neutralise the virus in several ways: by blocking virus binding, aggregation, complement activation, target the virus for destruction, or prevent endosomal escape of the virus (**Figure 9**) [163, 164].

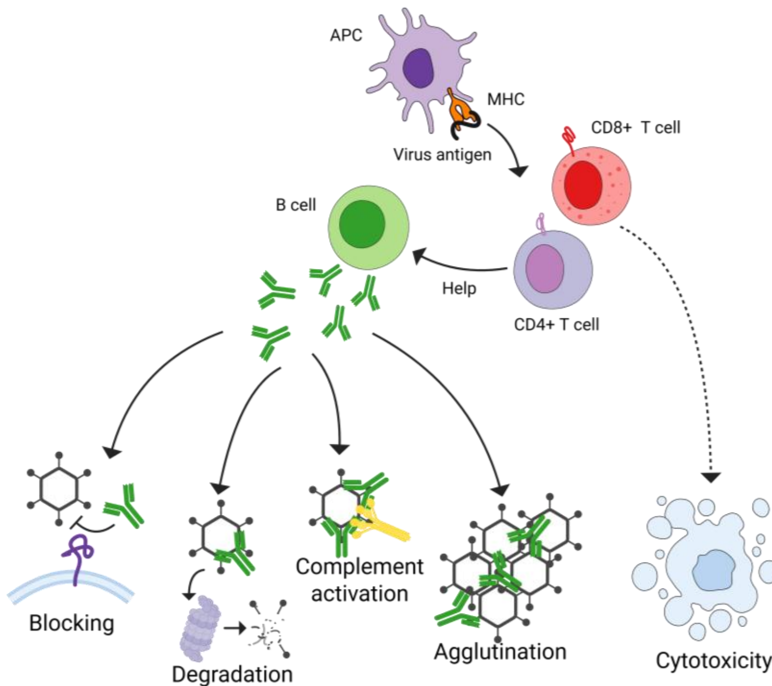

Figure 9. *Adaptive immune sensing of adenoviruses. B cells and T cells constitute the adaptive immunity, where T cells either help B cell activation, or act directly on infected tissue by releasing effector molecules. Plasma B cells release immuno-globulins (Igs), which bind to the capsid and inhibit HAdV in multiple ways, either by blocking interactions, targeting the virus for proteasome degradation, complement activation, or agglutination of virions [164]. Image created with BioRender.com (2026).*

NAbs against HAdVs are produced against all the major capsid proteins: penton, fiber and hexon, but they exhibit different mechanisms and neutralising properties. Fiber antibodies cause aggregation of the virions and prevent attachment to a primary receptor, but they cannot neutralise virus already bound to cells [165]. Penton antibodies can similarly block attachment, and together with fiber antibodies they can neutralise the virus [166]. The hexon protein is a major target for NAbs as hexon-specific NAbs outnumber fiber-specific ones by up to 10-fold [167]. The anti-hexon NAbs can be directed toward either the type- or group-specific epitopes on the surface of the virion [168], and have several modes of action, including blocking, preventing uncoating and endosome escape [164, 168]. The anti-hexon NAbs are on their own able to effectively neutralise infection, and HAdV-C5 can, as an example, allegedly be neutralised by as few as 1.4 anti-hexon IgG antibodies per capsid [2, 168-170].

5. Adenoviruses as viral vectors

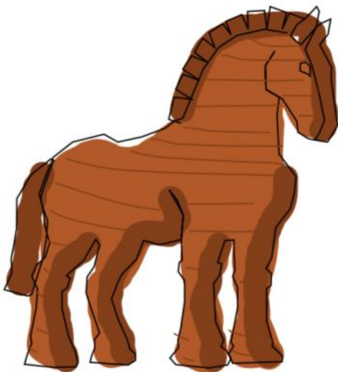

Figure 10. *The Trojan horse has become a metaphor for deception and hiding things in plain sight.*

After a ten-year stalemate between the Greeks and the city of Troy, the Trojan War culminated in a legendary feat of deception: the Greeks constructed a wooden horse (known as the Trojan Horse (**Figure 10**)) masquerading as a peace offering, while hiding a group of soldiers ready to launch an ambush within the city. As a metaphor it works perfectly in a number of biological settings, from cancer cells excreting exosomes, to passing of small molecules through the blood-brain-barrier. In virology, the parable is quite literal, mirroring what viruses are attempting to do on a daily basis. Naturally, it is their expertise in breaking-and-entering that has led us to repurpose viruses as Trojan Horses for the benefit of modern gene therapy.

Development of AdV vectors has evolved through generations to improve safety, reduce immunogenicity, and increase transgene capacity. AdV vectors were initially developed to treat cystic fibrosis due to their respiratory tropism, but today most gene therapy trials using AdV vectors

are for oncolytic purposes, where HAdV-C5 is the most common backbone. Solid tumours allow for intratumoral injections, which can prevent activation of the complement system as well as generation of NAbs. Oncorine, a HAdV-C5 based E1B-55K/E3-deleted oncolytic vector, has been approved for clinical use in China for treatment of solid tumours [171, 172]. Ofranergene obadenovec is another HAdV-C5-based vector in clinical trials, with dual function; it has a tissue-specific promotor which permits expression only in angiogenetic blood vessels, and it encodes a Fas-Tumor necrosis factor receptor 1 transgene, which becomes expressed on the transduced cells to induce apoptosis [173-175]. Recently, HAdV-C5-based Adstiladrin (developed by Ferring Pharmaceuticals) was approved as treatment for bladder cancer [176, 177]**.** AdVs have also been used as vaccine vectors against Ebola, under the name Zabdeno (HAdV-D26-based) [178, 179], and SARS-CoV-2 under the name AZD1222 (ChAdV-based) [180], and are in clinical trials as vaccines against, for example, malaria (HAdV-C5-based) [181] and RSV (ChAdV- and HAdV-D26-based) [182, 183]. Other HAdVs from species D, including HAdV-D56, -D48, and -D49, have been vectorised and had immune profiles assessed in different animal models [133, 161].

AdV vectors can be divided into categories based on the techniques used for generating them (**Figure 11**).

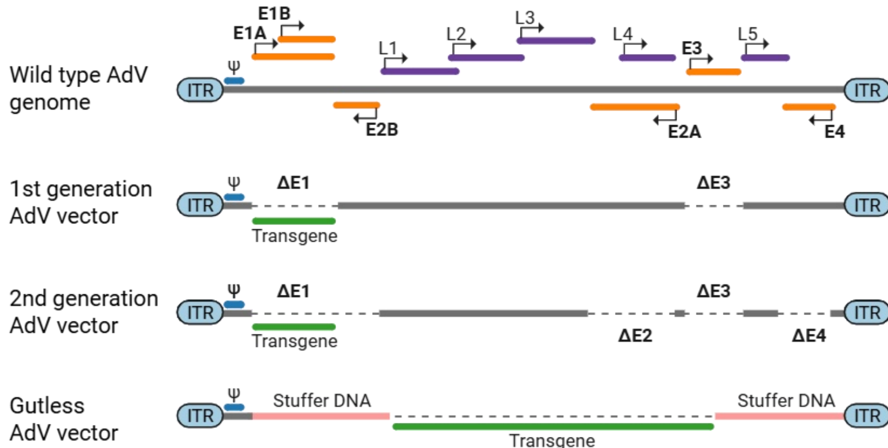

Figure 11. *HAdV vectors. Wild type HAdV genome contains four early (E) gene clusters (orange), and five late (L) gene clusters (purple). First generation HAdV vectors has deleted E1 for insertion of transgenes and can contain additional deletions of E3. Second generation HAdV vectors are deleted in all E regions. Gutless vectors contain only the ITR and packaging signal (ψ) with stuffer DNA to reach optimal size. Image created with BioRender.com* (2026).

