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Environmental risk assessment of replication competent viral vectors in gene therapy trials

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Abstract

Environmental risk assessment of replication competent viral vectors in gene therapy trials

The National Institute for Public Health and the Environment (RIVM) has developed a method to estimate the risks for man and the environment of the application of replication competent viral vectors in cancer therapy. Since such a method did not exist, this report will be a significant aid in the risk assessment of replication competent viruses and in guiding applications for a gene therapy license involving the use of these viruses through the regulatory process in the Netherlands.

Dutch scientists are planning to initiate clinical trials in which genetically modified replication competent viruses will be applied. These viruses are able to specifically replicate in cancer cells leading to their destruction.

Potential adverse effects of viral therapies are related to the exposure of man and the environment to the virus. In the Netherlands, exclusively clinical studies making use of replication deficient viruses have been permitted thus far. These 'crippled' viruses can only infect a limited amount of cells and are thereby able to repair the effects of a genetic defect, for instance in a patient with a metabolic disease. Using risk assessment it has been concluded that in most cases the environmental risks of this type of application are negligible.

Replication competent viruses, however, retain characteristics that make them able to multiply within a cancer patient and therefore a basic principle in the risk assessment of these viruses should be that there is a chance of spreading of the virus from the patient into the environment. The report gives points to consider for the environmental risk assessment of replication competent viruses taking into account the viral characteristics, the effects of the genetic modifications on the virus, the current clinical applications and future developments.

The report is expected to provide guidance to risk assessors and regulatory officers as well as to applicants for a gene therapy license.

Key words:
environmental risk assessment, virus, genetic modification, gene therapy, cancer

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Rapport in het kort

Leidraad voor de milieurisicobeoordeling van genetisch gemodificeerde replicatiecompetente virussen in gentherapiestudies

Het RIVM heeft een methode uitgewerkt waarmee de risico's voor mens en milieu van replicatiecompetente virussen als kankertherapie kunnen worden beoordeeld. Zo'n methode bestond nog niet. De verwachting is dat de risicobeoordeling, en daarmee de vergunningverlening, van klinische studies die gebruikmaken van genetisch gemodificeerde replicerende virussen hierdoor kan worden bespoedigd.

Nederlandse onderzoekers zijn van plan om klinische studies te starten waarbij gebruik zal worden gemaakt van virussen die in staat zijn zich te vermenigvuldigen (replicatiecompetent). Dit zijn genetisch gemodificeerde virussen die zich in kankercellen kunnen vermenigvuldigen en ze op die manier kunnen vernietigen.

Mogelijke schadelijke effecten van gentherapieën met een virus zijn gekoppeld aan de mate waarin mens en milieu aan het virus worden blootgesteld. In Nederland zijn tot nu toe uitsluitend virussen die zich niet meer kunnen vermenigvuldigen als therapie toegepast. Deze ‘kreupele’ virussen infecteren een beperkt aantal cellen en kunnen bijvoorbeeld een genetisch defect in een patiënt met een stofwisselingsziekte opheffen. Uit de risicobeoordeling blijkt dat in de meeste gevallen de risico's hiervan voor mens en milieu verwaarloosbaar klein zijn.

Een belangrijk uitgangspunt in de risicobeoordeling van replicatiecompetente virussen is dat er een zekere kans is dat de toegepaste virussen zich vanuit de patiënt in het milieu verspreiden. Replicatiecompetente virussen hebben immers eigenschappen waardoor ze zich binnen een patiënt kunnen vermenigvuldigen. In de aanbevelingen voor de milieurisicoanalyse wordt rekening gehouden met deze eigenschappen, de eventuele effecten van de genetische modificaties op het virus, de huidige klinische toepassingen en toekomstige ontwikkelingen.

Het rapport biedt handvatten voor zowel risicobeoordelaars en beleidsmakers als voor aanvragers van een introductie in het milieu vergunning om gentherapiestudies uit te mogen voeren.

Trefwoorden:
milieurisicobeoordeling, virus, genetische modificatie, gentherapie, kanker

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List of Abbreviations

AdV	Adenovirus
CAR	Coxsackie-adenovirus receptor
CCMO	Central committee on research involving human subjects
CD	Cytosine deaminase
CEA	Carcino embryonic antigen
CIK	Cytokine induced killer
Cor	Coronavirus
CPA	Cyclophosphamide
CPE	Cytopathic effect
CVA	Coxsackievirus A
DLT	Dose limiting toxicity
EC	European Communion
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
EMEA	European medicines agency
ERA	Environmental risk assessment
EV	Echovirus
FDA	Food and drug administration
GALV	Gibbon ape leukemia virus
GFP	Green fluorescent protein
GM	Genetically modified
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMO	Genetically modified organism
HAdV	Human adenovirus
HIV	Human immunodeficiency virus
HMEC	Human mammary epithelial cell
HSP	Heat shock protein
HSV	Herpes simplex virus
IA	Intra-arterial
IBC	Institutional biosafety committee
IH	Immunohistochemistry
IL	Intralesional
IL12	Interleukin 12
IND	Investigational new drug application
InflA	Influenza A
IP	Intraperitoneal
IRES	Internal ribosomal entry site
ISH	In situ hybridization
IT	Intratumoural
IV	Intravenous
Ives	Intravesicular
LacZ	β-galactosidase
MLV	Murine leukemia virus
MTD	Maximum tolerated dose
MU	Mumps virus
MV	Measles virus
Myx	Myxoma virus

NDV	Newcastle disease virus
OBA	Office of biotechnology activities
OVM	Oncolytic virus meeting
p	protein
Parvo	Parvovirus
PCR	Polymerase chain reaction
pfu	Plaque forming units
PKR	Protein kinase R
Polio	Poliovirus
PSA	Prostate specific antigen
RAC	Recombinant DNA advisory committee
RCR	Replication competent retrovirus
RCVV	Replication competent viral vector
RDVV	Replication deficient viral vector
(c) RGD	(cyclic) Arginine-glycine-aspartic acid
Reo	Reovirus
RT-PCR	Reverse transcriptase polymerase chain reaction
SIN	Sindbis virus
TK	Thymidine kinase
Tm	Temozolomide
vp	Viral particles
VSV	Vesicular stomatitus virus
VV	Vaccinia virus
WNV	West Nile virus

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Summary

The objective of environmental risk assessment (ERA) for the application of a genetically modified organism (GMO) is to identify and evaluate potential adverse effects of the GMO on public health and the environment. In the Netherlands the ERA of GMOs in clinical trials is performed according to Directive 2001/18/EC of the European Union that provides the general principles, methodology and a framework for an ERA on the deliberate release of GMOs in the environment (1, 2). The approach is based on the principle that potential adverse effects of a GMO and mechanisms through which these effects may occur will vary from case to case.

Replication competent viral vectors (RCVVs) represent a new and potentially important modality for treatment of cancer that is expected to be applied in clinical studies in the Netherlands in the near future (3). In the approval process of these vectors it has to be considered that spread of the vector into the environment may occur. The ERA of gene therapy products especially evaluates the risks to those most likely to be exposed to the GMO, including persons that handle or administer the gene therapy product, those involved in patient care, but also relatives and 'the general public'. Therefore the ERA will need to focus on the possible effects of the RCVV on public health, taking the consequences of the genetic modifications on the viral life cycle and interaction between virus and (human) host and the conditions of use into detailed consideration.

An ERA has to take into account the relevant and scientific details regarding characteristics of the parental organism, the genetic modifications, the GMO, the intended release, the potential receiving environment and the interaction between these (2). Presently, however, there is no specific guidance to perform the ERA of clinical studies that make use of RCVVs.

Current (pre-)clinical strategies are aimed at increasing the potency and the number of replicating cycles of RCVVs *in vivo*. This has gradually resulted in the development of RCVVs with more complex modifications and application of combination treatments that promote vector replication. Given the complexity of RCVVs and their proposed use, the aim of this report is to identify points to consider in the ERA and to present the ERA in a format that shows the association between the different steps.

In the present report an overview is provided on characteristics regarding the use of RCVVs in clinical trials. These include properties of the applied RCVV backbones, the types of genetic modifications that are being applied within these backbones and the influence of the modification on the properties of the backbones. Moreover, clinical strategies may involve a variety of combination treatments that directly interact with the applied genetically modified viral vector and that should be considered in the ERA. This overview has lead to a stepwise methodology for the ERA of the deliberate release of genetically modified RCVVs which is presented in the form of a general ERA template in paragraph 4.2. This ERA for RCVVs should be applicable to all currently used vector backbones and strategies. Based on published safety data, basic features and assumptions, the ERA has been tested for the systemic use of the adenoviral vector Onyx-015 and the conclusion is that although environmental spreading of this vector can not be excluded, the environmental risks of the use of this vector are negligible. This specific example shows how the ERA can be approached in actual practice.

The structured ERA is expected to provide guidance to both regulatory officers and applicants about which information is needed to perform the ERA for RCVVs, and why this information is needed. The proposed ERA is expected to be of significant aid in guiding applications involving

RCVVs through the regulatory process in the Netherlands and may be implemented in the current ERA structure in other countries.

1 Introduction

Genetically modified (GM) viruses are being applied for the treatment of an increasing amount of hereditary disorders and diseases in the clinic. In the Netherlands, exclusively clinical studies that make use of genetically modified replication deficient viral vectors (RDVVs) or vectors that are replication deficient in the human host (e.g. canary pox virus) have been permitted thus far. However, outside of the Netherlands various clinical trials have been performed with GM replication competent viral vectors (RCVVs). Preclinical research and clinical trials abroad have shown that RCVVs are a promising new entity in medicine, especially in cancer (gene) therapy. Therefore, it is not surprising that several proposals involving the use of RCVVs in clinical trials will be submitted in the Netherlands in the near future.
The objective of an environmental risk assessment (ERA) is to identify and evaluate potential adverse effects of the application of a GMO on public health and the environment. An important factor in the ERA of clinical studies involving RDVVs is that if spreading of infectious virus from the patient occurs, which may take place (in most cases at low levels) for a short period of time after administration, the replication deficient particles are not likely to lead to productive infections. Thus, the exposure will be less than that associated with the wild type strain.
RCVVs vectors are able to (conditionally) replicate in the human host meaning that these vectors can infect cells thereby producing new viral particles that in turn may infect neighbouring cells. In the approval process of these vectors it has to be considered that spread of the vector into the environment may occur; RCVVs are more likely to spread more effectively and for a longer period into the environment compared to RDVVs. The risk assessment of such studies therefore needs to take into account the replication competency and other properties of RCVVs and their proposed conditions of their use (i.e. the clinical protocol).
Presently, there is no specific guidance to perform the ERA of clinical studies that make use of RCVVs. The goal of this report is to provide background information about developments in this field and recommendations for the present and future ERAs of clinical studies making use of RCVVs. For this purpose the following information is included in this report:

- A description of the general ERA methodology according to Annex II of Directive 2001/18/EC (chapter 2);
- A review of RCVVs focusing both on vectors that are being developed preclinically (paragraph 3.2 and Appendix A) and on vectors that have already been applied in clinical studies (paragraph 3.3 and Appendix B);
- A survey of safety data from clinical studies (paragraph 3.3 and Appendix B);
- Predictions about future developments in the application of RCVVs (Appendix B and supplementary document (4));
- A methodology for the ERA of RCVVs based on identified elements that are possibly involved in spreading and environmental effects of RCVVs (chapter 4);
- The ERA according to this methodology for an example RCVV (Appendix D).

2 Material and Methods

2.1 General principles of the ERA according to Annex II of Directive 2001/18/EC

Directive 2001/18/EC describes in general terms the objective to be achieved, the elements to be considered and the general principles and methodology to be followed to perform the environmental risk assessment concerning the deliberate release of GMOs (2). Annex II of this Directive describes the aim and general principles of the ERA and provides a framework for the ERA methodology.
The ERA should evaluate the environmental risks of the GMOs in their interaction with the environment, in order to identify the need for risk management during the release and to identify the most appropriate risk management methods to be used. The ERA is performed on a case-by-case basis, in an iterative process. Identified characteristics of the GMO and its use which have the potential to cause adverse effects should be compared to those presented by the non-modified organism from which it is derived and its use under corresponding situations. The ERA should be performed in a scientifically sound and transparent manner based on available scientific and technical data and if new information on the GMO becomes available the ERA may need to be readdressed to determine whether the risk has changed.
Ideally an ERA takes into account quantitative data. However, most of the information that is important in an ERA may be qualitative for the reason that quantification is often hard to accomplish, and not necessary to make a decision. An ERA does not have to be based on the scenario that is expected to occur, but may also be based on a worst case scenario (5). A worst case scenario will especially be applied in cases where there is a high degree of scientific uncertainty. It is anticipated that the worst case scenario will be more often applicable to the use of RCVVs compared to RDVVs, because of scientific uncertainty. Although there are many parallels in the risk assessment of RDVVs and RCVVs and the general methodology for the ERA is the same, spreading and effect scenarios of RCVVs are expected to be more complex compared to those of RDVVs, given the (relative) replication competency of RCVVs. Moreover, the fast pre-clinical developments in the field of RCVVs that are mainly aimed at increasing their potency and the limited availability of both quantitative and qualitative safety data add to this notion (see also chapter 3).

2.2 The ERA methodology

Annex II of Directive 2001/18/EC provides a framework for the technical and scientific information that is required for an ERA (2). The information that is needed for a specific application is decided upon on a case-by-case basis. The information should according to the Annex contain a description of the following characteristics:

- the parental organism;
- the genetic modification; be it inclusion or deletion of genetic material, and relevant information on the vector and the donor;
- the (expected) new physiological traits (phenotype) of the GMO;

- the intended release or use, including its scale;
- the potential receiving environment;
- and the interaction between all of the above.

The ERA is performed according to the following steps described in Annex II.

Step 1: Identification of characteristics that may cause potential adverse effects and evaluation of the potential consequences of each adverse effect (Hazard identification)

A hazard is defined as a potential harmful effect on human health or the environment, caused by features of the GMO and its application. Effects on human health or the environment are differentiated into direct and indirect effects. Direct effects occur due to an interaction between the GMO itself and its direct environment. Indirect effects are the result of a more extensive causal chain of events. Moreover, both direct and indirect effects may be either immediate or delayed. Immediate effects occur during the time period of the release, and can usually be attributed to the release in a straightforward manner, whereas delayed effects may become apparent at a later stage, after termination of the release, and are consequently less easily attributed to an effect caused by the GMO. Effects that are to be identified as potentially harmful will vary from case to case and may include:

1. Persistence and invasiveness of the viral vector in natural habitats under the conditions of the proposed release(s).

2. Selective advantages or disadvantages conferred to the GMO and the likelihood of this becoming realized under the conditions of the proposed release(s).

3. Potential for gene transfer to other species under conditions of the proposed release of the GMO.

4. Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the GMO and persons working with, coming into contact with or in the vicinity of the GMO release(s).

5. Possible immediate and/or delayed effects on animal health including toxic and allergenic effects and consequences for the feed/food chain resulting from consumption of the GMO and any product derived from it, if it is intended to be used as animal feed.

6. Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO with non-target organisms, including impact on population levels of competitors, prey, hosts, symbionts, predators, parasites and pathogens.

7. Possible immediate and/or delayed, direct and indirect environmental impacts of the specific techniques used for the management of the GMO where these are different from those used for non-GMOs compromising prophylactic or therapeutic medical, veterinary, or plant protection treatments, for example by transfer of genes conferring resistance to antibiotics used in human or veterinary medicine.

8. Possible immediate and/or delayed effects on biogeochemical processes resulting from potential direct and indirect interactions of the GMO and target and non-target organisms in the vicinity of the GMO release(s).

9. Altered susceptibility to pathogens facilitating the dissemination of infectious diseases and/or creating new reservoirs or vectors.