In the first-generation AdV vectors the E1 region, which is essential for the viral life cycle, is replaced with a transgene, and the vector can be produced in an E1-containing packaging cell line, such as HEK293. Additional deletion of E3 is sometimes included, as it increases transgene capacity. E3 proteins suppress host immune responses, and are involved in lysis, but is non-essential for the virus life cycle [146, 151]. Second-generation AdV vectors include additional deletions in the E2 and E4 regions, where the E4 proteins are associated with several functions including gene regulation and apoptosis, and E2 unit codes for the AdV DNA-binding protein (DBP), TP, and DNA polymerase [146, 184]. Modern, gutless AdV vectors only contain the inverted terminal repeats (ITRs), and the packaging signal (ψ), which maximises the transgene capacity. Gutless vectors can either be helper virus-dependent using a helper virus to supply viral proteins in *trans*, or helper virus-independent, which uses helper plasmids instead.

Drawbacks of using AdV vectors, especially in the case of HAdV-C5, includes high prevalence of pre-existing NAbs in the population [2]. Despite this, HAdV-C5 remains popular as vector backbone as its ability to elicit a potent adaptive immune response is unmatched by other genotypes [185]. As HAdV-C5 dampens the innate type I IFN response, it is able to generate more viral transcripts and a sustained transgene expression, which results in an increased number of antigen-presenting cells and by virtue a continued CD8+ T cell response [186]. To further circumvent virus neutralisation there have been several attempts at modifying the hexon protein to prevent binding of NAbs [187-190]. The modifiable aspect of AdVs is indeed an advantage of using them as vectors and have thus far mainly been done for cancer-targeting AdVs.

It is relatively easy to modify the capsid and thereby change the tropism and/or immunogenicity of the vector. Some examples of capsid modifications in relation to targeting and immune masking is presented in **Figure 12**.

Modifications include replacing the whole fiber, or the knob domain, with that of different AdVs, alternatively replacing the hexon HVRs to that of rarer AdVs with lower immunogenicity (as the HVRs is a major target for NAbs) [188]. Integrin-binding RGD-motifs, or antibody fragments inserted in the fiber or hexon can target the virus to cancer tissue, and in some cases reduce unwanted interactions, such as that with FX [191-194]. Insertion of lysine residues at the fiber knob, RGD-motifs at the hexon or fiber knob, or

specific muscle targeting peptides in the hexon have been shown to enhance uptake of HAdV vectors in muscle tissue [195-197]. Lysine residues in the capsid can be targeted by polyethylene glycol (PEG) for covalent binding to the capsid. PEGylation of the AdV capsid can then mainly reduce immunogenicity as it shields the immunogenic epitopes from recognition [198-203]. Covalent attachment of a Designed Ankyrin Repeat Protein (DARPin) adapter to the HAdV-C5 fiber has been shown to enhance binding to Her2-expressing HEK293 cells [204]. Encapsulating the virus in a membrane vesicle can slow tumour clearance and enhance oncolytic capacity of AdV vectors [205], and albumin coating can partially block NAbs upon systemic delivery [206]

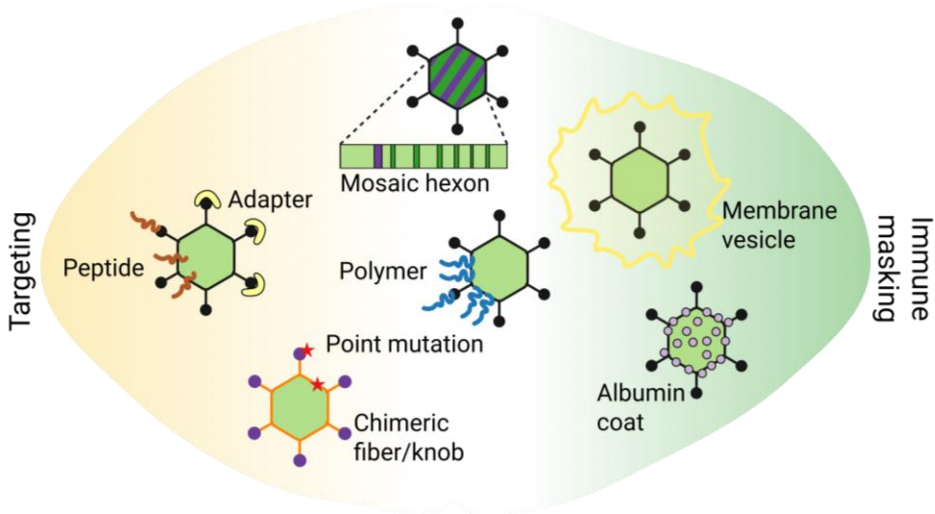

Figure 12. *Engineering adenovirus vectors for improved targeting and immune masking can be done in several ways. Examples of targeting include attachment of targeting peptides or adapters; examples of immune masking include coating with albumin or cloaking with membrane vesicles; Examples of dual effects include attachment of polymers, exchanging fibers, penton bases, or hexon hypervariable regions. Image created with BioRender.com (2026).*

Aims

The goal of viral vector development is to generate new treatments for genetic diseases and improve vaccine delivery strategies. To create efficient and safe viral vectors we need to understand virus biology to identify their limitations and advantages. The overarching aim of this thesis work is to explore adenovirus-host interactions in the context of tropism and viral vector development.

Specific aims:

Study I.

Investigate receptor usage and binding partners of the newly identified HAdV-20-42-42 and evaluate its potential as a viral vector.

Study II.

Identify the primary binding partners or receptors used by HAdVs from species D.

Study III.

Investigate the role of lactoferricin as an enhancer of HAdV-C5 infection in human skeletal muscle cells.

Methodological considerations

Following is an overview of the key methods used in this thesis. Detailed experimental procedures can be found in the Materials and Methods sections of each publication (I–III).

1. Main methods

1.1. Detection of adenovirus infection/transduction

1.1.1. Viral vectors

A common way to measure AdV transduction is to use vectorised viruses, where one or several of the early (E) genes are replaced by a reporter gene (e.g. GFP or luciferase), under the control of a promoter (typically cytomegalovirus (CMV)). Fluorescence microscopy or flow cytometry can then be used to analyse GFP expression, and luminescence measurements for luciferase. This offers a rapid, live-cell readout that reduces artefacts by eliminating the need for fixation or staining. However, the CMV promoter is not necessarily representative of natural virus expression. It also requires further modifications, such as the simian virus 40 poly(A) signal, to act as a stop for the cellular machinery, ensuring that the mRNA is correctly processed.

1.1.2. Immunostaining

When performing infection experiments with wild type adenoviruses, we have used antibodies to stain for uptake and replication, mainly MAB8052. This antibody detects all human adenoviruses via its binding to a common hexon epitope located on the inside of the hexon capsomer. The free hexons produced during translation of structural proteins are detected by the antibody, and a secondary antibody conjugated to a fluorophore can be used to fluorescently image infected cells. As intact virus particles cannot be detected with this antibody, it is necessary for the virus to initiate translation of capsid proteins for the antibody to work. Therefore, the use of this antibody is restricted to later time points in the infection cycle. Additional drawbacks include the potential for artefacts by unspecific binding of primary or secondary antibody.

1.1.3. qPCR

As our primary adenovirus antibody requires production of free hexons to work, we used qPCR to measure virus binding (an assay during which the

virus does not have time to initiate replication). A conserved region in the hexon gene is amplified, which enables use of a single primer pair to detect all HAdVs. To ensure specificity, we used probe-based qPCR. The probe is designed to bind to a region between the primers and is covalently attached to a fluorophore (FAM) on one end, and on the other end a quencher (TAMRA) is bound which supress the signal from the fluorophore. Upon amplification the polymerase breaks the probe, separating the quencher from the fluorophore. This allows the fluorophore to release a signal that can be detected. qPCR is a highly sensitive method and is as such ideal for analysing samples with minute differences. A specific AdVantage with qPCR for HAdV detection is that it can be performed directly on the extracted DNA, avoiding the reverse transcription step necessary when working with RNA. The method still requires normalisation of input samples, for example loading the same amount of DNA, and/or using a housekeeping gene (in our case GAPDH) as loading control. This increases the time and material needed for analysis.

The output from a qPCR is a cycle threshold (CT) value, that can be used for quantification. We used the ΔΔCT method, in which viral DNA is normalised against GAPDH to generate a ΔCT value. The ΔCT is then compared to a control sample which gives a ΔΔCT, a quantitative measurement in arbitrary units (AU) and can be presented as fold change compared to baseline. Unlike using absolute quantification, there is no need for a standard curve which allows for more samples analysed per run. However, it only gives the result as a ratio between untreated and treated samples, so comparisons can only be done within the same experiment. It also assumes that the amplification efficiency of your target gene and your housekeeping gene are equal, and that the housekeeping gene expression is unaffected by treatments.

1.2. Adenovirus neutralisation

We used neutralisation assays to test whether lactoferricin could prevent binding of neutralising antibodies against the adenovirus capsid. We pre-incubated the virus with Lfcin and added it to serum with HAdV-neutralising properties. The serums were selected form a cohort of patients vaccinated against SARS-CoV-2 with the AstraZeneca ChAdOx1 vector. The neutralising properties of the serums was estimated by how well HAdV-C5 was able to infect A549 cells in the presence of an increasing amount of serum. Titrating a serum with HAdV neutralising capacities gives a sigmoidal curve where a high serum concentration completely blocks

HAdV infection and reaches a point where the neutralising capacity is fully reduced. From this, the neutralisation titer at 50% (NT50) can be calculated, which corresponds to the concentration required to neutralise 50% of the virus infection. Neutralisation assays typically require a sigmoidal dose-response curve to generate accurate NT50 values. In our experiments, where we aimed to test if Lfcin could prevent the effect of NAbs, we utilised A549 cells due to their high susceptibility to HAdV infection compared to muscle cells. Although this assay identifies changes in neutralisation, it does not provide any mechanistic insights. Further, the serum cohort used was selected not for cross-reactivity against ChAdOx1, but because we assumed it provided a source of HAdV-C5 antibodies.

1.3. Flow cytometry

Flow cytometry is commonly used to analyse proteins, glycans, or other moieties on the outside of the cellular membrane. The cytometer itself is primarily designed to analyse individual cells, where cells in suspension are injected into a flowline. The flowline is crossed by lasers that detect the size and complexity (or granularity) of the passing cells. Fluorescently coupled antibodies are used for detection of specific epitopes, and lasers can excite the fluorophores and detect their signal. In this way, cells can be characterised and sorted based on expression of specific cell receptors or glycans. We have used flow cytometry to detect the presence of protein and glycans on the surface of cells and to detect virus bound to the cell surface. The virus is covalently labelled with a fluorophore (in our case green fluorescent Alexa fluor 488) prior to incubation with cells. By counting several thousands of cells individually in each sample, we can get an idea of what the proportion of virus bound to cells is. Flow cytometric analysis requires single-cell suspension, which requires detachment if using adherent cells. To avoid the cleavage of surface receptors by trypsinisation, we employed EDTA as a non-enzymatic alternative. EDTA promotes detachment by chelating the Ca^{2+} and Mg^{2+} ions in cell junctions. Although this method preserves receptor proteins, its lower efficiency necessitating longer incubation times may affect cell health or surface protein distribution. Additionally, it is important to note that direct labelling of virions can potentially interfere by blocking key binding motifs on the capsid.

1.4. Knock-out cells

We generated CRISPR/Cas9 plasmids that knock out surface proteins used by AdVs as receptors. This was done to assess how well AdVs were able to infect cell in the absence of said surface proteins. For this, we designed guide RNAs (gRNAs) that targeted the genes *DSG2*, *CD46* (*MCP*), *CXADR* (CAR), and *CMAS* (cytidine monophosphate N-acetylneuraminic acid synthetase). The gRNAs were cloned into a backbone plasmid (pSpCas9 (BB)-2A-Puro (PX459)), and plasmids were transformed into Stbl2 cells, from which the final CRISPR/Cas9 plasmids were produced. A549 cells were transfected with three CRISPR plasmids targeting the same gene, and selection of transfected cells was done using puromycin. Single clones were isolated and propagated for further use. Verification of the knockouts was done with antibody staining and analysed with flow cytometry. CRISPR technology has simplified the generation of knock-out cell lines. However, the design of the gRNAs dictates the effect of the knock-out. Especially membrane-bound proteins are complex to knock out, and there can be residual functional mRNA. If the exact virus binding site on the protein is unknown, it also becomes more complex. Additionally, knock-outs can generate off-target effects, that lead to higher or lower expression of other proteins.

1.5. Cell viability

We used the Promega kit CellTiter Glo for viability measurements of cells treated with different compounds. It is a bioluminescent assay that quantifies the amount of ATP in a sample, which is proportional to the number of metabolically active cells. It depends on efficient lysis of cells, for the ATP to be released, and does not require a solid monolayer of cells, like the MTT assay. If there are additional sources of ATP in the samples, or anything that interferes with the luminescent signal, this could affect the results. Further, for a fair comparison it requires that the cells are metabolically similar. If cells become metabolically inactive, the results may show the cells as dead.

1.6. Purification of adenovirus fiber knob

Fiber knob sequences obtained from GenBank were amplified and inserted into pET24 vectors. A His-tag was added to facilitate purification. The plasmids were transformed into Rosetta DE3 bacterial cells which were induced to produce fiber knobs, which were purified via Ni-NTA affinity chromatography. For applications requiring the removal of the affinity tag,

a TEV protease cleavage site was utilised. The cleaved product was purified by re-application to a Ni-NTA column, which retained the His-tag and allowed for collection of the tag-free fiber knob in the flow-through. An advantage of using recombinant fiber knobs is that they can be produced in high amounts, and because of the His-tag to a high degree of purity, reducing the risk of bacterial contaminations in the final product. A general disadvantage of using bacterial expression for non-bacterial proteins is that the expression may result in misfolding or alternate glycosylation patterns. It is not possible to produce the very large trimeric hexon protein using bacterial expression.

1.7. Purification of adenovirus hexon

During virus infection hexons are produced in excess, and allegedly only 20-30% are used for assembly of complete viral particles. Thus, virus harvesting present an opportunity to isolate these free hexons. We harvested infected cells via lysis and centrifuged it over a caesium chloride (CsCl) gradient. The virus will sediment in the gradient, but individual proteins are too small to be affected by the centrifugal force. Thus, hexons will be left in the "top phase" that sits above the CsCl. The top phase consists of several cellular and viral proteins, that can be separated using HPLC. The hexons have a net negative charge and will therefore bind to positively charged molecules, which is why we used anion exchange chromatography in the first purification step. The hexons were further purified by size exclusion chromatography (SEC), which removes additional impurities that are either smaller or larger than the hexon. In the end, this gives pure hexons that we used for measuring protein-protein interactions. Purity of the hexons was determined via SEC and SDS-PAGE and was largely dependent on the efficiency of the anion exchange chromatography step. HVR1 of species C is, for instance, longer and more negatively charged (rich in Asp and Glu) than that of species D. This results in a stronger binding affinity to the anion exchange column and a higher elution threshold, giving a purer final product. Hexons are stable proteins, which mean they withstand the purification process and subsequent storage.