According to Annex II adverse effects may occur directly or indirectly through a number of mechanisms that may include:
- the spread of the GMO in the environment;
- the transfer of genetic material to other organisms;
- phenotypic and genetic instability;
- interactions with other organisms.

The second part of the hazard identification concerns the identification of the potential consequences of each adverse effect, if it occurs. The magnitude of the consequences is likely to be influenced by the environment in which the GMO is released and the manner of release. For each adverse effect that is identified the consequences for the environment need to be qualified in qualitative terms ranging from high, moderate, low to negligible.

Step 2: Evaluation of the likelihood

The next step in an ERA deals with the evaluation of the likelihood of the occurrence of the described adverse effects and their consequences, i.e. whether the potential adverse effect associated with the application of the GMO will be effectuated. All characteristics that could contribute to the actual occurrence of the hazard must be identified. For the determination of the likelihood it is important to consider the manner of release and the surrounding environment. If no quantitative data are available to determine the likelihood, the likelihood should be described in qualitative terms ranging from high, moderate, low to negligible, based on an expert judgement that considers the available data[1]. In case of a high degree of scientific uncertainty a worst case scenario may be applied, meaning that the likelihood will be equal to 1.

Step 3: Risk estimation

An estimation of the risk to human health or the environment posed by each identified characteristic of the GMO which has the potential to cause adverse effects should be made by multiplying the likelihood of the adverse effect occurring and the consequences, if it occurs. The risk should be described in qualitative terms ranging from high, moderate, low to negligible, based on previous steps and expert judgement.

Step 4: Application of risk management strategies

Under circumstances that the estimated overall risk is not negligible, risk management strategies should be applied that are adequate to reduce the level of risk. It should be assessed whether the application of risk management affects the characterized hazards and the estimated likelihoods.

Step 5: Determination of the final risk

An evaluation of the overall risk of the GMO should be made taking into account any proposed risk management strategies. The determination of the final risk should conclude an ERA based on the previous steps in ERA described under step 1 to 4. This step in an ERA concludes whether the overall environmental impact is acceptable or not. Preferably the final evaluation should be expressed as a summary of the overall risks that are connected to the specific application.

[1] Criteria for the qualitative description of the likelihood of a hazard: negligible = theoretically possible, but never observed in practice, low = observed in practice, but with very low frequency, moderate = observed in practice, but with moderate frequency, high = observed in practice, with high frequency. In practice, the applicant will make the initial judgement about which qualitative term is applicable and the risk assessor will evaluate the argumentation.

2.3 Generation of the overview of replication competent viral vectors and the ERA for these vectors

The information on oncolytic viruses and their applications was primarily gathered by literature searches in Pubmed (6) and by visiting the Oncolytic Virus Meeting 2007 in Carefree, Arizona, USA. Additional information on ongoing clinical trials and clinical protocols involving replication competent viruses was found by visiting the web pages of biotechnology companies (7-11) and by studying the minutes of the RAC meetings (from 30/03/1990 till 11-12/03/2008) (12). The risk assessment method presented in this report was developed in conjunction with the supervising committee from the GMO office/RIVM. A draft version of this report and the proposed ERA was evaluated by means of interviews with Dutch experts involved in gene therapy and virology research (see acknowledgements). Their input has been incorporated in the entire report and more specifically their vision on future developments has been worked out in Appendix B.5. However the author takes full responsibility for the views expressed in this report.

3 Results

3.1 The need for guidance for the environmental risk assessment of replication competent viral vectors in clinical trials

As discussed in the previous chapter, Annex II of Directive 2001/18/EC provides the general principles and the framework for the ERA of GMOs. The ERA of gene therapy products, including RDVVs and RCVVs, should especially evaluate the risks to those that are most likely to be exposed to the applied product. These include persons that handle or administer the gene therapy product and other hospital personnel, but also close relatives and the general public. The ERA should also establish whether there might be potential adverse effects on other organisms. In the case of gene therapy studies the dose and the conditions under which the products are administered to the subject may determine the magnitude and duration of the potential exposure. It should be emphasized that the ERA of gene therapy products does not consider the safety of the trial subject. In the Netherlands the risks for the human subject are assessed by the CCMO according to the Central Assessment of Medical Research (Human Subjects) Decree.
GM RCVVs are a relatively new entity in clinical research and presently there are no specific guidance documents to perform the ERA for RCVVs[2]. Potential adverse effects of a GM viral vector may result from altered properties compared to the parental virus regarding specific aspects of the viral life cycle and the interaction with the host. Such properties include changes in tissue tropism and host range, altered infectivity and pathogenicity, changes due to genes that have been inserted for specific purposes of the gene therapy, and recombination and complementation resulting in the development of new recombinant viruses. Indirect effects may occur e.g. by dissemination of the GMO from the site of injection, shedding of the GMO, infection of another host and effects of the GMO on the other host.
This project was initiated to identify elements that play a role in spread and effect scenarios for RCVVs and that should be considered in the ERA. The ERA should be applicable for vectors that are currently applied in clinical studies and for future more complex vectors. Therefore up to date scientific data knowledge about the vector backbones, the genetic modifications that are being applied, and the applications of the vectors is necessary. Moreover, an overview of safety data of RCVVs may be of aid in the generation of spreading and effect scenarios for these vectors.
To give points to consider for the ERA of RCVVs in present and future clinical studies an overview of the types of genetic modifications that are being applied and tested in pre-clinical models, and the influence of the modifications on the viral properties has been generated (Appendix A and supplementary document (4)). Moreover, the specific applications and strategies that are being developed to optimize the use of RCVVs are evaluated, as these parameters may also influence the ERA (Appendices A and B and supplementary document (4)). In paragraphs 3.2 and 3.3 a survey of these appended data and general conclusions are provided. The identified elements and other considerations have been worked out in a structure for the ERA of RCVVs and a general ERA template (paragraphs 4.1 and 4.2).

[2] The Gene Therapy Working Party of the European Medicines Agency has published a draft version of a guideline on scientific requirements for the environmental risk assessment of GMO containing gene therapy medicinal products as required for marketing authorization (http://www.emea.europa.eu/pdfs/human/genetherapy/12549106en.pdf.). This guideline applies to GMO containing gene therapy medicinal products in general and contains some of the considerations also addressed in this paper.

3.2 Replication competent viral vectors: strategies and (pre)clinical developments

Genetically modified RCVVs are currently almost exclusively being applied for use in cancer therapy. RCVVs can be roughly divided into two categories, based on their application as either a tumour vaccine or as an oncolytic virus. The first category of GM RCVVs comprises viral vaccine strains, most often based on the immunogenic vaccinia virus, that are modified by insertion of single or multiple tumour antigens and / or immunostimulatory molecules. These vectors are able to promote an immune response against a tumour through their immunostimulatory properties (13, 14). It should be noted that besides for cancer therapy there are examples of replication competent GM viral vaccines (besides vaccinia also based on for instance VSV and HAdV) that are being developed for treatment of other diseases (e.g. HIV) (15-17). Many vaccinia based viral tumour vaccines have been extensively tested in clinical studies (phase I-III) abroad and these vectors have a well established safety profile. Several of the oncolytic viruses (see below) also fall into the viral vaccine category (e.g. oncolytic vectors based on vaccinia virus) since these viruses partially elicit their anti-tumour activity by acting as a tumour vaccine, because of the immune stimulating properties of the viral backbone and introduced inserts.
The second and much broader category of RCVVs is comprised by the GM oncolytic viruses. Oncolytic viruses are defined as wildtype, or genetically modified viruses that more or less selectively replicate in tumour cells leading to the specific lysis of these cells. Ideally an oncolytic virus should have the following hallmarks: it should specifically replicate in, propagate in and kill tumour cells, have a low toxicity for normal cells, be able to be inactivated by an antiviral agent, and not pose a danger for the general population (13, 14, 18-20). Many of the wildtype viruses that are candidates to be applied as an oncolytic virus in clinical studies do not have all of these properties. A way to overcome this problem is the genetic modification of candidate viruses in order to increase their specificity, potency and/or safety. Moreover, viruses that do not have strong oncolytic properties by themselves can be turned into an oncolytic agent by genetic modification (19, 21-23). Additional hallmarks of an ideal oncolytic virus therefore are ease of genetic manipulation and genetic stability.

RCVV backbones

Table I provides an overview of the wildtype and GM replication competent viruses that are being used in pre-clinical studies and in clinical trials, the types of genetic modifications that have been applied in each of the viral backbone(s), and the possible applications as either wildtype or GM virus. In the overview viruses that are now being applied in their wildtype form have been included because it can be predicted from past developments that, when technically possible, these viruses will also be subjected to similar genetic modifications in the future. The rationale behind the most important strategies that have been undertaken in the context of replication competent vectors are described in Appendix A in more detail.
Table I shows that a limited number of replication competent wildtype and GM viruses (i.e. non-recombinant reovirus, CVA and NDV and genetically modified HAdV, HSV, VV and MV have been subjected to extensive clinical trials abroad (24), but that viruses belonging to many different families are candidates to be applied in experimental medicine and are currently being tested in a preclinical setting.
Table II shows an overview of some of the specific advantages and disadvantages associated with the applied viral backbones (see also Appendix A). These properties are important for their potential anti-tumour efficacy as well as for estimation of the environmental risks associated with their use. All of the applied viruses have basically shown efficacy in animal models (4).

Table I. Overview of replication competent viruses and viral vectors; applications of wildtype and GM viruses and types of modifications applied.

Species	**AdV**	**HSV-1**	**VV**	**Myx**	**Aut P**	**MLV**	**VSV**	**MV**	**MU**	**NDV**	**InflA**	**CVA**	**EV1**	**Polio**	**Cor**	**SIN**	**Reo**
Type virus	DNA	DNA	DNA	DNA	DNA	Retro-virus	RNA (-)	RNA (-)	RNA (-)	RNA (-)	RNA (-)	RNA (+)	RNA (+)	RNA (+)	RNA (+)	RNA (+)	RNA ds
Applications WT[a]																	
Oncolytic virus		x	x	x	x		x	x	x	x		x	x			x	x
Vaccine			x					x	x	x				x			
Applications GM																	
Oncolytic virus	x	x	x	x	x	x	x	x		x	x			x	x	x	x
Tumour vaccine			x							x							
HIV vaccine	x						x										
Conditional replication																	
Mutation	x						x				x						x
Single gene deletion	x	x	x								x						
Double gene deletion	x	x	x														
Transcriptional targeting	x	x			x	x											
Translational targeting	x													x			
Transgene																	
Marker	x	x	x	x		x	x	x		x					x	x	x
Prodrug	x	x	x			x	x	x		x							
Cytokine	x	x	x			x	x	x		x							
Fusogenic glycoprotein	x	x				x	x			x							
Angiogenesis	x	x				x		x		x							
Apoptosis[b]	x	x															
Antigen	x		x				x	x								x	
Heat shock protein	x																
Safety insert	x		x			x	x										
Tissue penetration	x																
Tropism																	
Modification native binding	x					x	x	x									
Antibody insertion	x						x	x		x					x		
Combinations	x	x	x			x		x									

No shading = existing preclinical vector, orange = clinical protocol submitted, blue = trial in progress or non published, green = published trial; [a] Wildtype strains also include spontaneously derived mutated strains.
[b] insertion of p53 is regarded as an apoptosis regulating transgene although this insertion may have various other effects besides apoptosis. Abbreviations not in the list of acronyms: ds = double-stranded.

Table II. Direct comparison of advantages and disadvantages of the main viral backbones that are applied as RCVVs.

Species	HAdV	HSV-1	VV	Myx	Aut P	MLV	VSV	MV	NDV	InflA	CVA	Polio	Reo
Type virus	DNA	DNA	DNA	DNA	DNA	Retro	RNA (-)	RNA (-)	RNA (-)	RNA (-)	RNA (+)	RNA (+)	RNA ds
Backbone (serotype, strain)	HAdV-2/5	KOS, F, 17, HF	Wyeth	Lausanne	H1	Moloney	Indiana	Edm	73T, HUJ PV701, MTH68	PR8	CVA21	Sabin	Dearing
Properties backbone													
Human host	+	+	+	-	-	-	+	+	-	+	+	+	+
Animal host(s)	-	-	-	+	+	+	+	-	+	+	-	-	-
Vector based on vaccine strain?	-	-	+	-	-	-	-	+	-	+	-	+	-
Pathogenic in humans (wt backbone)	+ (mild)	+	+	-	+ (mild)	-	+ (mild)	+ (mild)	-	+ (mild)	+ (mild)	+	-
Replication cycle	24h	19h	8h				2h		6h		<10h		18h
Tumour specificity	-	-	-	+/-	-	+/-	+/-	+/-	+/-	-	+/-	-	+/-
Specific antiviral available	+/-	+	+/-	-	-	+	- (ifn)	- (ifn)	- (ifn)	+	-	-	+/-
Fusogenic insert	-	-	-	-	-	-	-	+	+	+	-	+	-
Genetic Stability	+	+	+	+	+	-	-	-	-	-	-	-	-
Mutation	-	-	-	-	-	+	+	+	+	+	+	+	+
Recombination	+	+	+	+	+	+	-	-	+/-	-	+	+	-
Reassortment	-	-	-	-	-	-	-	-	-	+	-	-	+
Integration in DNA	-	-	-	-	-	+	-	-	-	-	-	-	-
Latent infections	+	+				+							+
Capacity for GM	~10kb	~30 kb	~25 kb		limited	limited	~4kb	~4kb	~4kb				
Stability insert	+	+	+		-	-	+/-	+/-	+/-			+/-	
Pre-existing immunity (humans)	80-100%	50-90%	>90%	0%	<10%	0%	<10%	90-100%	0%		<10%	>90%	100%
Suitability for systemic administration	-	+/-	+	+		-	+	+	+		+	-	+/-
References	(4, 24, 34, 35)	(4, 24, 36)	(4, 24)	(4, 37)	(4, 38, 39)	(4, 40-43)	(4, 44-46)	(4, 47)	(4, 24, 48-51)	(52-54)	(4, 55, 56)	(4, 57, 58)	(4, 24, 59, 60)

In case of an empty field no specific data were available or were found. (+) = yes, (-) = no, (+/-) = relative (tumour specificity, stability) or maybe (antiviral available, suitable for systemic administration). Abbreviations not in the list of acronyms: ifn=interferon, h = hour, kb = kilobase. Adapted, modified and expanded from (24).

However pre-clinical studies in which the anti-cancer activities of oncolytic viruses based on different viral backbones are directly compared are very rare (25, 26) and up to date no direct comparison has been made in clinical trials. Each oncolytic virus may have unique biological characteristics that may determine the tumour types against which it may be applied (18, 20). Therefore, it is at this moment very difficult to predict which viruses will be applied successfully in clinical trials in the future. Based on the overview of current and future clinical trials (see Appendix B), the number of hits for each virus in Pubmed (table III), the trends apparent from the oncolytic virus meeting 2007, recent reviews (20, 27, 28) and the opinion of the interviewed experts, the most important GM viruses that are expected to be further explored into clinical trials are based on the DNA viruses HAdV-5, HSV-1, VV and on the RNA viruses MV, VSV and Reovirus (table III).