1.8. Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is an optical technique used to measure real-time molecular interactions by immobilising a ligand on a sensor chip and passing a soluble analyte over the surface. Binding events alter the angle of total internal reflection, which is recorded as a sensogram to

determine binding kinetics and affinity. We used SPR to measure the interactions between HAdVs or specific capsid proteins and CD46 or CAR. Using whole virions allowed for the assessment of avidity, while individual proteins provided information about affinity. SPR is optimal for measuring the affinity between ligands and analytes of similar sizes. If the analyte is significantly larger or smaller than the ligand it can result in a decreased signal-to-noise ratio which makes it more difficult to detect the interaction. Furthermore, unlike solution-based methods such as Isothermal Titration Calorimetry (ITC) and analytical ultracentrifugation, SPR requires surface immobilisation, which may result in heterogeneous orientation of the ligand.

1.9. Negative stain EM

An adenovirus particle is about 90-100 nm in diameter, which is too small to be seen in any regular inverted microscope. Thus, to study the appearance of virus particles we used negative stain electron microscopy. This technique allows for visualisation of samples as small as 10 nm. The prepared virus samples, diluted in PBS, were pipetted onto carbon-coated grids and stained with uranyl acetate. The uranyl acetate gets embedded in the virus capsid and provides contrast due to it being electron dense. Still, differences in sample homogeneity and methods for preparing the samples can cause large differences in contrast, so it is important that samples are prepared in the same way. Negative stain EM is not necessarily a quantitative method. In our case we wanted to study the virus distribution within different samples, and in some we observed large clusters of viruses. These clusters made it difficult to perform any type of quantification of the data, and we used the technique to visualise rather than to quantify data. Other techniques such as Dynamic Light Scattering (DLS) or SEC can also be used to determine sizes of particles, and can be used to quantify sizes, but they do not provide information regarding appearance of samples.

2. Additional methods

Additional methods performed by co-authors include 1) whole genome sequencing, 2) AdV vector cloning and production, 3) ELISPOT to detect IFN-γ producing T cells, 4) *in vivo* biodistribution experiments, and 5) cytokine profiling.

Results and discussion

The very first virus to be identified by humans was the tobacco mosaic virus, first reported on in 1892 [207]. But as far as we can assume, viruses have been around for much longer, and a fun fact is that it is estimated that the human genome today consist of about 9% retroviral DNA [208]. Their omnipresence is a result of their ability to efficiently enter cells and utilise the host machinery to replicate exponentially, most within only hours. By virtue of this proficiency, viruses serve as excellent platforms for intracellular delivery, and we can exploit their evolution and design to turn them from disease-causing agents to tools for modern medicine in the form of viral vectors. But to generate efficient and safe vectors requires finding a close-to perfect virus to use as backbone, and a deep understanding of their biology.

This thesis consists of three studies, where study I discusses the identification of a new HAdV belonging to species D, which was vectorised and analysed biologically and functionally. Study II discusses the receptor preferences of species D HAdVs. Study III discusses how an endogenous human peptide can enhance the infectivity of HAdV-C5, one of the most commonly vectorised AdV used in clinical trials and applications.

(I) A novel adenovirus vector

In this study, I contributed by purifying hexon proteins from HAdV-20-42-42, and HAdV-C5 to be used in SPR experiments assessing binding to CD46.

Many species D HAdVs have been isolated from AIDS patients and otherwise immunosuppressed individuals, where co-infections of multiple HAdV-D types can occur. This can result in recombination in or around the genes encoding the major capsid proteins: fiber, penton base, and hexon. The recombination ability of species D has allowed an array of chimeric viruses to be produced. Serological testing commonly determines species D to have a lower seroprevalence than for example the more commonly vectorised HAdV-C5. Therefore, species D HAdVs have become widely used as AdV-based vectors.

In this study, we identified a new chimeric HAdV-D virus HAdV-20-42-42 resulting from recombination between HAdV-D20 and -D42. The hexon and fiber were closely related to HAdV-D42, and the penton base related the closest to HAdV-D20. HAdV-20-42-42 was vectorised with a β-galactosidase, luciferase, or GFP reporter, and the vector design is shown in **Figure 13**.

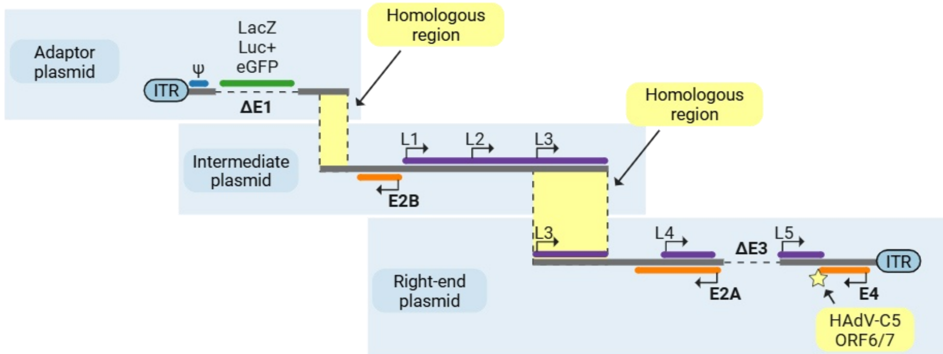

Figure 13. *Representation of the HAdV-20-42-42 vector. A three-plasmid system consisting of an adaptor, intermediate and right-end plasmid (with deleted E1 and E3 genes) were linearised and co-transfected into packaging cell line HEK293. Overlapping regions allowed homologous recombination. E and L represent the "early" and "late" gene cassettes of the AdV genome, ITRs are the inverted terminal repeats at the 5' and 3' ends, and ψ is the packaging signal. Image created with BioRender.com (2026), modified from figure 3A in paper I.*

The seroprevalence of HAdV-20-42-42 was assessed using a serum cohort from U.S citizens, and it was found to have much seroprevalence compared to HAdV-C5 and was comparable to that of HAdV-B35 (**Figure 14**).

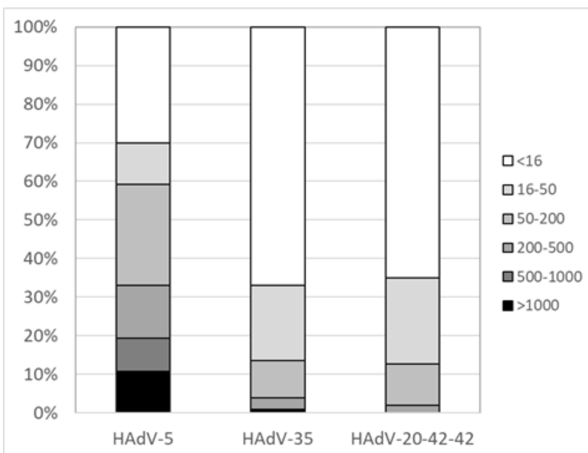

Figure 14. *Human serum samples showing presence of neutralising antibodies against HAdV-C5, HAdV-B35, and HAdV-20-42-42. Serum dilution giving 90% neutralisation were divided into categories: <1:16 (no neutralisation), 1:16–1:50, 1:50–1:200, 1:200–1:500, 1:500–1:1000, and >1:1000. Modified from Figure 2 in paper I.*

Biologically, the vector was analysed for receptor-binding properties and was found to use CAR and CD46 as receptor. The most common isoforms of human CD46 are BC1, BC2, C1, and C2, and they differ in the length of the STP region [99]. To assess the importance of each isoform, HAdV-20-42-42 was used in infection experiments with Chinese hamster ovary (CHO) cells expressing each isoform. As CHO cells are naturally refractory to HAdV infection, the level of infection displayed the ability of HAdV-20-42-42 to enter cells when each isoform was expressed. The results showed that HAdV-20-42-42 was able to infect CHO cells expressing all different isoforms of CD46, compared to CD46 negative CHO-K1 control cells (**Figure 15**).

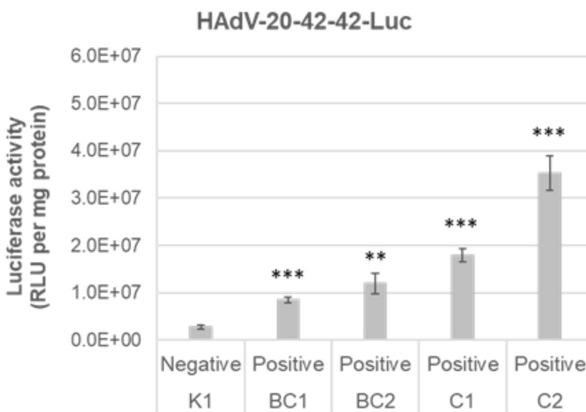

Figure 15. *CHO cells lacking CD46 (K1) or expressing different CD46 isoforms (BC1, BC2, C1, or C2) were infected with HAdV-20-42-42-Luc. Luciferase activity is presented as relative light units per milligram of protein. Error bars are presented as standard errors of the means (SEM). Statistical significance was calculated compared to K1 with two-tailed Student's t test; *, P < 0.05; **, P < 0.01; ***, P < 0.005. Modified from Figure 4B in paper I.*

HAdV-D56 has previously been shown to engage CD46 via the hexon [79]. We measured the affinity between HAdV-20-42-42 hexon and CD46 (BC1 isoform) to be at 13.5 µM using surface plasmon resonance (SPR) (**Figure 16**). This is significantly weaker than the nM affinity reported for the HAdV-C5 fiber knob-CAR interaction [29, 92, 95]. Some species D HAdVs have lower affinity, for example the HAdV-D9 fiber knob-CAR affinity is 7.9 µM, and the HAdV-D26 fiber knob-CAR affinity is 21 µM [92, 95], and it is inconclusive whether CAR function as attachment factor for species D HAdVs. From a structural perspective, fiber knobs are present in 12 copies per virion, and hexon trimers in 240 copies per virion. Thus, fiber knobs presumably require higher affinity to ensure attachment, whereas the hexons could achieve functional binding through avidity.

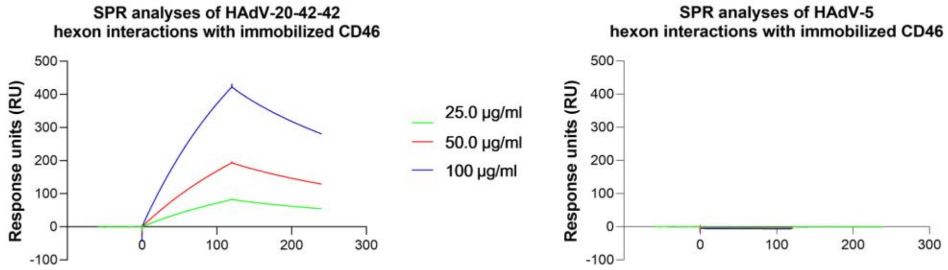

Figure 16. *Surface plasmon resonance analysis of immobilised CD46 and HAdV-20-42-42 (left), or HAdV-C5 (right) hexon as analyte. Modified from Figure 4C-D in paper I.*

To address the immediate immune response to AdV-20-42-42, levels of cytokines were measured 6 hours post injection in mice, and elevated levels of IFN-γ, IL-10, IL-2, IL-6, TNFα, IL-27, MCP1, and MIP2 were detected. Using mouse splenocytes, we could also show that the vector induced T cell responses, indicated by release of IFN-γ. Mice were infected with HAdV-20-42-42 to assess the biodistribution of the virus. Two days post injection the virus accumulated mainly in the spleen. It has previously been reported that in mice, HAdVs accumulate in the liver and spleen [209], functioning as a way for viral clearance. Biodistribution is also affected by the presence of entry receptors. Although human and mouse CAR are orthologues, and HAdVs can bind both [210], studies have shown that rodents only express CAR in olfactory bulb, skeletal muscles, brain and heart in neonatal stage, after which it is reduced to undetectable levels [211-213]. CD46 is, in humans, expressed on all nucleated cells, but in mice it is restricted to the testis and eyes [214]. This discrepancy in receptors means that biodistribution might appear different in humans compared to mice.

Lastly, we could show that the presence of FX enhanced HAdV-20-42-42 transduction in human saphenous vein endothelial cells. The saphenous vein function as both a model for vascular cells and is sometimes used for transplantation in coronary artery bypass operations. HAdV-20-42-42 thus represents a candidate for targeted gene therapy in the heart. As it can additionally use CAR as a receptor, it could also be used in targeting neurons, where CAR expression is increased [215].

In summary, we developed a vector of the newly discovered chimeric HAdV-20-42-42. Biological evaluation revealed properties that are advantageous for clinical applications. Further optimisation of the HAdV-20-42-42 vector could generate an important tool for gene therapy in the heart and/or brain.

(II) A main species D adenovirus attachment factor

In study II we continued investigating species D HAdVs. However, here we looked at them in a broader context and attempted to identify a common receptor used by the group. For HAdVs in general, the fiber knob is considered as the main mediator of primary receptor engagement, and different types are known to bind CAR, DSG2, CD46, and sialic acids using different areas of the knob. CAR interacts with the sides of the HAdV-A12 knob, CD46 with the sides of the HAdV-B11 knob, DSG2 interacts with the centre top of the HAdV-B3 knob, and sialic acid interacts with the central cavity of the HAdV-D37 knob (**Figure 17**). In addition to this, the HAdV-D56 hexon can bind CD46. The exact residues mediating the attachment are unknown, but increased density was observed in the central cavity between the hexon trimers (**Figure 17**) [79], implying this as the binding site.