Types of genetic modifications in RCVVs

Genetic modification of replication competent viruses is currently being performed on an extensive scale and the modifications (see supplementary document (4) for specific references) include:

- functional deletion of single or multiple viral genes, that are more (e.g. HAdV E1B-55kD) or less (e.g. HAdV E3 genes) essential for replication in normal cells; functional deletion can be accomplished by complete or partial deletion of genes
- modification of gene function by deletion of domains in genes or introduction of point mutations
- insertion of foreign sequences that regulate expression of endogenous viral genes / proteins or inserted transgenes / proteins:
  - sequences involved in transcriptional regulation (e.g. PSA promoter in adenoviral vectors)
  - sequences involved in translational regulation (e.g. IRES from rhinovirus in poliovirus vectors)
- marker genes (e.g. GFP, lacZ)
- therapeutic transgenes from a variety of donor organisms:
  - prodrug converting enzymes (e.g. CD, TK)
  - cytokines (e.g. GM-CSF, IL12)
  - tumour antigens (e.g. CEA)
  - fusogenic viral glycoproteins (e.g. GALV envelope)
  - angiogenesis regulating genes (e.g. platelet 4)
  - genes involved in apoptosis (e.g. p53)
  - genes that promote tissue penetration (e.g. relaxin)
  - safety inserts (e.g. interferon, TK)
- a variety of insertions and manipulations of viral coat proteins that alter the viral tropism:
  - insertion of bispecific antibodies (e.g. antibodies binding EGFR)
  - insertion of motifs that enhance tropism (e.g. RGD motif in HAdV)
  - ablation of native tropism (e.g. HAdV fiber knob replacement)
- insertions that shield the vector from the immune system (e.g. insertions in pIX in HAdV vectors)
- almost any combination of the above modifications (see Appendix A).

Conclusions

Changes in aspects of the viral life cycle (e.g. entry, transcription, translation, assembly, replication, release, tropism, transmission) and / or the interaction with the host (e.g. immuno-modulation, apoptosis, extracellular and intracellular signalling, pathogenesis, recombination and

adaptation) are regarded as mechanisms that may lead to specific environmental hazards in the use of viral vectors (2, 29, 30). It is apparent from the overview in Appendix A and tables I and II that many of the applied strategies are either directly aimed at, or may accidentally alter, specific or multiple aspects of the viral life cycle and the interaction with the host. Moreover, in a vector with multiple alterations, each of the single alterations may have a different effect on each of these aspects. It is therefore important to consider each of the aspects as a separate element in the ERA. Elements that may influence the ERA are not only associated with the properties of the viral backbone and the genetic modifications in this backbone. Also external factors (e.g. conditions of use) that may influence the viral life cycle and the interaction with the host have to be integrated in the structured ERA for RCVVs. This includes information about the combination treatments of which examples are mentioned in Appendix A5.3, and that in most cases are aimed to increase tumour cell killing by the applied vector by enhancing the number of replicative cycles or by additive toxic effects.

Table III. Overview of number of publications, RAC reviewed clinical protocols and published clinical trials for replication competent viruses / viral vectors.

Virus or viral vector	**Number of hits in combination with term 'oncolytic' in Pubmed (06-02-2008)**	**Number of RAC reviewed protocols with RCVVs (19-11-2007)**	**Published clinical trials with RCVVs (journals, OVM meeting 2007) (06-02-2008)**
Adenovirus	428	28[a]	29
Herpes simplex virus	233	16	8
Vesicular stomatitis virus	55	0	0
Newcastle disease virus	49	NA (WT)	4 (WT)
Measles virus	46	3	1
Reovirus	43	NA (WT)	4 (WT)
Vaccinia virus (OV)	35	5	5
Vaccinia virus (TV)	ND	46	ND
Parvovirus	22	0	0
Coxsackievirus	19	NA (WT)	1 (WT)
Poliovirus	18	1	0
Myxoma virus	10	0	0
Influenza virus	8	0	0
Retrovirus	8	0	0
Sindbis virus	4	0	0
Mumps virus	3	0	0
Echovirus	2	0	0
Coronavirus	1	0	0

NA (WT) = not applicable, so far only wildtype virus applied in clinical trials, ND = not determined, OV = oncolytic virus, TV = tumour vaccine. [a]27 oncolytic vectors and one live vaccine vector have been reviewed.

3.3 Clinically tested replication competent viral vectors and available safety data

This paragraph provides a short survey of the clinical trials that have been performed with RCVVs abroad thus far, and of published clinical safety data. A more exhaustive overview of the specific vectors applied in clinical studies can be found in Appendix B and in the supplementary document (4). For specific vectors also an overview of available pre-clinical safety data is provided in these documents. Clinical and pre-clinical safety data play an instrumental role in risk determination in an ERA; the availability and quality of these data determines the scenarios on which the ERA will be based. In case of insufficient data and scientific uncertainties a worst case scenario may be applied.

RCVVs in clinical trials

In table I the viral vector backbones and types of modifications that have entered the clinic or that are currently under regulatory review are indicated. Among the wildtype replicating viruses that have been applied in clinical trials are specific strains of VV, NDV, CVA and reovirus. Genetically modified variants of NDV, CVA and reovirus are under pre-clinical development and may be applied in the future.
Genetically modified viruses that have been or that are currently applied in clinical trials are based on HAdV, HSV-1, VV and MV. In case of VV and MV, the RCVVs are respectively based on the naturally attenuated vaccine strains Wyeth and Edmonston containing additional therapeutic or immunogenic insertions. Most of the applied recombinant HAdV, HSV-1 and VV RCVVs are conditionally replicating vectors containing either deletions of single (the HSV vector HSV1716, the VV vector JX-594) or multiple genes (the adenoviral vectors Onyx-015 and H101 and the HSV-1 vectors G207 and NV1020). In case of HAdV also conditionally replicating transcriptionally targeted type 2 CRAds (i.e. CV706, CV787, CG0070) are being applied in clinical trials. Besides vectors without therapeutic insertions a number of conditionally replicating vectors containing donor insertions have entered clinical trials. These include vectors containing the CD/TK suicide gene (Ad5-CD/TKrep), a lacZ marker insert (G207) and the immunostimulatory cytokine GM-CSF (the HSV-1 vector Oncovex^{GM-CSF}, the VV vector JX-594 and the HAdV vector CG0070). In 2007, the first clinical trial making use of a tropism modified vector was initiated in the USA (AdΔ24-RGD in patients with ovarium cancer).

Safety data from clinical trials

Safety data from clinical trials with RCVVs, when published, may include (table IV and V, (4)):
- information about occurrence of adverse events including toxicity;
- establishment of maximum tolerated dose (MTD) and dose limiting toxicity (DLT);
- systemic distribution data (PCR, plaque assay on blood);
- biodistribution data (PCR, EM, plaque assay on normal tissues versus tumour biopsies);
- data about ongoing viral replication (PCR or plaque assay at different time points on tumour biopsies);
- shedding data (PCR, plaque assay on samples from injection site, urine, stool, sputum).

Table IV. Summary of safety data and results from individual (oncolytic) HAdV clinical trials

Viral vector (HAdV-2/5)	Trial -phase/ country -no of patients	Treatment dose -max pfu[a]	Treatment regimen -s/m -no of a in period -no of c	Route	Combi-treatment	Tumour type	Shedding? (PCR) -tissue -tpa	Shedding? (live virus) -tissue -tpa	Virus in circulation? (PCR) -tpa	Virus in circulation? (live virus) -tpa	Virus in tumour tissue? -tpa	Virus in normal tissue? -tpa	Repli-cation	Toxicity -DLT or MTD? - grade 3/4?	CR	Ref.
Onyx-015	I/UK 22	10^{11}	s, m 1a in 4w ≤5c	IT	no	head/ neck		no, inj-s, pharynx, d0-29	no, d0-29		yes, d8, d22	no, d8, d22	+/-	no no	+/-	(61)
Onyx-015	II/USA 40	10^{10}	m 5-20a in 3w ≤8c	IT	no	head/ neck			c1: yes, d0-17, no, d22 c2: yes, d0-10 no, d15		yes, d1-10, no, d14-17	no, d1-17	+/-	no yes	+/-	(62, 63)
Onyx-015	II/USA 37	10^{10}	m 5a in 3w ≥1	IT	chemo	head/ neck					yes, d0, d10	no, d0, d10	+/-	no yes	+	(64)
Onyx-015	I/USA 23	10^{11}	m 1a in 3w ≤5c	IT	no	pancreas			yes, 15 mi, no, d1-15		no, d22		no	1 DLT yes	+/-	(65)
Onyx-015	I-II USA 21	$2x10^{11}$	m ≤8a in 57d	IT	chemo	pancreas			no		no		no	no yes	+/-	(66)
Onyx-015	I-II USA 6	10^{10}	m 5a in 1m ≤6	IT	chemo	sarcoma			yes, d0-7		yes, d0	no, d0	yes	no no	+/-	(67)
Onyx-015	II/UK 15	10^{10}	s	IT	no	oral					yes, 24h-d14	yes, 24h-d14	+/-	no no		(68)
Onyx-015	I/USA 24	10^{10}	s	IL	no	glioma								no no	no	(69)
Ad/L523S	I/USA 13	$400x10^{9}$ vp	m 2a in 56d	IM	prime/ boost	lung cancer								no no		(70)
H101	I/China 15	$1.5x10^{12}$ vp	m 5a per c	IT	no	various	no, urine, inj-s, pharynx		yes				+/-	no no		(24, 71)
H101	II/China 50	$5x10^{11}$ vp	m 5a in 3w	IT	chemo	various			yes, 30 mi		yes, d16		+/-	no yes	+/-	(24, 72)
H101	III/China 160	$1.5x10^{12}$ vp	m 5a in 3w	IT	chemo	various									+	(73)
Ad5 CDTKrep	I/USA 16	10^{12} vp	s	IT	prodrug	prostate		no, urine, d0-1y	yes, d0-76, no, d76-1y	no, d0-1y	yes, d14		yes	no no	+/-	(74)
Ad5 CDTKrep	I/USA 15	10^{12} vp	s	IT	prodrug radio	prostate			yes, d0-45	no, d0-45	yes, d14, 21, no, d28		+/-	no no	+	(75)
Ad5γCDmut TKrep-ADP	I/USA 9	10^{12} vp	s, m 1-2a in 22d	IP	prodrug radio	prostate			yes, d0-96					no yes	+	(76)

Table IV (continued)

Viral vector (HAdV-2/5)	Trial -phase/ country -no of patients	Treatment dose -max pfu[a]	Treatment regimen -s/m -no of a in period -no of c	Route	Combi-treatment	Tumour type	Shedding? (PCR) -tissue -tpa	Shedding? (live virus) -tissue -tpa	Virus in circulation? (PCR) -tpa	Virus in circulation? (live virus) -tpa	Virus in tumour tissue? -tpa	Virus in normal tissue? -tpa	Repli-cation	Toxicity -DLT or MTD? - grade 3/4?	CR	Ref
Onyx-015	I/UK 16	10^{11}	m 5a in 3w ≤4c	IP	no	ovarian	yes, d5, 15, 354		no, 15mi-24h				+/-	1 DLT yes	-	(77)
Onyx-015	II/USA 20	$3x10^{10}$	s, m ≥1a in 3w ≤6c	IP, IT	no	hepato-biliairy	yes, bile, ascites, d1-5, d1-9	yes, ascites, d1-6, no, urine, d1-14			yes, 37h, d7	yes, 37h, d7	yes	no yes	+/-	(78-80)
Onyx-015	I/USA 22	10^{11}	m ≤20a in 12w	MW	no	oral					yes, d3		+/-	no no	+/-	(81)
Onyx-015	I-II/Egypt 16	$3x10^{11}$	m 3-6a per c ≤4c	IA, IV, IT	chemo	liver					yes, d14		+/-	no no	+/-	(82)
Onyx-015	II/UK 5	$3x10^{11}$	m, 6a in 30d	IV+IT	no	liver			no, 4h		yes			no no	+/-	(83)
Onyx-015	I/USA 10	$2x10^{13}$ vp	m, 3a in 21d ≤8c	IV	chemo	metast. to lung			c1-2: yes, 6h-d7		yes, d5 no, d134	no	yes	no no	-	(84)
Onyx-015	I/USA 10	$2x10^{12}$ vp	m 6a in 6w	IV	chemo, IL2	metast.			yes, 48h		yes	no	+/-	no	-	(85)
Onyx-015	I/USA 11	$2x10^{12}$ vp	m ≤9a in ≤200d	IA	chemo	crc liver			yes, 0-6h, d4				+/-	no yes	+/-	(86-88)
Onyx-015	II/USA 27	$2x10^{12}$ vp	m ≤9a in ≤200d	IA	chemo	crc liver			c1: yes, 0-6h, d3-4 c4: yes, d1-d8				yes	no yes	+/-	(87-89)
Onyx-015	II/USA 18	$2x10^{12}$ vp	m 2a in 4w ≤4.5c	IV	no	crc liver			yes, 6h, 24h, 72h			yes, spleen, liver, 56h	yes	no yes	+/-	(90)
Onyx-015	I/USA 9	$1x10^{12}$ vp	M 4a in 4w 2c	IV	enbrel	solid tumour			yes, d3, d8				+/-	no no	-	(91)
CV706	I/USA 20	$1x10^{13}$ vp	s	IT	no	prostate		yes, urine, d2, 8, no, d15, d29	yes, d0-d15		yes, d4, d22, 3m		yes	no yes	+/-	(92)
CV787	I/USA 23	$6x10^{12}$ vp	s	IV	no	prostate		no, urine, d0-29, yes, saliva, d4-8	yes, d0-29				yes	no yes	+/-	(93)

[a] dose is in plaque forming units (pfu) unless otherwise indicated. Replication column: (+/-) = evidence for prolonged viral presence but no sufficient data to prove ongoing replication. CR (clinical response) column: (+) = minor, mixed, partial or complete response in >50% of patients, (+/-) in <50% of patients, (-) = no responses observed. In case of empty fields no data were reported. Shedding data are highlighted in grey.
List of abbreviations: a = administration(s), c = cycles, chemo = chemotherapy, crc = colorectal cancer, d = days, DLT = dose limiting toxicity, h = hours, IA = intra-arterial, IL = intralesional, inj-s = injection site, IP = intraperitoneal, IT = intratumour, IV = intravenous, m = multiple treatments, mi = minutes, mo = months, MTD = maximum tolerated dose, MW = mouthwash, no = number, radio = radiotherapy, ref = references, s = single treatment, tcid50 = 50% tissue culture infective dose, tpa = time post administration, vp = viral particles, w = weeks, x = times, y = years. Adapted, modified and expanded from (24).

Table V. Summary of safety data and results from individual oncolytic clinical trials with HSV-1, VV, Reovirus, Measles virus, NDV and picornavirus vectors.

Viral vector	Trial -phase/ country -no of patients	Treatment dose -max pfu[a]	Treatment regimen -s/m -no of a in period -no of c	Route	Combi-treatment	Tumour type	Shedding? (PCR) -sample -tpa	Shedding? (live virus) -sample -tpa	Virus in circulation? (PCR) -tpa	Virus in circulation? (live virus) -tpa	Virus in tumour tissue? -tpa	Virus in normal tissue? -tpa	Repli-cation	Toxicity -DLT or MTD? - grade 3/4	CR	Ref
1716 (HSV-1)	I/UK 5	10^3	m, 1-4a in ≤8w	IT	no	melanoma			no		yes, d8, d14		+/-	no no	+/-	(24, 94)
1716	I-II/UK 12	10^5	s	IT	no	glioma			yes, d5		yes, d4-9 (lv), yes, d251, no, 2.5y	no	yes	no no	+/-	(95-97)
1716	I/UK 12	10^5	s	IL	no	glioma (rim)			yes				+/-	no no	+/-	(98)
G207 (HSV-1)	I/USA 21	$3x10^9$	s	IT	no	glioma		no, saliva d4, 1m, 3m, 6m, 1y		no, d4, 1m, 3m, 6m, 1y	yes, d56, d157		+/-	no no	+/-	(99)
NV1020 (HSV-1)	I/USA 12	$1x10^8$	s	IA	no	crc	yes, saliva, d3	no, saliva, urine, conj, vagina, d0-28	yes, <60mi d4, d6	yes, <60m no, d4, d6			+/-	no yes	+/-	(100)
NV1020	I-II USA (PE)	$1x10^8$	m, 1a in 4w	IA	chemo	crc								no	+/-	(101)
Oncovex GM-CSF (HSV-1)	I/UK 30	10^8	s, m 1a in 2-3w ≤3cycles	IT	no	skin metast.	yes, urine, 8h-1w	yes, inj- s, <2w, no, cold s, 48h	yes, 1h-1w		yes, 3w	no	yes	MTD[b] yes	+/-	(102)
VV (Wyeth)	I/USA 4	10^8	m, 3a	IVes	no	bladder					yes, 24h	yes, 24h		no no	+	(103)
VV-IL2 (NYCB)	I/AUS 6	10^7	m, 12a in 12w	IT	no	meso-thelioma	no, sputum, urine	no, sputum, urine	no		yes, ≤3w (lv)		+/-	no no	-	(104)
JX-594 (NYCB)	I/USA 7	$8x10^7$	m, 18a in 6w ≥1c	IT	no	melanoma					yes, 18h		+/-	no no	+	(105)
JX-594	I-II KOR 10, PE	$3x10^9$	m 1a in 3w ≤8c	IT	no	liver			yes, <60mi, d3-22				yes	no no	+/-	(106)
VV-HPV (Wyeth)	I-II/UK 49	10^6	s, m 2a in 4w	SC	prime/ boost	ano-genital		yes, scab, 4w, no, genital area, throat					+/-	no no	+/-	(107-109)

Table V (continued)

Viral vector	Trial -phase/ country -no of patients	Treatment dose -max pfu[a]	Treatment regimen -s/m -no of a in period -no of c	Route	Combi-treatment	Tumour type	Shedding? (PCR) -sample -tpa	Shedding? (live virus) -sample -tpa	Virus in circulation? (PCR) -tpa	Virus in circulation? (live virus) -tpa	Virus in tumour tissue? -tpa	Virus in normal tissue? -tpa	Repli-cation	Toxicity -DLT or MTD? - grade 3/4	CR	Ref
Reovirus	I/USA 18	$3x10^{10}$ tcid50	s, m 1a in 1m ≤7c	IV	no	various								no	+/-	(9)
Reovirus	I/UK 33	$1x10^{11}$ tcid50	m ≤5a in 4w ≥1c	IV	no	solid	no, urine, stool, sputum d0, d7		no, 0h, 7d		yes		+/-	no yes	+/-	(9, 110)
Reovirus	I/UK 23	10^{10} tcid50	m, ≤6a	IT	radio	solid	no, urine, stool, sputum		no					no no	+/-	(9, 111)
Reovirus	I/CAN 12	10^{9} tcid50	s	IT	no	glioma								no yes	-	(9, 112)
Reovirus	I/CAN 6		s	IT	no	prostate									+/-	(9)
Reovirus	I/CAN 18			IT	no	various								no	+	(9)
MV-CEA	I/USA 15 (PE)	10^{7} tcid50		IP	no	ovarian	no, sputum, urine		yes					no	+/-	(113)
NDV PV701	I/USA 79	$144x10^{9}$	s, m 1-3a in 28d 6a in 21d ≤34c	IV	no	solid		yes, sputum, d0-14, c1 yes, urine, <3w, c1-6			yes, 2w in c8		+/-	7 DLT MTD[c] yes	+/-	(114)
NDV PV701	I/USA 16	$120x10^{9}$	m 6a in 21d ≤16c	IV	no	solid		yes, urine, c1, 2, no, c3					+/-	no yes	+/-	(49)
NDV PV701	I/CAN 18	$120x10^{9}$	m 6a in 21d ≤16 c	IV	no	solid		yes, urine c1-3	yes, c1-2				+/-	no yes	+/-	(33)
NDV HUJ	I-II/Israel 14	$55x10^{9}$ iu	m 2-5a in 7-14d ≥2c	IV	no	glioma		yes, urine, saliva, 6, 24, 72h, c1-7		yes, d0-d9 c1-7	yes, d5 (lv)		+/-	no no	+/-	(48)
NTX-010 (Picorna)	I-II/USA			IV	no	solid		yes, sputum, faeces[d]	yes		yes	no	yes	no		(115)

[a] dose is in plaque forming units (pfu) unless otherwise indicated, [b] MTD = 10^7 pfu in HSV seronegative patients, [c] first (desensitizing dose) MTD = $12x10^9$ pfu, second dose MTD = $120x10^9$ pfu. [d] Levels of shedding were reported to be higher at lower compared to higher doses! Replication column: (+/-) = evidence for prolonged viral presence but no sufficient data to prove ongoing replication. CR (Clinical response) column: (+) = minor, mixed, partial or complete response in >50% of patients, (+/-) in <50% of patients, (-) = no response. In case of empty fields no data were reported. Shedding data are highlighted in grey. List of abbreviations: a = administration(s), c = cycles, chemo = chemotherapy, cold s = cold sore, crc = colorectal cancer, d = days, DLT = dose limiting toxicity, h = hours, IA = intra-arterial, IL = intralesional, inj-s = injection site, IP = intraperitoneal, IT = intratumour, IV = intravenous, IVes = intravesicular, iu = infectious units, lv = live virus, m = multiple treatments, mi = minutes, mo = months, MTD = maximum tolerated dose, no = number, radio = radiotherapy, ref = references, s = single treatment, SC = scarification, tcid50 = 50% tissue culture infective dose, tpa = time post administration, vp = viral particles, w = weeks, x = times, y = years. Adapted, modified and expanded from (24).

However, as shown in table IV and V not all of these parameters are assessed in each study. Moreover, currently there is no uniformity in shedding analysis in clinical gene therapy studies (e.g. standardization of protocols and time points at which parameters are assessed)(31). To demonstrate viral presence (or absence) techniques as diverse as PCR, RT-PCR, ISH, EM, IH, and plaque assays are currently being applied (table IV and V). Most publications use one or more of these techniques to demonstrate selective presence of virus in the tumour, to prove ongoing replication or to monitor shedding. However, the data that are presented are most often incomplete. For instance to demonstrate ongoing viral replication by PCR on viral genomes present in for example the blood, sufficient time points after the last time of administration should be measured in order to demonstrate cycles of replication by presence of multiple peaks.
A second example is the use of PCR to monitor shedding from urine, saliva etc. In case of positivity by PCR, additional plaque assays should be performed in order to distinguish between shedding of live virus or of viral breakdown products. From a safety standpoint also the publication of negative data concerning viral presence is interesting. When these data are presented it is often not clear for how long measurements were continued.
The publication of other data that are interesting from the biosafety standpoint is extremely rare. The assessment of total viral biodistribution can only be performed if materials from autopsies are available. Specific parameters may have been measured in case of risks associated with the use of certain viruses (e.g. reactivation of latent HSV in the use of HSV vectors). Data concerning transmission of replicating viruses to hospital and family members are rarely mentioned in publications suggesting that there is no active monitoring. If monitoring of transmission to thirds or potential recombination/mutation is reported in trials, no actual data or monitoring protocols have been presented. Also information about the risk management strategies that are being applied during clinical trials is often very limited or is not provided at all.

Conclusions from the available safety data

Some general conclusions regarding toxicity, replication, systemic distribution and shedding can be drawn from the overview of safety data from clinical trials with the current generations of RCVVs under their current conditions of use (table VI). The reported toxicities in trials with the current HAdV, HSV-1 and VV vectors are mostly of grade 1 or 2. Incidentally higher toxicities were observed, which in most cases were transient and could be attributed to disease progression or other treatments. These limited toxicities may be due to the attenuated nature of the specific vectors applied so far in clinical studies (32). Only in case of NDV (wildtype strain), substantial toxicities associated with the first viral dose have been observed in several patients, which could be controlled by a stepwise desensitization protocol and a slower infusion rate (33).
For AdV, HSV-1, VV and NDV vectors data suggesting ongoing replication and systemic appearance of viral genomes have been presented, also in case of local administration. Clear presence of circulating live virus has only been demonstrated for NDV. Furthermore, it appears that HSV vectors are rapidly inactivated upon systemic reappearance. For other viruses the situation is less clear because data are lacking (e.g. for HAdV after IV administration).
Shedding of wildtype NDV is likely to occur following IV administration. In contrast, shedding of GM VV or HSV vectors in their current application (mainly IT delivery) is not likely to take place, except for direct shedding from the injection site, for instance in case of local administration to skin tumours. Again, for the HAdV vectors applied in current protocols the situation is less clear; the occurrence and period of shedding of HAdV (but also of other viruses) may be very much related to the route of administration. Based on the currently available data, HAdV shedding may take place by any route, both after local or systemic administration.

Table VI. General conclusions from published clinical safety data from different categories of replication competent viral vectors.

Vector category	**HAdV E1B deleted[a]**	**HAdV Type 2 CRAd[a]**	**HSV-1 (attenuated)**	**VV Wyeth (wt, TK$^-$)**	**NDV (wt)**	**Reovirus (wt)**
Route	IT, IV, IP	IT, IV, IVes	IT	IT	IV	IV, IT
Toxicity - DLT/MTD - Grade 3/4	+/- (2 / 23) (11 / 13)	+/- (0 / 2) (2 / 0)	+/- (1 / 6) (2 / 4)	- (0 / 5) (0 / 5)	+/- (1 / 3) (3 / 1)	+/- (0 / 5) (2 / 1)
Biodistribution	W (1)	ND	ND	ND	ND	ND
Replication	+ (6 / 13 / 2)	+ (2 / 0 / 0)	+ (2 / 4 / 0)	+ (1 / 3 / 0)	+/- (0 / 4 / 0)	+/- (0 / 1 / 0)
Systemic virus	+/- (12 / 6)	+ (2 / 0)	+/- (4 / 2)	+/- (1 / 1)	+ (2 / 0)	- (0 / 2)
Recombination / Mutation	ND	- (0 / 1)	ND[b]	ND	ND	ND
Shedding[c]	+/- (2 / 3)	+ (2 / 0)	+/- (1 / 2)	+/- (1 / 1)	+ (4 / 0)	- (0 / 2)
Transmission	ND	ND	ND	- (0 / 2)	ND	ND

For each safety aspect the overall conclusion is shown: (+) = yes, (-) = no, (+/-) = varied or conflicting data. Numbers indicate respectively the total number of studies with evaluable and convincing data showing either the (+) or (-) result (see tables IV/V and Appendix B.4 for details). In the replication column the three numbers indicate respectively a (+), (+/-) or (-) score in table IV and V.

[a] Distinguishing between type 1 and type 2 CRAds may be arbitrary since the physical particle is similar. Conditions of use (e.g. route of administration) may be more important than genetic differences. [b] Proof for absence of reactivation of latent HSV-1 has been reported in a number of studies. [c] Trials in which viral DNA could be detected by PCR in bodily secretions, but in which at the same time live virus was not detected, are regarded as a (-), as opposed to trials in which only PCR was applied with a positive result regarding presence of viral DNA. List of abbreviations: IT = intratumour administration, IP = intraperitoneal administration, IV = intravenous administration, IVes = intravesicular administration, ND = no data reported, TK = thymidine kinase, W = widespread, wt = wildtype.

4 Discussion

4.1 Points to consider for the ERA of replication competent viral vectors

Based on the safety data for the current generation of -mostly attenuated- RCVVs and their proposed use (see paragraph 3.3, Appendix B) it can in many cases not be excluded that vector replication, shedding and subsequent spreading of the vector or a recombinant vector may occur, which may result in environmental effects. This may be even more likely for future more potent generations of RCVVs with more complex modifications (see Appendix A). In the review of RCVVs and their (pre)clinical applications (paragraph 3.2, Appendix A and B) elements have been identified that may play a role in spreading and effect scenarios for RCVVs. These elements include properties of the viral backbone itself (e.g. specific properties of RNA / DNA / retroviruses), the applied genetic modifications (deletions of viral genes and a variety of insertions and mutations) and the proposed conditions of use (i.e. dose and way of administration, specific combination treatments). These elements have been worked out in a structured ERA for RCVVs taking the following notions into account:

1) Shedding should be regarded as a mechanism through which spreading, and effects on the environment and public health may occur. Shedding is an important factor in the exposure of the environment to the GMO (31). It is often seen as a hazard, and identified as such in the first step of the ERA. High shedding resulting in high exposure however not necessarily results in an adverse effect. Vice versa low exposure might even lead to a high risk as a result of severe consequences that might be characterized in the first step of an ERA. The degree of exposure through shedding depends on the duration of the infectious state of the gene therapy product which may be increased by replication after administration to the patient.

2) The ERA should make use of separate spreading and effect scenarios. Like shedding, subsequent spreading and transmission of the GMO should not be necessarily seen as a hazard, but as a mechanism through which hazards may occur. Therefore spreading scenarios and effect scenarios should be separated in the ERA. To make it possible to make the distinction between spreading and effect scenarios, information about the impact of the modifications on the viral life cycle, the interaction with the host and possible recombination should be presented in the early steps of the ERA, as these are mechanisms through which both spreading and adverse effects may occur.

3) Recombinants should be separately considered in the ERA. Replicating vectors may encounter wildtype viruses, for instance after spreading from the injection site or the tumour to organs where wildtype viruses may persist. By mechanisms like recombination (in case of both DNA and RNA viruses) or reassortment (in case of RNA viruses with a segmented genome) novel viral variants may arise that may have different properties compared to the applied vector. Therefore, the evaluation of such recombinants/mutants of the applied vector, in the early steps of the ERA, is very important.

4) The effect of each modification should be evaluated in the context of the RCVV and other modifications. In the present ERA that has been applied for RDVVs (Appendix C) the risks

associated with each of the singular insertions are first separately considered and subsequently a conclusion on the combined risk of the insertions is drawn. This may not be the most logical approach since context is often more important than the modification itself. E.g. the risk of applying the CMV promoter can only be considered if the characteristics of the insert driven by this promoter are known and vice versa. And in case of multiple modifications of the tropism separate risk evaluation of each insertion is timely and inefficient. A report by van Haren and Spaan has addressed whether it would be possible to order viral inserts in viral vectors into 'safe' and 'unsafe' categories. The conclusion was that each viral insert has to be evaluated in the context of the viral vector and the host system and that generalization into safe and unsafe categories of inserts outside of this context is not possible (29). This conclusion may also be extrapolated to non-viral inserts and deletions of viral sequences in the viral backbone itself; also with these modifications the total context is important. Not only insertions, but also deletions should be evaluated as these may also impact on the viral life cycle and may interact with the insertions.

5) The ERA should be performed according to a clear stepwise approach. In the current ERA (Appendix C) there is no strict definition of what is meant with terms like persistence, invasiveness, pathogenicity, virulence, infectivity, host range, tropism etc. Moreover, it is important to evaluate these terms in the ERA in a more stepwise approach that shows the connections between the different elements and how these elements influence each other. The stepwise approach ensures that essential elements are not being overlooked in the ERA.

The previous considerations have been worked out in the ERA template for RCVVs (paragraph 4.2). The overall setup of the ERA is shown in Figure 1 that gives an overview of the questions asked in the ERA and their connections.

Figure 1 (next page). Explanation of concurrent steps in the ERA for replication competent viral vectors.