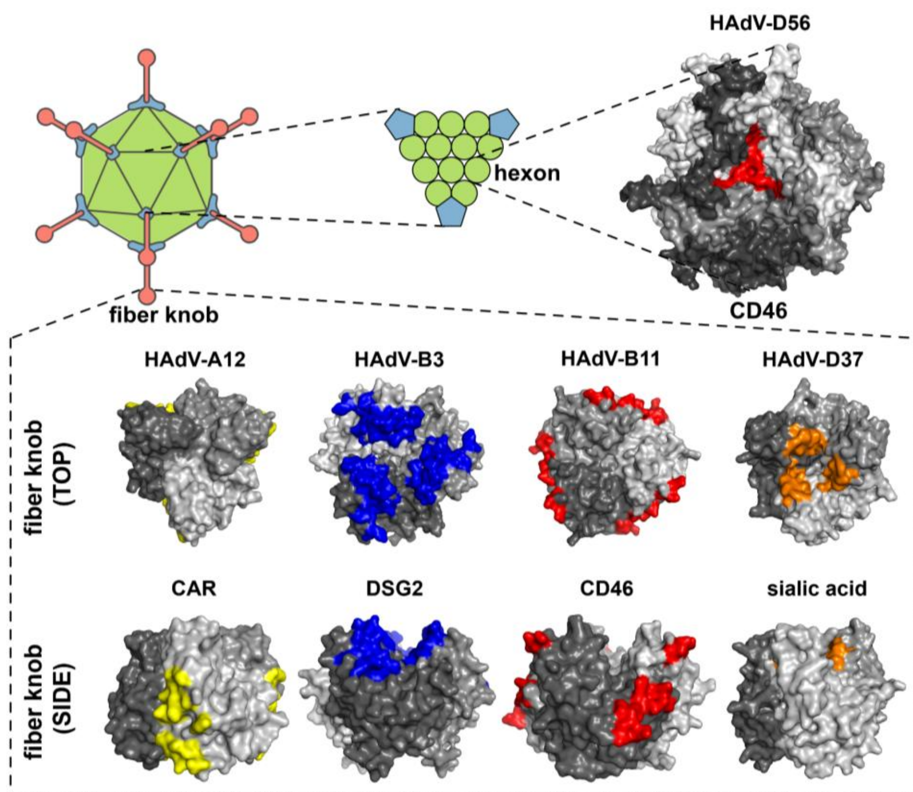

Figure 17. *Predicted binding sites on the HAdV-A12, -B3, -B11, or -D37 fiber knobs to CAR (yellow), DSG2 (blue), CD46 (red), or sialic acid (orange), respectively. Predictions based on references [31, 72, 98, 216]. Chains in the fiber trimers are coloured in different grey tones. Image created with BioRender.com* (2026), *modified from Figure 1 in paper II.*

Using CRISPR/Cas9 in the human epithelial lung cell line A549, we knocked out the expression of DSG2, CAR, CD46, and sialic acid to evaluate their importance during infection of 18 different species D HAdV types plus control types known to use each molecule for attachment. We observed that species D HAdV infection was markedly reduced in A549 cells lacking CD46 (A549-ΔCD46) (**Figure 18**), implying that CD46 has a role during infection. Out of 18 species D HAdVs tested, only three, HAdV-D13, -D32, and -D39, showed a non-significant reduction of infection. HAdV-D13 and -D32 remained infectious across all knockout backgrounds, suggesting alternative mechanism(s) of attachment and entry.

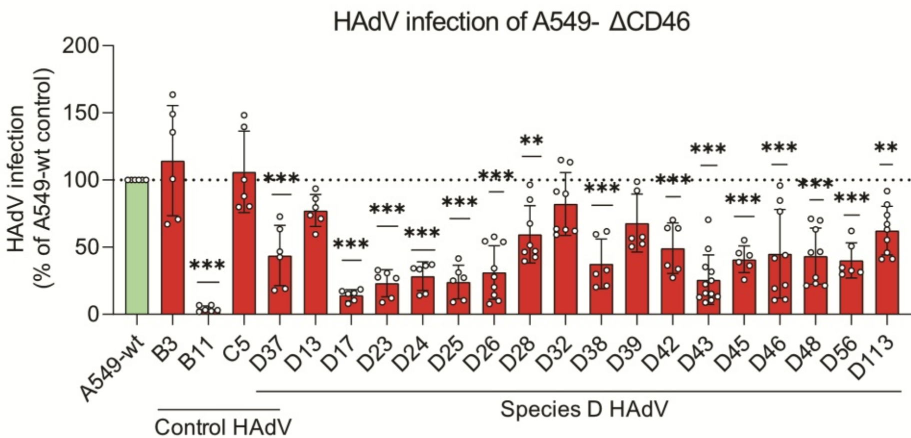

Figure 18. *CD46 knock-down reduces Species D HAdV infectivity in A549 cells. HAdV-B3, -B11, -C5, and 18 HAdV-D types were analysed for infection on A549-ΔCD46 cells. Data is shown as percent infection normalised to A549-wt. Results are presented as mean ± SD. Statistical significance was determined with Student's t test; *, P < 0.05; **, P < 0.005; *** P, < 0.001. Modified from Figure 4b in paper II.*

Species D HAdVs showed variable reduction in infectivity (20–80%) following CD46 knockout in A549 cells, unlike HAdV-B11 that was completely inhibited (**Figure 18**). To address the stage at which CD46 was important we performed binding experiments with selected HAdVs on A549-ΔCD46 cells. Here we observed a reduction in binding for some, but not all HAdVs tested, suggesting that the role of CD46 could be compensated for by other surface proteins (**Figure 5 in paper II**). Previous studies provide inconsistent findings regarding attachment factor preferences, possibly due to the use of pseudotyped viruses, or non-permissive cell lines

transfected with the attachment factor of interest [95, 97, 217]. If a virus can engage multiple binding partners, this may provide a skewed perspective.

We performed SPR experiments on either whole virus or purified HAdV hexons or fibers from a subset of selected types to address the direct interaction of species D HAdVs to CD46 (**Figure 19a**). Using whole virus, we observed a stronger binding to CD46 (picomolar to femtomolar range) compared to CAR (nanomolar range) (**Figure 6b in paper II**). We also demonstrated that it was the hexon, and not fiber, that interacted with CD46 (**Figure 19b**), which is unusual as the fiber is normally considered the main mediator of primary interactions.

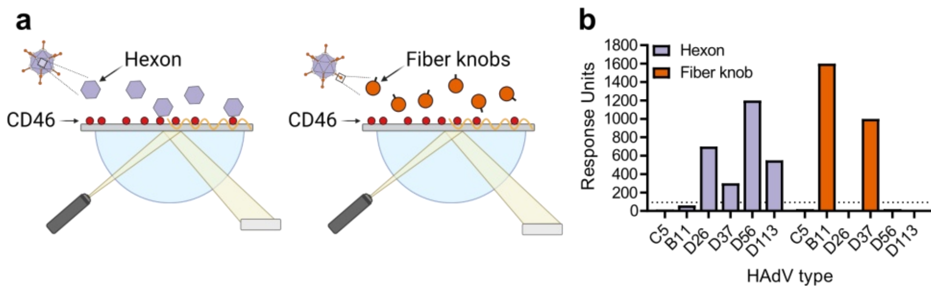

Figure 19. *SPR analysis of affinity between HAdV hexons or fibers. (a) Experimental set-up of SPR experiments with hexon (left), or fiber knob (right) as analyte over immobilised CD46. (**b**) Bar graph of response units (RU) between purified HAdV-D hexon or fiber knobs over immobilised CD46. Modified from Figure 6 in paper II.*

CD46 is a regulatory protein of the complement cascade, serving as co-factor for the serine protease factor I, which facilitates inactivation of complement C3b and C4b and protection from immune-mediated destruction. Its ubiquitous expression has led to pathogens evolving the capacity use CD46 for infection. Apart from species B HAdVs, bovine viral diarrhea virus, measles virus, and human herpesvirus 6, as well as bacteria *Neisseria gonorrhoeae* and *Neisseria meningitides* bind CD46 [218]. The HAdV-B11 fiber knob engages SCR1 and SCR2 of CD46 via its HI, DG, and IJ loops [98], but the specific knob residues are spread over the loop regions [95]. This prevents the drawing of direct parallels between fiber knob-mediated and hexon-mediated CD46 binding, and the residues mediating interaction between the HAdV-D hexon and CD46 remain to be identified.