The chart gives an overview of the steps in the ERA and their connections. It should be emphasized that parts A and B of the chart are not a 'decision tree', but show the consecutive steps. In the ERA spread and effect scenarios are separated. The complete ERA is available in the form of a template in Word (see paragraph 4.2). Part A, General information: This part contains the technical and scientific details regarding the characteristics of the wildtype virus on which the RCVV is based, the RCVV itself and the intended scale and nature of its use / release (steps 1-3). Information about the clinical trial protocol (e.g. status of the patient, way and dose of administration, combination treatments) is provided in order to establish whether the clinical trial protocol could influence the behaviour of the RCVV in the subject (part B). Part B: Identification of mechanisms that may lead to adverse effects. In part B, all mechanisms (steps 4-13) are evaluated (including those mentioned in Annex II of Directive 2001/18/EC) that are relevant for conducting the ERA for current and future RCVVs in clinical trials. The stated mechanisms are included based on (a) the properties of the RCVVs that are currently being developed preclinically and that may be used in future clinical studies and (b) the intended current and future use of RCVVs vectors. The mechanisms are compared to those presented by the wildtype virus from which the RCVV is derived and its use under corresponding situations. Mechanisms are evaluated in a logical stepwise manner, indicated by the arrows: e.g. effects of insertions / deletions on the viral life cycle (5), like effects on replication (5d) and tropism (5f) and effects on the interaction with the host (6), like immunomodulation (6a) may influence the biodistribution in the subject (7) that is together with various other elements (like the proposed conditions of use (3)) involved in the likelihood of recombination (4/13) which may result in new variants of the RCVV that should be evaluated in the ERA. The biodistibution may also influence the period / routes of shedding from the subject (8) and the likelihood of transmission of the RCVV and its descendants to thirds (10), vertical transmission (11) and transmission to animals / cross species transfer (12). Part C: ERA: This part (steps 14-16) contains the integral evaluation of all the mechanisms addressed in part B on the identification and evaluation of potential hazards for public health and the environment. In practice, the individual likelihoods of the mechanisms addressed in part B will be used to determine the overall likelihood of the hazards evaluated in step 14. Potential hazards for RCVVs include effects on human and animal health and medical and veterinary practice and on population dynamics. Hazards for the patients subjected to the RCVV in the clinical trial are not part of the risk assessment in part C.

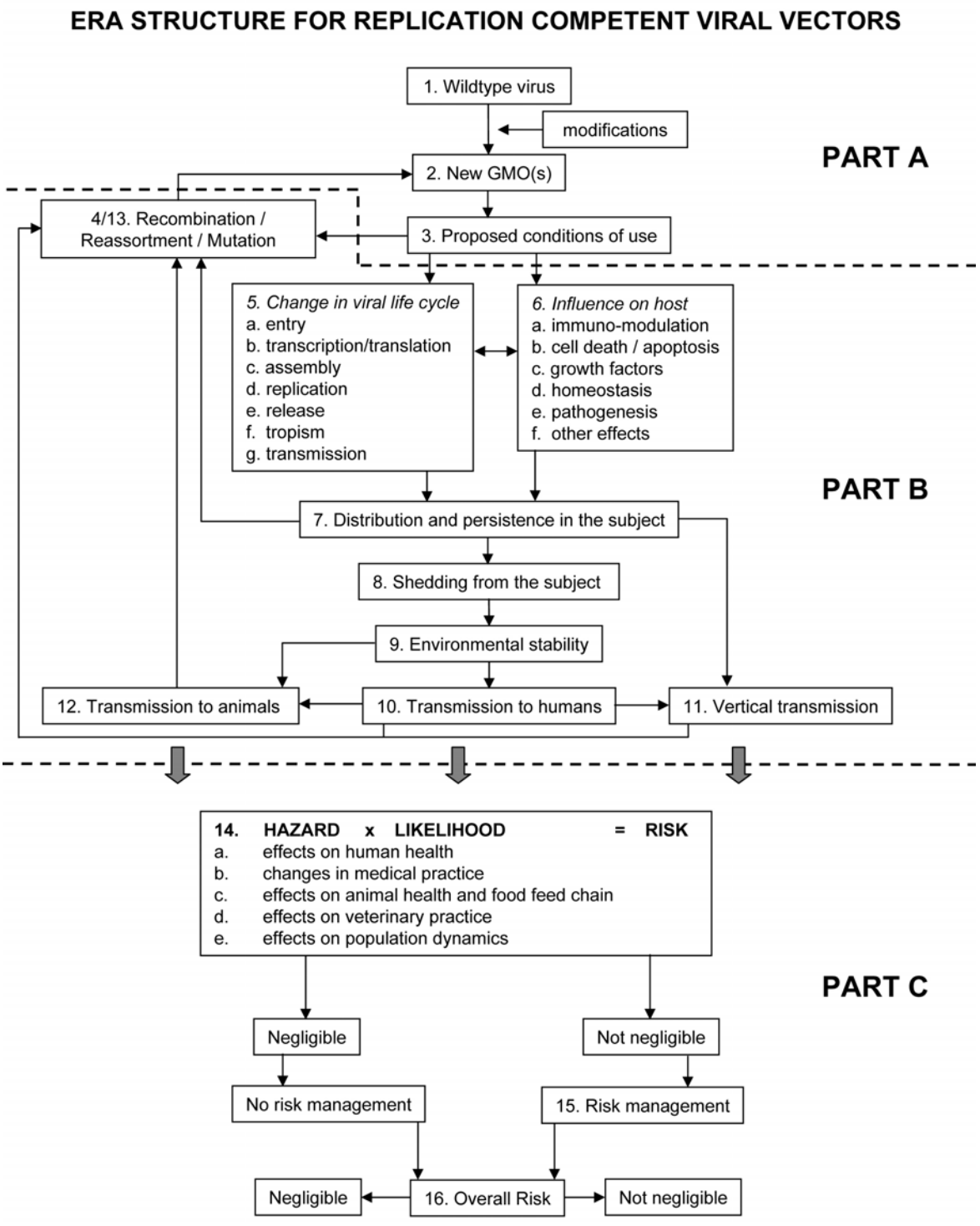

Figure 1. Explanation of concurrent steps in the ERA for replication competent viral vectors.

The ERA is divided in three parts. Part A contains general information about the RCVV and its conditions of use. In part B all mechanisms through which adverse effects may occur and that may influence the likelihood are evaluated in a logical stepwise manner. The mechanisms have been included based on the properties of RCVVs that may be applied in future clinical studies and predictions on the intended use of RCVVs. Step 5 and 6 of the ERA are based on the methodology for the risk assessment of viral sequences as proposed by Van Haren en Spaan (29). The mechanisms are compared to those presented by the wildtype virus from which the RCVV is derived and its use under corresponding situations. It should be emphasized that parts A and B of the chart are not a 'decision tree', but show the most logical order of the consecutive steps. Part C contains the actual ERA containing the steps according to Annex II of Directive 2001/18/EC:
- Step 14-1: Identification of potential adverse effects (Hazard identification)
- Step 14-2: Estimation of the likelihood
- Step 14-3: Risk estimation = effect x likelihood
- Step 15: Risk management
- Step 16: Overall risk

The ERA is structured in such a way that it is conceivable that applicants will draw conclusions regarding the likelihood of environmental hazards in part B of the ERA. It should be emphasized that part B addresses the individual mechanisms that are used to determine the overall likelihood of the hazards evaluated in step 14. Because of this setup the same arguments will inherently be encountered twice. If an argument has already been addressed in detail at a previous step in the ERA it is acceptable that the argument is readdressed quickly at subsequent steps. For instance if shedding can be excluded based on experimental data at step 8, the evaluation of all subsequent steps should be performed to complete the ERA, but some of these steps may be addressed quickly referring to the evaluation in step 8.

The ERA template for RCVVs has been tested for the HAdV vector Onyx-015 in Appendix D. We have chosen this vector as an example since a variety of published safety data is available on which the scenarios could be based. The ERA of Onyx-015 should be seen as an example of how the ERA can be addressed making use of the available information and safety data. According to the outcome of the ERA spreading of Onyx-015 can not be excluded, but no adverse effects are identified following spreading of this vector into the environment, meaning that no risk management measures are necessary for environmental safety. For reasons of clarity, table VII that shows the ERA and the outcome of each of the steps in summary has been added to Appendix D. Although a table is not part of the ERA template as presented in paragraph 4.2 applicants are free to use a table similar to table VII or other schemes as an aid in the evaluation process.
The proposed ERA for RCVVs, although structured, remains to be a case by case analysis that is influenced by many different specific factors. It is therefore conceivable that the ERA will have a very different outcome for other RCVVs. For some RCVVs the likelihood of shedding will be negligible, and therefore subsequent steps in the ERA may be taken quickly based on the neglibility of environmental exposure to the RCVV. However for RCVVs that enter the clinic for the first time, less experimental data may be available, worst case scenarios may have to be applied, risks may be identified, and specific risk management measures associated with the nature of the RCVV involved and the proposed conditions of use may be required. In some cases the identification of risks may lead to the disapproval of the protocol, if the proposed risk management measures do not lead to a reduction of the final risk. In special cases, a decision may be taken that potential benefits outweigh the risks. However, such a conclusion is of a political nature, and is outside of the scope of this report. The presented ERA for RCVVs is expected to provide guidance to both regulatory officers and applicants about which information is needed to perform the ERA for RCVVs, and why this information is needed. In case of initial disapproval of a protocol the ERA may help to pinpoint the specific experimental data that are lacking in the original submission.

4.2 A general ERA template for replication competent viral vectors

A) GENERAL INFORMATION ON THE WILDTYPE VIRUS, THE GENETICALLY MODIFIED REPLICATION COMPETENT VIRAL VECTOR AND THE INTENDED USE.

1. Information about wildtype virus

All relevant scientific information (including references) about the wildtype virus(es) and/or the non-modified strains on which the RCVV backbone is based on should be presented by addressing each of the points below.

1a. General information

1b. Host-range

1c. Pathogenicity

1d. Biodistribution and persistence

1e. Tissue tropism

1f. Cell lysis and lateral spreading

1g. Horizontal transmission

1h. Vertical transmission

1i. Physical stability

1j. Genetic stability

1k. Availability of anti-viral treatment

2. Information about the RCVV

All relevant scientific information (including references) about the RCVV should be presented by addressing each of the points below.

2a. General information about the RCVV

2b. Role of deleted or mutated genes / sequences

2c. Role of inserted transgenes / sequences

2d. Replication of the RCVV in normal cells

2e. Availability of preclinical models for monitoring effectivity, toxicity or biodistribution

2f. Information about systemic distribution after *in vivo* administration

2g. Information about the biodistribution after *in vivo* administration

2h. Toxicity

2i. Environmental shedding
Environmental shedding is defined as excretion of the RCVV from the subject into the environment via excreta (i.e. faeces, urine, saliva) or the injection site.
Shedding in the circulation is not considered under this point but is considered at step 2f.

2j. Horizontal transmission

3. Overview of the proposed conditions of use

The proposed conditions of use of a GMO will determine to a large degree the environmental exposure and should always be evaluated in the ERA. For this ERA the proposed condition of use is the clinical setting. The clinical setting (i.e. immune status of the subject, dose and way of RCVV administration, combination treatments, etc.) may influence recombination and mutation of the applied RCVV (4), the viral life cycle (5), the interaction of the RCVV with the subject (6), biodistribution and persistence (7), shedding (8) and transmission (10-12) and therefore the magnitude and duration of the potential environmental exposure. In a clinical trial the surrounding environment of the subject includes medical staff members, relatives and other social contacts as well as the general public. It is necessary to address all scenarios that may lead to an exposure of the surrounding environment after application of the RCVV to the subject[3].

3a. What is the number of patients, and the dose, route, way and timing of administration?

Information needed:
Provide the total number of patients, the exact dosages (in pfu or vp), the route (e.g. systemic, intralesional, intracranial), way (e.g. oral spray, direct injection) and timing of treatment (single or multiple treatment and time between administration).

Point of attention:
The dose, route, way and timing of administration may influence the likelihood of recombination, the biodistribution, persistence and shedding of the RCVV and should be taken into account at points 4, 7 and 8.

[3] Procedures performed by hospital personnel (e.g. preparation of the GMO, administration of the GMO, handling of test samples from patients and waste disposal), and the risk management methods to prevent exposure of hospital personnel in the controlled hospital environment during these procedures are not the focus of this project/ERA.

3b. What is the immune status of the subjects?

Information needed:
Is the subject immunocompetent or (relatively) immunodeficient?

Point of attention:
The immune-status of the subjects may influence the viral life cycle and the effects of the RCVV on the subject, for instance biodistribution, persistence and shedding; therefore when the subject is immunocompromized, points 4, 5d, 6a, 6e, 7 and 8 should be evaluated taking this into account.

3c. Which treatments are applied in combination with the viral vector?

Information needed:
List the regimen of all combination treatments being applied in combination with the RCVV (e.g. chemotherapy, radiotherapy, revaccination, angiogenesis inhibitors, immune-modulating agents etc.)

Point of attention:
Combination treatments may influence the likelihood of recombination, the viral life cycle, the interaction of the RCVV with the host (subject), biodistribution, persistence and shedding; therefore when a combination treatment is applied the effects should also be evaluated at points 4, 5, 6, 7 and 8.

3d Information about the direct environment of the subjects

Information needed:
Any particular circumstance in the direct environment of the patient (hospitalization, housing, profession) that could influence the risk assessment should be mentioned.

Point of attention:
In case of application of modified animal viruses the environment could play a considerable role in the possibility of transfer to for instance pets, cattle or poultry. Step 12 and 13 should be evaluated considering this environment.

3e. Information about GMO production

Information needed:
An overview of the production process (i.e. the origin of the production cell lines and the genetic components contained within these cell lines, steps and methods used in quality control and the criteria used to reject a batch) should be provided.

Point of attention:
The production process may influence the likelihood of complementation and recombination and should be considered in the evaluation at step 4.

B) IDENTIFICATION OF MECHANISMS THAT MAY DIRECTLY OR INDIRECTLY LEAD TO ADVERSE ENVIRONMENTAL EFFECTS.

4. Recombination and mutation of the GM vector in the subject (human host)

Potential effect:

Recombination may occur if wildtype viruses related to the RCVV are able to meet and interact with the RCVV in the subject. Under these circumstances sequences may be exchanged and deletions of viral genes in the vector may be repaired by homologous recombination or by re-assortment. Presence of wildtype virus may also result in (temporal) complementation of deleted functions.

Some viruses do not efficiently replicate when foreign sequences are inserted and in many cases only a moderate increase in size is permitted. Instability of viruses may result in loss of both inserted sequences and sequences from the viral backbone, especially in case of presence of more copies of a particular sequence. Whether mutation of RCVVs may occur depends on the properties of the parental virus itself and this should be evaluated taking into account what is known about genetic stability of the parental virus (RNA/DNA virus); RNA virus replication lacks a proofreading mechanism causing a high mutation rate and short replication times allowing RNA virus to rapidly adapt to a new environment. If such mutations occur in key viral proteins this may for example influence the tropism, host range (5f) and pathogenicity (6e) of a virus. DNA viruses are less prone to mutate.

Mutations and recombination may result in (transient) presence of viral variants that have different properties compared to the applied RCVV. This may influence the viral life cycle (5), interaction with the host (6), biodistribution and persistence (7), shedding (8) and vertical and horizontal transmission (10-12). The resulting recombinant RCVVs should be separately evaluated.

Information needed:

- *Information about the purity of the manufactured RCVV;*
- *Information about the possibility of recombination or reassortment of the RCVV with wildtype virus;*
- *Information about the possibility of temporal complementation of missing functions in the RCVV by wildtype virus;*
- *Information about the genetic stability and the mutation rate of the manufactured vector.*

Extra points of attention:

- *Recombinant, re-assorted and mutated vectors may arise during the vector manufacturing process;*
- *Combination treatments may influence the rate of mutation of DNA and RNA viruses;*
- *Combination treatments, subject status and the way and dose of administration may influence viral replication (5d) and biodistribution/persistence (7) and therefore the likelihood of recombination;*
- *The likelihood of recombination, functional complementation and mutation should, if appropriate, be re-evaluated after completing steps 5, 6, 7, 10 and 11;*
- *Possible recombination / functional complementation and mutation of the vector in animals (13) will be considered later in the ERA after completion of steps 4-12.*

Evaluation:

4a. Recombinant GMO: Yes/No.
4b. Complemented GMO: Yes/No.
4c. Mutated GMO: Yes/No

If the answer is yes these variants should be evaluated at each of the steps below next to the introduced GMO.

5. <u>Do the modifications in the GMOs affect the viral life cycle by changes in virus structure or by non-structural changes in the context of the subject (human host)?</u>

Extra point of attention:
If the natural host of the virus where the RCVV is based on, is or includes an animal species (especially cattle or pet animals), each of the points below should, if applicable, also be considered in the context of these hosts. See also steps 12 and 13.

5a. Changes in binding and entry compared to the parental virus

Potential effect:
Entry of the virus into the cell is mediated by viral surface proteins that bind to cellular receptors. Changes in the mechanism of viral entry can influence the viral tropism and host range (5f); depending on the change the tropism and host range can be broadened or narrowed. Changes in entry can have large consequences on public health and the environment (14 and onward), mostly in cases that result in an increase of the viral tropism.

Information needed:
- *Information about whether the proteins involved in viral entry are present and expressed in a normal manner in the RCVV or recombinants.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on viral entry.*

Extra points of attention: *Especially the modification of viral surface proteins and the application of temporal (physical) or permanent (genetic) shielding techniques that may influence virus entry should be taken into account.*

Evaluation:

- GMO
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

5b. Changes in transcription and translation compared to the parental virus

Potential effect:
Transcription and translation of viruses are complex processes that are driven by the interaction of viral and cellular transcription and translation factors with the intracellular transcription /

translation machinery. The pace at which transcription / translation take place and the cell and tissue specificity of transcription / translation determine to a large extent the pace and cell and tissue specificity of replication (5d) and the effectiveness of assembly (5c). Transcription and translation may also partly determine the tropism of a virus (5f). Especially increased transcription/translation may have consequences on public health and the environment (14 and onward).

Information needed:
- *Information about whether the proteins involved in viral transcription and translation are present and expressed in a normal manner in the RCVV or recombinants.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on viral transcription and translation.*

***Extra points of attention**:*
Modifications and treatments that may influence the efficiency and specificity of transcription and translation should be taken into account; especially deleted and mutated viral genes and inserted foreign regulatory sequences.

Evaluation:

- GMO
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

5c. Changes in assembly compared to the parental virus

Potential effect:
Once all viral components have replicated, the virus is assembled. Sufficient quantities of structural protein have to be produced to pack all the genetic material. Modifications that influence packaging signals or the effectiveness of protein production and viral assembly could alter replication (5d) and pathogenicity (6e). In most cases an increased effectiveness of assembly may have consequences on public health and the environment (14 and onward).

Information needed:
- *Information about whether the proteins involved in viral packaging and assembly are present and expressed in a normal manner in the RCVV or recombinants.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on packaging and assembly.*

Evaluation:

- GMO
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

5d. Changes in replication compared to the parental virus

***Potential effect**:*
Replication of a virus is determined by many different viral, cellular and extracellular factors. Changes in replication time and / or replication specificity for certain cells or tissues compared to the parental virus may alter pathogenesis (6e), persistence (7), the likelihood of recombination and complementation (4) and shedding (8) from the subject into the environment. In most cases an increased replication pace or time or a decrease of specificity may have consequences on public health and the environment (14 and onward).

Information needed:
- *Information about whether the proteins involved in viral replication are present and expressed in a normal manner in the RCVV or recombinants.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on viral replication, compared to wildtype virus.*

***Extra points of attention**:*
Deleted viral genes, inserted foreign regulatory sequences, the status of the subject (immunocompromized or not) and combination therapies may influence the pace and specificity of replication and the number of replicative cycles. Changes in replication should be re-evaluated after completing step 6 (evaluation of the interaction with the host).

Evaluation:

- GMO
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

5e. Changes in release and cell to cell spreading compared to the parental virus

Potential effect:
Enveloped viruses are released by budding from the plasma membrane or via vesicle transport. Non-enveloped viruses assemble in the nucleus, cross or disrupt the nuclear membrane and are released following lysis of the cell or apoptosis. Other viruses may spread applying cellular junctions or by inducing fusion of cells. Modifications or treatments that promote viral release and spreading may increase the pathogenicity of the virus (6e) and may have consequences on public health and the environment (14 and onward).

Information needed:
- *Information about whether the proteins involved in viral release and spreading are present and expressed in a normal manner in the RCVV or recombinants.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and the influence of combination treatments, on viral release and spreading, taken into account spreading and release of the wildtype virus.*

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

5f: Changes in cell, tissue and host tropism compared to the parental virus

***Potential effect**:*
Infectivity refers to the ability of a virus to infect a certain organism, tissue or cell. Tropism refers to the specific targeting of particular organisms, tissues or cell types by a virus. Many factors are involved in determining viral infectivity and tropism.
Genetic changes that influence the structure of the virus or alter surface proteins may alter the tropism (receptor tropism). The interaction of viral surface structures with receptors present on the surface of the host cell is a commonly known determinant of viral specificity (see step 5a). For some viruses, tropism is partly determined by cellular nucleases and proteases that are involved in cleavage of viral surface proteins that promote viral entry and release after cleavage.
However, depending on the virus, intracellular mechanisms may be equally or even more important in determining the specificity (post-entry tropism); these include specific interactions with intracellular signalling cascades (6d), and effects on transcription and translation (5b).
In this step the overall result of all modifications and treatments that may influence the viral tropism (receptor and post-entry) is evaluated: to what extent do these influence the specificity of the virus for certain cells, tissues and hosts?
Modifications that change the viral tropism of the RCVV compared to the wildtype vector, especially an increase of infectivity / tropism, may lead to an increased pathogenicity for humans (6e and 14a). Altered tropism may influence viral persistence and biodistribution and thereby the route and period of viral shedding (8), the likelihood of recombination (4, 13), and transmission (10, 12). Modifications that increase the host range of the vector may lead to novel or more severe disease symptoms in animals (14c). Therefore alteration of tropism may have serious consequences on public health and the environment (14).

Information needed:
- Information about whether the proteins involved in the viral receptor and / or post-entry tropism are present and expressed in a normal manner in the RCVV or any recombinant.
- Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and the influence of combination treatments, on cell, tissue and host tropism, taking into account the main tropism determining mechanisms of the host virus.

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

5g. Changes in route of transmission compared to the parental virus

***Potential effect**:*
Viruses have specific routes of transmission that are based on the tissue tropism, the structural features of the virus and the functions of specific viral proteins. Changes in tissue tropism (5f) may directly influence the route or efficiency of virus transmission. Small genetic changes in viral proteins (e.g. as observed for influenza virus) may affect viral transmission. A change in the route or efficiency of transmission may for example influence the likelihood of transmission to the non-target human population and animals (10 and 12) and the pathogenicity (6e, 14a, 14c) and have serious consequences on public health and the environment (14).

Information needed:
- Information about whether the proteins involved in viral transmission are present and expressed in a normal manner in the RCVV or recombinants.
- Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on viral transmission, taking into account the transmission route of the host virus.

Evaluation:

*- **GMO***
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

6. <u>What are the effects of the GMO compared to wildtype virus on the subject (human host)?</u>

Extra points of attention:
- If the natural host of the virus where the RCVV is based on, is or includes an animal species (especially cattle or pet animals), each of the points below should, if applicable, also be considered in the context of these hosts. See also steps 12 and 13.
- If transgenic insertions are present in the vector the effects of these insertions on the host should be evaluated in the context of the viral vector. First of all, the level of transgene expression and therefore the magnitude of the effect will depend on the endogenous viral or ectopic promoter present in the vector. Second, in case of for instance effects of a vector on immunomodulation, an attempt should be made to describe the overall effects of the entire vector on the immune system taking both the effects of the transgene and the viral genes involved in immunomodulation into account.
- If the effects on the host can not be placed under category 6a-e, the other effects category can be applied (6f).

6a. Changes in immuno-modulation compared to the parental virus

***Potential effect**:*
Viruses have evolved various mechanisms to modulate the immune system of the host. A major mechanism is the prevention of immune-mediated apoptosis of infected cells. Genetic changes in the immuno-modulatory properties of a virus or treatments that influence the normal immune

response may for example directly influence viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8). Especially a decreased immune response may have consequences on public health and the environment (14).

Information needed:
- *Information about whether the viral proteins involved in modulating the host-immune response are present and expressed in a normal manner in the RCVV or any recombinants.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on immuno-modulation.*
- *Evaluation of the effects on e.g. viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) and when appropriate, other mechanisms.*

Extra point of attention:
Modifications and combinatorial treatments may result in a decreased immune response against the RCVV. Some insertions may result in a hyperactive immune response. Both situations may influence the pathogenicity (6e) of the virus and should be evaluated. The immune status of the subject should also be considered.

Evaluation:

- ***GMO***
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

6b. Changes in induction of cell death (apoptosis and necrosis) compared to the parental virus

Potential effect*:*
Viruses have evolved various mechanisms to circumvent or to induce apoptotic or necrotic cell death of host cells. Modifications and combination treatments may directly influence these properties and may result in for example altered viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) and may have consequences on public health and the environment (14).

Information needed:
- *Information about whether the viral proteins involved in modulating cell death and apoptosis are present and expressed in a normal manner in the RCVV or any recombinant.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on cell death and apoptosis.*
- *Evaluation of the effects on e.g. viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) and when appropriate, other mechanisms.*

Extra point of attention*:*
While an increase of cell death may increase pathogenesis it may also limit viral replication (5d), depending on the timing and onset of cell-death inducing mechanisms.

Evaluation:

- ***GMO***
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

6c. Changes in growth factor, cyto-/chemokine signalling compared to the parental virus

***Potential effect**:*
Growth factors, cytokines, and chemokines have a wide range of effects on their target cells that include regulation of proliferation, differentiation and cell death. Modifications in the RCVV and combination treatments may directly influence growth factor, cytokine and chemokine (receptor) expression and responses and may result in for example altered viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) and may have consequences on public health and the environment (14).

Information needed:
- *Information about whether the viral proteins involved in modulating growth factor or chemokine signalling are present and expressed in a normal manner in the RCVV or any recombinant.*
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on signalling. Evaluation of the effects on e.g. viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) and when appropriate, other mechanisms.*

Evaluation:

- ***GMO***
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

6d. Changes in cell intrinsic signalling and intracellular homeostasis compared to the parental virus

***Potential effect**:*
Disturbance of intracellular homeostasis and intrinsic signalling can have a wide range of effects on the cell. Modifications in the RCVV and combination treatments may directly influence intrinsic signalling and homeostatic processes and may result in for example altered viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) and may have consequences on public health and the environment (14).

Information needed:
- *Information about whether the viral proteins involved in modulating intracellular homeostasis and intrinsic signalling are present and expressed in a normal manner in the RCVV or any*

recombinant.
- *Information about the influence of the modifications (deletions, insertions and mutations) in the RCVV and recombinants, and combination treatments, on intracellular signalling and homeostasis.*
- *Evaluation of the effects on e.g. viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) and when appropriate, other mechanisms.*

Evaluation:

- ***GMO***
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

6e. Pathogenesis compared to the parental virus in the subjects

***Potential effect**:*
Increased pathogenesis may inherently have large consequences on public health and the environment (14 and onward).

Information needed:
- *Information about whether the viral proteins involved in inducing pathogenic effects are present and expressed in a normal manner in the RCVV or any recombinant.*
- *Information about the influence of the modifications in the RCVV and recombinants, and combination treatments on pathogenesis.*

***Extra point of attention**:*
- *The information supplied at steps 6a-d should be considered in the evaluation.*
- *When applicable genotoxic effects (e.g. in case of retroviral vectors) may be considered at this step.*

Evaluation:

- ***GMO***
- *Recombinant GMO(s): when applicable (see step 4a)*
- *Complemented GMO(s): when applicable (see step 4b)*
- *Mutated GMO(s): when applicable (see step 4c)*
- *Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)*

6f. Other effects in the host

***Point of attention**:*
Mention here any other effect of the RCVV on the host which can not be placed under categories 6a-e. This effect should be considered in the evaluation at step 14.

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

7. Biodistribution and persistence in the proposed clinical setting

Potential effect:
Spreading of the RCVV to the circulation, the biodistribution and the persistence of the RCVV may for example influence the likelihood of recombination (4), the route and period of shedding (8) of the RCVV, and should if appropriate be considered at these steps.

Information needed:
Information about the influence of the modifications in the RCVV and recombinants, and combination treatments / status of the subject on viral distribution and persistence.

Extra point of attention*:*
Biodistribution and persistence should be evaluated taking into account each of the points addressed in the information about the clinical setting (3).

7a. Does the GMO spread to the circulation?

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

7b. What is known about biodistribution after administration of the GMO?

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

7c. What is known about persistence of the GMO?

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

8. <u>Can shedding from the subject into the environment occur? For how long and via which ways?</u>

Potential effect:
If the modifications in the RCVV affect aspects of the viral life cycle (5) and its interaction with the host (6) in such a manner that it may influence public health and the environment (14), then shedding becomes important, since shedding determines the likelihood of transmission (10) and exposure of the environment to the RCVV. If no hazards of the RCVV are identified shedding becomes less important.

Information needed:
- Information about shedding (period, route) of the RCVV or any recombinant from the subject taking into account the dose and route of administration and the status of the subject.

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

9. <u>What are the effects of the modifications in the GMO on the stability in the environment?</u>

Potential effect:
Stability of the RCVV, once shed, can influence whether transmission to thirds (10) or animals (12) may indeed occur.

Information needed:
Information about stability of the RCVV or any recombinant in the environment compared to wildtype virus.

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)

- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

10. <u>Can transmission of the GMOs to the human non-target population occur and is further replication and transmission possible?</u>

***Potential effect**:*
Horizontal transmission may have large effects on public health, e.g. if the RCVV is able to induce disease in thirds (14a). Transmission to the non-target population could influence the likelihood of recombination, reassortment and mutation and should when applicable be considered at step 4.

Information needed:
Information about the likelihood and possible period of transmission of the RCVV or recombinants to the surrounding environment and about further replication and spread in thirds. In a clinical trial the surrounding environment includes medical staff members, relatives and other social contacts as well as the general public. It is necessary to assess all foreseen procedures (3) that may lead to an exposure of the surrounding environment. These procedures do not only include administration of the RCVV but also production and preparation of the RCVV and waste disposal.

10a. Is transmission possible to thirds?

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

10b. Is replication possible in thirds?

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

10c. Is after infection of thirds further spread possible?

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)

- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

11. Can vertical transmission occur?

***Potential effect**:*
Vertical transmission may have large effects on public health and the environment, since it may for example lead to novel inherited diseases in men and in animals (14). Vertical transmission could influence the likelihood of recombination, reassortment and mutation and should when applicable be considered at step 4.

Information needed:
Information about whether vertical transmission of the RCVV or any recombinant can occur.

Points of attention:
- The conditions of use (3) and biodistribution (7) should be considered in establishing the likelihood of vertical transmission.
- If the natural host of the virus where the RCVV is based on, is or includes an animal species (especially cattle or pet animals), vertical transmission should, if applicable, also be considered in the context of these hosts. See also steps 12 and 13.

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

12. Can transmission to animals / cross species transfer occur?

***Potential effect**:*
Harmful effects on animals may occur if the vector is able to spread from the subject or thirds to animals. This may especially be the case with the application of RCVVs based on animal viruses. Natural occurring viruses tend to be restricted to their natural host species. Occasionally viruses cross the host-range barrier and in very rare cases this is followed by the establishment and persistence of a virus in the new host species, which may result in disease (zoonosis). The adaptation process often involves the acquisition of an altered tropism or host range. Especially RCVVs with an altered tropism or host range are a major concern as they virtually constitute a new viral pathogen with the potential of a new disease manifestation. Cross species transfer may have large effects on public health and the environment especially if the virus is able to adapt and induce disease in the novel animal or human host (13 and 14).

Information needed:
Information on whether transmission to animals / cross species transfer and further adaptation of the RCVV or any recombinant may occur taking into account all earlier steps, but especially points 3, 4 and 5f addressed earlier in the ERA.