In summary, we evaluated the receptor preferences of species D HAdVs and found that when knocking out CD46 the overall infectivity was reduced. All HAdV-D types bound CD46 with markedly higher affinity than CAR,

presumably owing to the fact that it engages CD46 via the hexon. Future structural studies, such as cryo-EM or X-ray crystallography, of the hexon-CD46 complex will be important to map the points of contact and provide additional insights to the findings presented here.

(III) An adenovirus infection-enhancing peptide

Human adenovirus C5 (HAdV-C5) is perhaps the most well studied AdV, and a lot is known about its biology. It has been evaluated as viral vector for vaccine and transgene purposes, but several attempts have been thwarted by unforeseen side effects. A notable clinical study attempted at developing a HIV vaccine using HAdV-C5 as backbone ended after it was discovered that the occurrence of HIV was higher in participants receiving the vaccine compared to the control group [154, 219]. Additionally, HAdV-C5 vectors injected intravenously have a tendency to accumulate in the liver as a result of interactions with vitamin K-dependent coagulation factors, primarily FX [220]. Despite this, modifications of the HAdV-C5 capsid have shown promising results in terms of eliminating unwanted tropism and adverse side effects. There is yet more to discover in the landscape of adenovirus-host interactions and finding new ways of altering the virus capsid for targeting and immune evasion will provide new possibilities when developing future viral vectors.

In study III we investigated the role of the endogenous peptide lactoferricin (Lfcin) during HAdV-C5 infection of human skeletal muscle cells. It has previously been shown that Lfcin enhance infection of HAdV-C5 in epithelial cells and T cells [40, 41], and from the perspective of HAdV-C5 as viral vector with potential to be used as vaccine vector, we decided that it was of interest to study whether this is true for muscle cells as well.

Proliferating skeletal muscle progenitor cells are called myoblasts. In humans, myoblasts are expressed throughout embryonic and foetal development [221]. They persist into adulthood as resting specialised stem cells known as muscle satellite cells, but become activated upon stimuli from exercise or mechanical injury [221]. Primary fusion between myoblasts results in formation of multinucleated cells called myotubes. Myotubes are senescent, multinucleated muscle cells more reminiscent of muscle tissue. The differentiation process we used in the lab is shown in **Figure 20**.

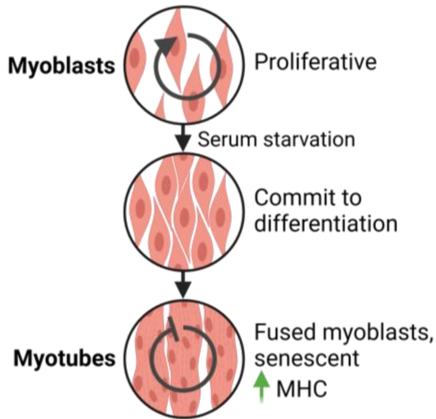

Figure 20. *Schematic image of the differentiation of myotubes from myoblasts, modified from Figure 2 in paper III.*

In our experiments we used a combination of proliferating myoblasts and senescent myotubes. We observed an enhancing effect of HAdV-C5 binding to muscle cells in the presence of Lfcin (**Figure 21a**). We additionally observed an increase of HAdV-C5 infection in both myoblasts and myotubes in the presence of Lfcin (**Figure 21b, c**). Titration of Lfcin demonstrated increased HAdV-C5 infection of myoblasts starting at 0.125 µM Lfcin with a peak around 1 - 4 µM, followed by decreased infection at 8 - 16 µM (**Figure 22a**).

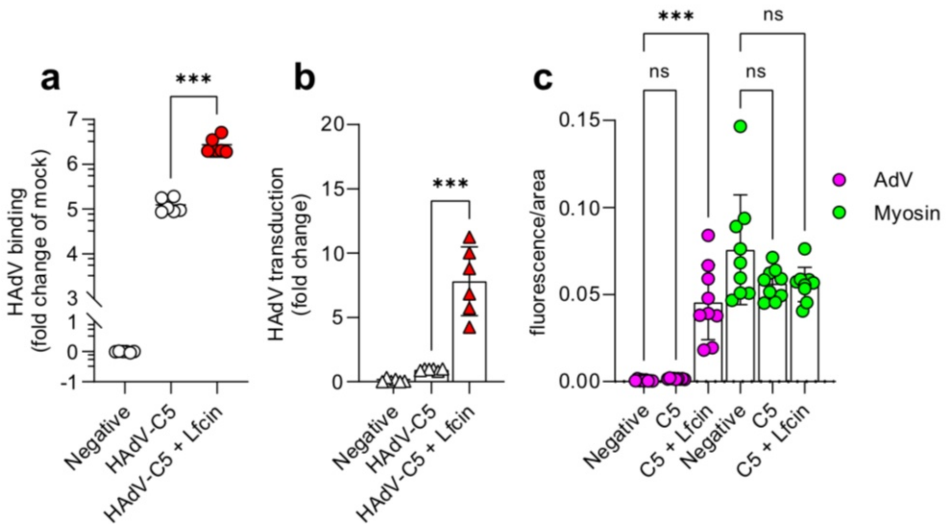

Figure 21. *Lficn-mediated HAdV-C5 binding to and infection of muscle cells.* ***a.*** *HAdV binding to myoblasts with or without Lfcin. Binding is normalised against GAPDH and is presented as log fold change of viral DNA compared to mock-treated cells.* ***b.*** *HAdV-C5 infection of myoblasts, presented as fold change in the presence versus the absence of Lfcin.* ***c.*** *HAdV-C5 infection of differentiated myotubes in the presence of Lfcin. Data is presented as fluorescence intensity of viral staining within myotubes (AdV) and fluorescence intensity of myosin staining (Myosin). Data is presented as mean ± SD. Statistical significance was determined with two-way ANOVA; ns, not significant; *** $P < 0.001$. Modified from Figure 1, Figure 2, and Figure 3 in paper III.*

We performed negative stain EM imaging to determine the cause of the decreased infection at Lfcin concentrations ≥8 µM. At 4 µM Lfcin, some larger clusters formed, but single virus particles were still present in abundance (**Figure 22b**). At concentrations at and above 8 µM however, we noted considerable particle clustering and only few single particles. The reduction of free virus particles at high Lfcin concentrations correlated with the decreased infection efficiency (**Figure 22a**).

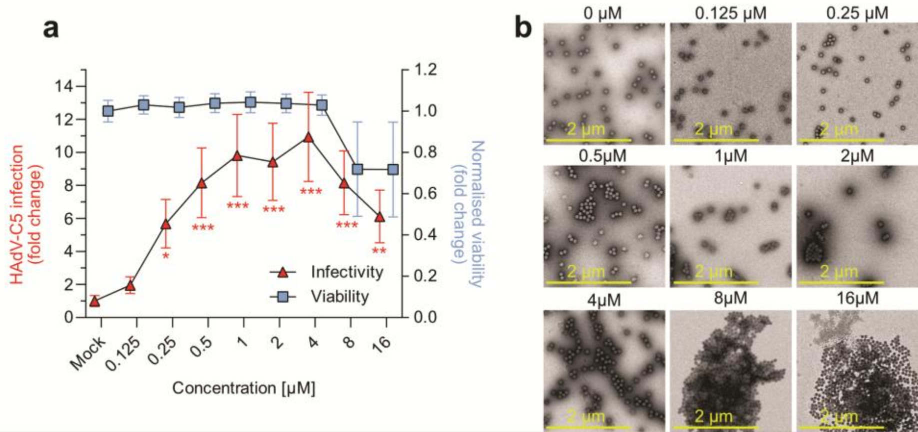

Figure 22. *Effect of increasing Lfcin concentration on HAdV-C5 infectivity.* ***a.*** *HAdV-C5 infection and cell viability in the presence of increasing concentrations of Lfcin. Data is presented as mean ± SD. Statistical significance was determined with unpaired T-test; *, P < 0.03; **, P < 0.002; *** P, < 0.001.* ***b.*** *Negative stain EM of HAdV-C5 with increasing concentrations of Lfcin. Modified from Figure 5 and Figure 6 in paper III.*

The effect was not unique to Lfcin as poly-L-lysine (PLL) also enhanced infection of HAdV-C5, however, Lfcin was less cytotoxic compared to PLL (**Figure 5 in paper III**). Finally, we could show that Lfcin prevented serum neutralisation of HAdV-C5 (**Figure 23**), showing a possible shielding mechanism, similar to how FX can protect HAdV-C5 from IgM and complement-mediated neutralisation [222].

We show that Lfcin can enhance the uptake of HAdV-C5 in human skeletal muscle cells, an important target for vaccines and gene therapy treatment. It has previously been shown that LF and Lfcin can enhance infection of HAdV-C5 in epithelial cells [41] and that neutrophils release LF during inflammation and via this enable HAdV infection in epithelial cells [223].

LF is produced in several body fluids, including saliva, which could additionally provide insights into why HAdV-C infects tonsils, that normally

are non-permissive to HAdVs, and form a latent infection there [14, 15]. It is not known why or how HAdVs enter T lymphocytes and remain in them as dormant, but one possible explanation is the interaction between the HAdV hexon and LF. It is known that other viruses, like Epstein-Barr virus, and more recently Rhinovirus can cause latent infections in T and B lymphocytes in the tonsils [224, 225], highlighting tonsils as a general target for viruses and also explains why tonsillectomy is a frequently performed surgical procedure worldwide.

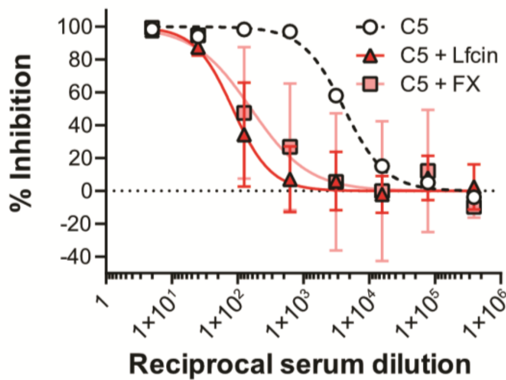

Figure 23. *Serum neutralisation of HAdV-C5. HAdV-C5 was preincubated with Lfcin or FX and subsequently incubated with heat inactivated serum before infection of A549 cells, presented as percent virus inhibition against log serum dilution, Data is shown as mean ± SD. Modified from Figure 7 in paper III.*

Two major hurdles for HAdV-C5-based vectors is targeting, and the prevalence of NAbs. Targeting is connected to making sure the vector transduces the correct cell types. There are several interesting examples of HAdV capsid modifications that alters tropism. For example, expressing PLL at the fiber knob enhances transduction in muscle cells [195], insertion of RGD motifs in the hexons enhances transduction in vascular smooth muscle cells [196], and insertion of phage-display selected muscle binding peptides in the hexon increases transduction in murine muscle cell line C2C12 [197].

In summary, we have evaluated Lfcin as a transduction enhancer of HAdV-C5. Our findings demonstrated that Lfcin significantly increased the infectivity of HAdV-C5 in both proliferating myoblasts and senescent myotubes, particularly when introduced during the early stages of infection. However, Lfcin concentrations exceeding 4 µM induced the formation of viral aggregates, which correlated with a decline in enhancement. While Lfcin successfully enhanced the infectivity of HAdV-C5, it remains to be explored whether it can be used as a genetic modification.

Conclusions and future perspectives

A growing topic in AdV research is their application as viral vectors in vaccine development, gene therapy, and oncolytic applications. HAdV-C5 was for a long time the go-to AdV backbone when designing viral vectors for vaccine use, as it elicits a potent adaptive immune response, essential for lasting immunity. HAdV-C5 is still an important vector used in cancer therapies, as evidenced by the recently approved Adstiladrin against bladder cancer. However, it is challenging to construct an effective HAdV-C5 vector. Several blood components interact with its capsid, including FX that targets the virus to the liver, and complement that marks the virus for opsonisation and destruction, as well as NAbs. Therefore, alternative AdV types have become increasingly important. This work focuses on AdV-host interactions in the context of developing AdV vectors.

In study I, we could show that the novel AdV type HAdV-20-42-42 have advantageous properties for vector development and application. These includes a relatively low seroprevalence, and the ability to induce T cell responses. The vector based on HAdV-20-42-42 could also effectively transduce saphenous vein endothelial cells, implying it as useful in cardiovascular gene therapy.

In study II, we highlighted CD46 as an important attachment factor for species D HAdVs on epithelial cells, demonstrating that it interacted with the hexon protein of the virus capsid. This could allow for multivalent interactions and clarifies the lack of consensus regarding primary receptors for species D HAdVs. Interactions via both fiber and hexon could compensate for each other, complicating the identification of a sole primary receptor. This information is important when developing viral vectors, as CD46 in humans is expressed on all nucleated cells and could make targeting to specific cells or tissues more complex.

The findings in study I and study II together contribute to the basis for a new project called iAds, funded by the European Innovation Council. iAds involves partners from France, Spain, Netherlands, United Kingdom, Sweden and Lithuania with the aim of engineering vectors using elements from rare HAdV types and non-human AdVs to facilitate development of stealth vectors that evade common antibodies and reduce interactions with host factors. Building upon the extensive body of existing AdV research has

great potential for guiding intelligent vector design, paving the way for more effective vaccines, as well as gene therapy and oncolytic treatments.

In study III, we demonstrated that the human endogenous peptide Lfcin acts as an enhancer for HAdV-C5 infection in human skeletal muscle cells. Muscle tissue is a target for vaccination and gene therapy and improving transduction efficacy means less vector is required, subsequently reducing the risk of potential harmful side effects. Future studies should evaluate the cytotoxicity of Lfcin and determine whether it is more effective as a free adjuvant or as a direct capsid modification, such as its integration into the fiber knob or hexon proteins.

Vaccine development advanced rapidly in correlation with the COVID-19 pandemic. With it, new mRNA and viral vector vaccines were developed. However, these achievements were accompanied by rare but significant adverse events. The Astra Zeneca COVID-19 vaccine ChAdOx1 was associated with increased risk of vaccine-induced immune thrombocytopenia and thrombosis [157, 158], caused by cross-reactive antibodies against pVII and platelet factor 4 [159]. The concurrent use of mRNA vaccines by Pfizer-BioNTech and Moderna was linked to cases of myocarditis, primarily in young males, driven by heightened chemokine and interferon responses [226]. Despite the challenges, these platforms have saved millions of lives globally. Regardless of whether one vaccine is better than another, the pandemic showed the necessity of maintaining a diverse repertoire of vaccine types, rather than relying on a single one. Having several alternatives when responding to future pandemics could be essential in saving lives.

Lastly, we should remember that AdVs are pathogens, and so the future of AdV-based vectors also depends on development of tools to study toxicity, safety, host immune interactions, and efficacy. There is a form of irony in the way that pathogens once causing disease today are engineered into tools for preventing it. By researching and developing new viral vectors, we progress toward better precision medicine. Perhaps in the future, as we reflect on these advancements, we will ask ourselves “Well, how did I get here?”

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