Extra point of attention:
The opportunities for transmission to animals / cross-species transfer of mammalian viruses have increased in recent years due to increased contact between humans and animal reservoirs. Especially in case of use of modified animal viruses transfer to pets, cattle or poultry (in rural areas) should be evaluated, taking the surrounding environment of the patient and all foreseen procedures that may lead to an exposure of the surrounding environment into account (3d).

Evaluation:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

13. <u>Can transmission to animals / cross species transfer lead to new (adapted) viral variants by recombination, reassortment or random mutation?</u>

***Potential effect**:*
The possibility of new variants of a RCVV arising in animals first of all depends on whether the RCVV or a recombinant / mutant of this vector that arose in humans is able to spread to and lead to a productive infection in an animal reservoir (12). The subsequent occurrence of homologous recombination, functional complementation or reassortment with a helper virus will depend on whether the wildtype parental or a related virus is present in the animal and if present, whether it is able to meet and interact with the RCVV. Whether adaptation by random mutation of RCVVs occurs will mostly depend on the properties of the virus itself (RNA/DNA virus). Recombination, mutation and genetic complementation whether temporal or permanent can result in (transient) presence of viral variants that have different properties compared the applied RCVV. This can influence the viral life cycle and the interaction with the animal and/or human host. If it can not be excluded that such variants arise, their properties should be considered at steps 5, 6 and at subsequent steps in the ERA. Step 3 in the ERA can be skipped since there are no proposed conditions of use for these viral variants.

Evaluation:

- GMO: Yes/No
- Recombinant GMO(s): when applicable (see step 4a): ***Yes/No***
- Complemented GMO(s): when applicable (see step 4b): ***Yes/No***
- Mutated GMO(s): when applicable (see step 4c): ***Yes/No***

C) INTEGRAL EVALUATION OF THE PREVIOUSLY ADDRESSED EFFECTS ON THE IDENTIFICATION AND EVALUATION OF POTENTIAL HAZARDS OF THE GMO FOR PUBLIC HEALTH AND THE ENVIRONMENT.

Identification of environmental hazards and their magnitude and estimation of likelihood and risk

14a Do the modifications in the GMO lead to an increased pathogenicity (including toxic and allergenic effects) compared to the parental virus in the human non-target population?

Pathogenicity is defined as the capacity of an organism (in this case a virus) to cause disease or damage in other organisms. The term virulence indicates the severity of the harmful effects that can be caused by a virus. Pathogenic effects on the non-target population may occur if the vector is able to spread from the subject to thirds (10). ***Effects on the subjects in the trial are not part of the ERA****. However, effects on the non-target population (including persons that handle or administer the gene therapy product, those involved in patient care, but also relatives and 'the general public') depend on the properties of the RCVV and the exposure to the RCVV and may be extrapolated from effects on the subjects (6e). Pathogenicity and virulence are influenced by many different factors. Points 5 and 6 of the ERA give an overview of the most important ones in the context of RCVVs. Genetic modifications that lead to an increase in pathogenicity, for instance by increased toxic and allergenic effects, are a potential risk for public health and the environment and the consequences can be large.*

14a1. Hazard:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

14a2. Estimation of likelihood:

14a3. Risk evaluation:

14b Are changes in medical practice possible due to the deliberate release of the GMO in the environment?

Application of a RCVV can lead to changes in medical practice by interfering with treatments for other diseases. The properties of the virus may also be changed in a manner that interferes with the actions of anti-virals or with medicines that are used for treating symptoms of the parental virus.

14b1 Hazard:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

14b2. Estimation of likelihood:

14b3. Risk evaluation:

14c Do the modifications lead to increased pathogenic effects including toxic and allergenic effects in animals? Are there possible immediate and/or delayed effects on the feed/food chain resulting from consumption of the GMO and any product derived from it, if it is intended to be used as animal feed?

Harmful effects on animals may occur if the vector is able to spread from the subject or thirds to animals (step 12). The effects on the health of animals depend on the properties of the RCVV in the context of the animal host (step 5, 6 and 11) and the exposure of the animal to the RCVV. Effects on the feed/food chain are relevant in case the animals to which the RCVV may be transmitted to are intended for consumption. In this case it should be evaluated whether consumption of expressed genes can cause health problems in human or animals.

14c1. Hazard:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

14c2. Estimation of likelihood:

14c3. Risk evaluation:

14d Are changes in veterinary practice possible?

Application of a RCVV may lead to changes in veterinary practice by interfering with treatments for other diseases. The properties of the virus may also be changed in a manner that interferes with the actions of anti-virals or with medicines that are used for treating symptoms of the parental virus in animals (i.e. if the vector is based on an animal virus).

14d1. Hazard:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)

- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

14d2. Estimation of likelihood:

14d3. Risk evaluation:

14e Are there effects on the population dynamics in the natural environment?

The term 'population dynamics' refers to the biological and environmental processes that cause immediate or delayed and long or short-term changes in the genetic diversity of one or several populations. Changes in a human, animal or microbial population may have profound effects on public health and the environment, e.g. by impacting on population levels of competitors, prey, hosts, symbionts, predators, parasites and pathogens, effects on the dynamics of populations of species in the receiving environment and the genetic diversity of each of these populations. Selective advantages or disadvantages due to gene transfer to other species are part of this evaluation.

14e1. Hazard:

- GMO
- Recombinant GMO(s): when applicable (see step 4a)
- Complemented GMO(s): when applicable (see step 4b)
- Mutated GMO(s): when applicable (see step 4c)
- Any novel viral variant that may arise in animals: when applicable evaluate the consequences for both the human and animal host(s) (see step 13)

14e2. Estimation of likelihood:

14e3. Risk evaluation:

15. Risk management

Risk management is only needed if environmental risks that are not negligible were identified in the previous steps. If no adverse effects were identified risk management is not necessary.
Risk management strategies include for example the use of contraception and the decontamination of urine. Isolation of treated patients can, according to the Dutch authorities, not reduce the risk for the environment since patients may leave the hospital at any time.

16. Determination of overall risk after risk management

In the final step of the ERA the overall risk of the application of the RCVV is evaluated taking into account the proposed risk management. When the risk is acceptable, the clinical trial can be carried out.

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Appendix A: Replication competent viral vectors and strategies applied for their genetic modification

A.1 Replication competent DNA viruses

The first viruses that were explored for oncolytic therapy or immunotherapy are the DNA viruses, HAdV-C, HSV-1 and vaccinia virus (VV). General advantages of DNA viruses are that they can be easily genetically modified and are genetically stable. Especially HSV-1 and VV have a large capacity for genetic modification (see table II). Since these viruses have humans as their natural host they have been well characterized and the (vaccine) strains, on which the viral vectors are based on, often have an established safety record in humans. Most of the current human population has pre-existing immunity against these viruses. While this may be good news from the standpoint of safety this may pose a problem for systemic administration (28).
To circumvent this problem replication competent viral vectors (RCVVs) based on DNA viruses that have other species as their host are being applied. Myxoma virus is a poxvirus that causes a lethal disease termed myxomatosis in its specific host (the European rabbit) but that is non-pathogenic to other species. This and the lack of pre-existing antibodies in the human population suggest that Myxoma could be an attractive agent for oncolytic therapy (37). Another animal DNA virus that is being explored is the rat parvovirus H-1 (38, 39, 116).
A drawback of the use of (modified) animal viruses are the specific environmental risks that may be associated with their use. Genetic modifications may for instance cause an altered pathogenicity of a virus in its natural host. Moreover, the spontaneous or engineered acquisition of an altered tropism may result in viruses crossing the host-range barrier and the establishment and persistence of a virus in the new host species, which may result in disease (zoonosis). The latter example is especially a concern in case of use of viruses not known to have been in contact with the human population previously (see also reference (30) and paragraph B.5).

A.2 Replication competent RNA viruses

Attenuated strains and vaccine strains from several RNA viruses including negative strand viruses (e.g. vesicular stomatitis virus, measles virus, Newcastle disease virus), positive strand RNA viruses (e.g. poliovirus, coxsackievirus A21, echovirus 1), and double stranded RNA viruses (reovirus) are being investigated for the treatment of cancer (4). The use of RNA viruses may have several advantages over the use of for instance HAdV (20, 24)(table II).
RNA viruses in general have a faster replicative cycle compared to DNA viruses such that tumour destruction may have occurred before the initiation of potentially neutralizing antiviral immune responses in the host, making them more suitable for systemic application. Some of the applied RNA viruses have potent anti-tumour effects that reside in the expression of fusogenic glycoproteins that elicit strong bystander effects (e.g. MV, NDV, coronavirus). Some RNA viruses appear to be relatively tumour selective in humans. This inherent relative tumour selectiveness is the result of genetic defects that are commonly found in tumours. For instance in the case of NDV and VSV, tumour selective replication is thought to be the result of defects in interferon antiviral defence pathways in tumour cells (23).
Several of the applied RNA viruses have additional advantages like the absence of pre-existing immunity in humans which increases the suitability for systemic administration, a low pathogenicity in humans and sensitivity to the anti-viral actions of interferon (table II, (4)). As a result of these properties several wildtype RNA viruses are ‘naturally smart’ and do no have to be modified to be used as an oncolytic virus (20). A by coming advantage of the use of wildtype viruses is that they do not fall under GMO legislations which may explain in part why several

wildtype RNA viruses (i.e. NDV, Reovirus, CoxA21 and SVV) are currently being tested in clinical trials in the USA, Europe and Israel (4).
Nevertheless, several RNA viruses are being subjected to genetic modification to enhance their oncolytic properties even further, to increase the potential for disease monitoring, to increase their safety or to make them more suitable for systemic administration. The most notable current examples are MV, VSV and poliovirus (table I)(4).
The main disadvantage of RNA viruses is their high mutation rate. Safety concerns are the potential arisal of viral variants that are more pathogenic to their natural host or adaptation of RNA viruses to humans because of the application (30, 117). In case of genetically attenuated variants like the poliovirus vector PVS-RIPO (118) reversion to a neurovirulent variant is a concern. Moreover, introduction into the environment of viruses pathogenic to animals can be risky, as recently shown by the outbreak of food and mouth disease following the outbreak of an FMDV vaccine strain (119).

A.3 Replication competent retrovirus

MLV based replication competent retroviral vectors (RCR) are under pre-clinical development (4). A pro of RCR compared to many other viruses is the ease of manipulation and virus production. This is important since for the main proposed purpose of these vectors, oncolysis, suicide or other therapeutic genes have to be included in the RCR vector. MLV has a built-in level of specificity for tumour cells in so far as it can only infect cells that are actively dividing. However RCR does infect dividing normal cells like normal fibroblasts and HMECs *in vitro*. Although this may be advantageous for *in vivo* therapy as these cells (when present in vicinity of tumours) may contribute to tumour growth and invasion, this poses a safety concern. There are however some data that suggest that spread of RCR is restricted to the immunosuppressive tumour environment (42, 120). Another safety concern for retroviral vectors is the occurrence of oncogenesis through insertional mutagenesis after treatment with retroviral vectors (121). However, insertional mutagenesis has only been shown to occur in treatment regimens were stem cells were *ex vivo* transduced with retrovirus. No pathology was for instance observed in primates after systemic administration of wildtype MLV and the virus is rapidly cleared from the bloodstream by the immune response in primates. A third potential problem with replicating retroviruses is their instability; inserts can be lost within three or fewer passages and it was found that the size of the insert should not exceed over 1.3 kb, or contain repetitive sequences, although experiments to improve the stability have been conducted with MLV based vectors (40, 120, 122).

A.4 Modification strategies in replication competent viral vectors

Virtually all genetic modifications that have been applied in replicating (and especially oncolytic) viruses have been explored in the adenoviral system (table I) and the underlying principles of the modifications are mostly similar for the different viral backbones. Therefore we will make use of adenoviral vectors to illustrate the different strategies sometimes using, when appropriate, viruses from other families as additional examples. A more exhaustive and detailed overview can be found in the accompanying supplementary document (4).

A.4.1 The adenoviral backbone

The HAdV-2 and HAdV-5 serotypes, members of the HAdV-C species, are widely applied as a backbone for viral vectors with oncolytic properties (4). HAdVs are double stranded DNA viruses with a linear genome of 36 kb which can be engineered to incorporate large enough stretches of DNA to permit the incorporation of foreign therapeutic genes. Adenoviruses are relatively mild,

class 2 pathogens and humans are their primary and almost exclusive host. In general, HAdV infections are asymptomatic but they can be associated with diseases of the respiratory, ocular and gastrointestinal system with HAdV-C viruses primarily causing mild respiratory and alimentary tract infections in children. Although approximately 80% of the human population is sero-positive for HAdV, no consequences of HAdV persistence are known. HAdVs are able to integrate in the host cell DNA but no adverse effects of this property are known. Despite considerable efforts scientist have not been able to create transgenic mice by direct injection of adenoviruses in the testis (123). Given all these properties, it is not surprising that HAdV vectors are the most commonly applied oncolytic vectors in both preclinical and clinical studies.
However, HAdV vectors also have some fundamental flaws which explain why especially these vectors have been extensively subjected to genetic modification. Several tumour types are not efficiently transduced by HAdV. HAdVs spread slowly and work poorly when administered intravenously, mostly due the pre-existing defence mechanisms present in most humans (124). At present there is a limited availability of proper immunocompetent models that can be applied to study pre-clinical efficacy, although the cotton rat and Syrian hamster are semi-permissive to HAdV infection and are currently being applied for pre-clinical safety studies (125, 126) and as pre-clinical models (126, 127).

A.4.2 RCVVs containing deletion(s) of essential viral genes

The HAdV genome has evolved a number of mechanisms involved in promoting viral replication and further spreading into the host. The adenoviral E1B-55kD protein inhibits p53 induced apoptosis and was recently shown to promote viral replication by stimulation of export of late viral RNAs (128). The E1A protein promotes entry of arrested cells into the cell cycle by binding to the cell cycle regulator pRb and E1A indirectly counteracts intrinsic and extrinsic cellular apoptotic pathways (23, 129). The E1B-19kD protein inhibits apoptotic stimuli of both the intrinsic (p53) and extrinsic (TNF/Trail-mediated) pathways (23). Deletion of these and other adenoviral genes can be applied to specifically target HAdV vectors to tumour cells. Tumour cells are unrestricted in their growth because they have accumulated mutations and other genetic defects that have led to increased proliferation and defective apoptosis. Many of these mutations occur in genes that have a role in the same cellular pathways as those targeted by the HAdV gene products, e.g. inactivating mutations in the tumour suppressor genes p53 and pRb, and activating mutations in the Ras proto-oncogene, that targets the interferon/PKR apoptosis pathway. For yet unknown reasons, tumour cells are also altered in their mechanisms of RNA transport. Therefore tumour cells (as opposed to normal cells) can, depending on type of mutation occurring in those cells, complement for the loss of function of specific viral genes in viral vectors, leading to tumour-selective viral replication (23, 129).
Vectors containing deletions in adenoviral genes are designated type 1 conditionally replicating adenoviral vectors (CRAds). The first GM oncolytic vectors that have been extensively studied in clinical phase I-III trials are E1B-55kD deleted vectors like Onyx-015 (130) and H101 (72). Despite a limited efficacy in these trials (see Appendix B), results were promising and this has resulted in the rapid development of other type 1 CRAds that appear to have an increased oncolytic potency in some models. An E1B-19kD deleted vector was shown to display enhanced anti-tumour potency and viral spread in preclinical *in vitro* and *in vivo* models compared to wildtype HAdV virus and Onyx-015 (23). Also AdΔ24 (131) and dl922-947 (132) that contain a small deletion in the E1A gene that abrogates binding to the pRb protein showed increased potency compared to Onyx-015 in preclinical studies (132, 133).
A general ‘problem’ of RCVV vectors is that they are not uniquely but only relatively specific for tumour cells. E1A-CR2 deleted vectors for instance are able to replicate in cycling normal cells (132), and E1B-55kD deleted vectors that were initially thought to be permissive for p53 deficient

cells only, do replicate to a certain extent in normal cells and in tumours without p53 mutation (134). The specificity observed in pre-clinical experiments depends on many factors (e.g. the choice of tumour and control cell lines, the setup and timing of the experimental assay and sensitivity of virus detection methods) and may not reflect the specificity *in vivo*.
To improve the relative specificity of AdV vectors, vectors with additional mutations have been generated. The CB1 and Adl118 / AxdAdB-3 adenoviral vectors contain deletions in E1A-CR2 plus E1B-55kD and E1A-CR2 plus E1B-19kD, respectively (134-136). These vectors show similar efficacy compared to control vectors in specific tumour cell lines and *in vivo* xenograft models, with an improved relative specificity for the tumour cells. CB1 was in contrast to AdΔ24 not able to replicate in dividing normal cells in a specific *in vitro* assay (135).
Similar to HAdV, single and compound deletion of viral genes involved in the promotion of viral replication has been applied in the context of the viruses HSV-1, VV, VSV and Influenza A in order to make these viruses replication competent under certain conditions (see table I and (4)).

A.4.3 RCVVs containing foreign regulatory sequences

An alternative strategy to specifically target HAdVs to tumours is to drive expression of essential viral genes by 'foreign' regulatory sequences that are hyper-reactive in certain tumour cells, a strategy also known as 'transcriptional targeting'. In a review from 2004, 26 of these type 2 CRAds were counted (129) and many more have been published since then. In most cases, expression of the essential E1A gene is placed under heterologous regulatory elements. Promoters from for instance the cellular genes telomerase reverse transcriptase (TERT) and E2F-1 can be used to target E1A expression and viral replication to many types of tumours, making use of the fact that most tumour cells have elevated levels of TERT and E2F-1. To target E1A expression and viral replication to specific tumour types, regulatory elements of for instance the cellular genes PSA (which is specific for prostate tumours) can be applied (129).
Like type 1 CRAds, type 2 CRAds replicate preferentially but most often not exclusively in tumour cells. Small amounts of E1A protein are known to be sufficient to initiate 'leaky' adenoviral replication and in most cases a small amount of replication is observed *in vitro* in normal cells. Moreover, promoters may be activated *in vivo* in certain cell types or under conditions that are difficult to test *in vitro*.
To increase the specificity of replication, additional essential viral genes (i.e. E1B, E4) may be placed under control of the same or different tumour-specific regulatory elements as those applied for the E1A gene. An example is the second generation vector CV764 (PSA promoter controlled E1A and hklk2 promoter controlled E1B) that in contrast to the first generation vector CV706 (PSA promoter controlled E1A) was found to be replication deficient in PSA negative cell lines (137). It should be noted that results regarding specificity of transcriptionally targeted viruses are difficult to predict and may depend on the viral and cellular context (138).
A different strategy to increase the tumour specificity is to combine transcriptional targeting with deletion of viral genes. For instance in the vector Onyx-411 the E1A-CR2 deletion is combined with an E2F-1 promoter driven E4 gene leading to reduced replication in normal cells, and a reduced hepatic toxicity, while retaining a tumour-killing efficacy similar to wildtype HAdV (139, 140).
Transcriptional targeting has besides for HSV-1 and HAdV also been applied in replication competent autonomous parvoviral and retroviral vectors (4).

A.4.4 RCVVs containing deletions of non-essential viral genes

Many of the first generation type 1 and 2 CRAds (e.g. Onyx-015, CV706) were also attenuated in their spreading and immune system avoiding properties (129) by secondary deletions in the

adenoviral E3 region. E3 region genes encode for proteins with very important anti-apoptotic and immuno-modulary functions. While these deletions may have increased the selectivity and the safety of the first generations of HAdV vectors, it has become clear that they may have compromised the efficacy of HAdV vectors as these vectors are more efficiently cleared by the immune system (23, 34, 141). Therefore in more recent type 1 and type 2 CRAds, E3 genes are often retained. Inclusion of E3 sequences in CV787 (rat probasin promoter controlled E1A, PSA promoter controlled E1B) resulted in increased *in vitro* and *in vivo* efficacy compared to CV764 (lacking E3 sequences), while retaining a greatly attenuated replication (more than 10.000 times compared to wildtype HAdV) in PSA negative cells (142).
It should be noted that deletion of non-essential genes can lead to faster viral clearance but may depending on the vector and its application also (temporally) enhance the anti-tumour effect of the oncolytic virus by increasing the immunogenicity. G47Δ is a vector derived from the HSV-1 vector G207 with an extra deletion in the ICP47 gene that normally inhibits TAP, a cellular transporter involved in tumour-antigen presentation. Compared to G207, G47Δ was more potent in immunocompetent and immunodeficient animal models while remaining non-toxic (143, 144).
An alternative strategy to improve the efficacy of GM oncolytic adenoviral vectors has been applied in the vector VRX-007. In this vector the E3 ADP gene is overexpressed due to the deletion of other genes in the E3 region, leading to enhanced cell lysis and cell to cell spreading. VRX-007 retains all essential genes and is therefore compared to wildtype adenovirus not increased replication selective (145).

A.4.5 Translational targeting of RCVVs

Translational targeting is a strategy that more recently has been developed to increase the tumour-selectivity and safety of oncolytic HAdV, poliovirus and coxsackievirus (table I).
A translationally targeted HAdV was developed based on the property of certain tumour cells having a constitutively active Ras-MAP kinase pathway that can lead to stabilization of certain cellular mRNAs. In this vector, Ad-E1A-Cox, the E1A gene is fused to the 3'-untranslated region (UTR) of the Cox2 gene. Upregulation of Cox2 in Ras-transformed tumours is in part mediated through selective stabilization of the Cox2 messenger through a region in the 3'-UTR that is activated by the MAP kinase pathway. This vector was preferentially oncolytic *in vitro* in tumour cell lines with an activated MAP kinase pathway and was as efficient as wildtype HAdV in an *in vivo* xenograft model (146).
PV1-RIPO and PVS-RIPO are intergeneric poliovirus type 1 recombinants bearing the internal ribosome entry site elements from a common cold human rhinovirus. This leads to an attenuated neurovirulence compared to the wildtype strain. The variants were shown to grow poorly in tissue culture cell lines of neuronal origin and were avirulent in CD155 overexpressing mice, and non-neuropathogenic to non-human primates. PVS-RIPO prolonged the survival in athymic rat xenograft models for glioma, breast cancer and intracranial disseminated breast cancer (57, 147-149).
Besides these strategies micro-RNAs were recently applied to mediate translational repression of CVA21 in normal cells as opposed to tumour cells (150).

A.4.6 'Arming' of RCVVs with therapeutic transgenes

The complexity of the processes leading to cancer formation necessitates that multiple treatment modalities (e.g. surgery, chemo- and radiotherapy) need to be combined for effective treatment. Oncolytic virotherapy can also be combined with additional agents with the aim to increase the therapeutic outcomes. The effects of combination treatments on viral replication will be discussed later (see paragraph A5.3). An alternative strategy which can be applied to enhance the probability

of tumour eradication is the direct genetic 'arming' of replicating viruses with (therapeutic) transgenes (21, 151). The different categories of transgenic inserts are discussed below.

Marker and reporter genes

Although marker and reporter genes will not enhance the oncolytic activity of replicating viruses, the treatment efficacy can be increased by more effective monitoring of the disease process. Reporter genes like eGFP (152), luciferase (153), Dsred2 (154) and the human sodium iodide symporter (hNIS) (155) have been included in oncolytic HAdV vectors as they are useful for monitoring viral replication, distribution and antitumour efficacy both in pre-clinical and clinical studies.

Therapeutic transgenes

Therapeutic genes as diverse as suicide genes, tumour suppressor genes, immune regulatory genes, apoptosis inducing genes, angiogenesis inhibitory genes and heat shock proteins have been inserted in both type 1 and type 2 CRAds (156)(table I). Some specific examples are given below.
A common transgenic insertion in AdV vectors in general has been the inclusion of suicide genes: enzymes that are able to convert non-toxic prodrugs into cytotoxic metabolites that in most cases are also able to spread to non-infected neighbouring cells. An example of an oncolytic adenoviral vector containing suicide genes is Ad5-CD/TKrep, an E1B-55kD attenuated, replication-competent HAdV containing a cytosine deaminase (CD) / thymidine kinase (TK) fusion gene (157, 158). Prodrug administration can either increase or decrease the anti-tumour efficacy of oncolytic vectors, mostly depending on the timing of administration.
Adenoviral oncolytic vectors have also been armed with immunostimulatory genes to increase their efficacy. Examples are Onyx-320, expressing TNF-α (a pro-inflammatory cytokine) instead of the ADP or E3B gene (159, 160) and YKL-IL12/B7, an E1B-55kD deleted HAdV vectors armed with CMV-IL12 inserted in the E1B region, and CMV-driven B7.1 (a T-cell stimulatory molecule) inserted in the E3 region, respectively (161).
AdΔ24 vectors have been armed with the tumour suppressor gene p53 and with TIMP-3 (an angiogenesis inhibitor) using respectively a SV40 early promoter and CMV promoter driven expression cassette inserted in the E3 region (162-164). Expression of siRNAs from oncolytic vectors can be used in order to target specific oncogenes expressed and involved in transformation, like the K-ras oncoprotein (140, 165). Heat shock proteins can be included in oncolytic viruses as they are able to increase immunogenicity by promoting antigen-presentation and since they are thought to promote viral replication through several intracellular mechanisms (10, 166, 167).
A notable insertion that has been explored in several vectors (HSV, Retrovirus, VSV) is the incorporation of fusogenic glycoproteins from other viruses. Fusogenic glycoproteins induce extensive syncytium formation and necrotic cell death by evoking cythopathic effects and triggering the immune response thereby also destroying neighbouring cells. This can enhance the oncolytic effect of viral vectors by a bystander effect that appears to be stronger than the bystander effect of already mentioned suicide genes. HSV vectors have been constructed containing the fusogenic GALV glycoprotein under the control of a strict late viral tumour-specific promoter (168-172). Similarly, a recombinant VSV vector rVSV-NDV/F(L289A), containing the syncytia inducing mutant (L289A) F protein from NDV in the noncoding region of the VSV/G gene, has been constructed. This vector showed superior oncolytic activity *in vitro* and *in vivo* compared to wildtype VSV (173-175). Insertion of fusogenic measles virus MV-H/F glycoproteins that determine the CD46 tropism and tumour selectivity of oncolytic MV may also be applied into HAdV vectors in the future (176, 177) since insertion of GALV glycoprotein was not successful in adenoviral vectors (172).

Table I gives an overview of the types of therapeutic transgenes that have been inserted into different oncolytic backbones. HSV-1, Poxviruses (VV and Myxoma), MLV, VSV, Coronavirus, Sindbis and NDV virus have been armed with similar types of marker and therapeutic transgenes as the ones applied in the adenoviral backbone.

Safety insertions

Currently there are no specific antiadenoviral agents approved for clinical use. Cidofovir is an antiadenoviral agent that has been applied with varied success (178). Therefore suicide genes like HSV-TK may be inserted in HAdV vectors as a failsafe mechanism (125, 179, 180). Failsafe mechanisms have also been applied in vectors based on other viruses. rVSV-IFN-β was generated to improve the safety of rVSV vectors since it was postulated that it would retain oncolytic activity in interferon-defective tumour cells and enhance the anti-tumour immune response, while activating the IFN pathway in surrounding normal cells leading to enforced protection against superfluous VSV (181).

Combinations of insertions

There are examples of replication competent viral vectors containing several (therapeutic) modifications. Type 1 and 2 CRAds containing two transgenes working in the same pathway (e.g. hTERT-AFP-Trail/Smac, in which Smac increases the effects of Trail) have been constructed (156). HSV-1 and VV have a large capacity for the insertion of foreign sequences. In the case of VV multiple combinations of cytokine genes, tumour-associated antigens and costimulatory molecules have already been applied to increase the activity as a tumour vaccine (4). TRICOM is a recombinant VV expressing three co-stimulatory molecules (B7.1, ICAM-1, and LFA-3) (182). PANVAC-V is a more recent recombinant vaccinia virus expressing B7.1, ICAM-1, LFA-3 and the tumour antigens CEA and MUC-1 (183).

A.4.7 Transductional targeting of RCVVs

HAdV-5 viruses use several receptor binding sites to gain access to the cell. Viral attachment is mediated by the knob of the fiber protein that binds to the cellular coxsackie virus and adenovirus receptor (CAR). Viral uptake into the cell is mediated by the RGD motif of the adenoviral penton protein that binds to cellular integrins. Moreover, lysine residues in the fiber shaft that bind to heparan sulfate glycosaminoglycans (HSG) are involved in viral binding and transduction. Tumour cells show a great variety in their CAR receptor expression level which can limit the efficacy of HAdV-5 vectors (184, 185). Moreover, HAdV-C viruses are able to infect a broad range of cells which may limit the viral fraction that becomes available for target cell transduction *in vivo*, especially upon systemic administration. Also other factors may limit the clinical efficacy of HAdV-5 vectors. Upon systemic administration, especially the liver but also other organs function as an adenoviral sink. Kuppfer cells in the liver are known to play a major role in adenoviral clearance in a non-CAR mediated process. Pre-existing titers of neutralizing antibodies against HAdVs are limiting the clinical efficacy of HAdV-5 vectors, especially in multiple dosing regimens. ‘Transductional targeting’ can be aimed at (a) enhancing the specific transduction of CAR-deficient tumour cells, (b) deleting the broad tropism of HAdV-5 in normal epithelial cells, (c) preventing systemic clearance by neutralizing antibodies, and (d) decreasing toxicity of HAdV-5 to the liver (186-189).
It should be noted that viral tropism is not necessarily exclusively determined by the expression of cellular receptors. Expression of specific proteases by tumour cells can for instance influence the tropism of measles virus (see below). Poxviruses can bind and enter most mammalian cells but

these viruses have a very specific host-range. It has been found that the ability to manipulate intracellular signalling pathways determines the ability to lead to productive infections. Alterations in such pathways in cancer cells also explain why a rabbit specific poxvirus can replicate in cancer cells (190). This also implies that tropism modification is possible by manipulating viral proteins involved in the regulation of intracellular pathways.

Tropism modification without ablation of native binding

Several strategies can be applied to redirect HAdVs to tumour cells without ablating CAR binding. In the E1B and E1A-CR2 deleted vectors Adv-E1BdB-F/K20 and Ad5.pK7-D24 extra lysine residues were incorporated into the C-terminus of the fiber protein leading to increased activity against glioma and breast cancer, respectively (191, 192). In AdΔ24-RGD an additional RGD motif was incorporated in the HI loop of the fiber knob allowing direct attachment of the virus to cell-surface integrins. This modification allowed more effective oncolysis of cancers with Rb pathway abnormalities and low CAR expression *in vitro* and *in vivo* (193-196). Incorporation of the cRGD peptide has also been applied to increase binding of oncolytic measles virus (MV) to the vasculature (197, 198).
AdV vectors can be programmed genetically to be directed to different target cells by incorporation of sequences encoding adapter molecules. This strategy is an extension of the strategy in which AdVs are retargeted by systemic co-administration of dual specific antibodies (186, 189). An example is AdΔ24-425S11, a CRAd that has a CMV driven expression cassette encoding a bispecific single-chain antibody directed towards the AdV fiber knob and the EGFR incorporated in its E3 region. This vector was more potent compared to AdΔ24 in destroying neuroblastoma cells (that overexpress the EGFR and that have low expression levels of CAR) *in vitro* and *in vivo* (199, 200).
Similar strategies that make use of single chain antibodies have been used in attempts to target recombinant MV to tumour cells overexpressing CEA, CD20 and CD38 (201-203) and the coronavirus MHV to tumour cells overexpressing non-native receptors (204), while maintaining the native tropism of these viruses. The tropism of measles virus can also be modified by adding C-terminal extensions to the H-glycoprotein. MVgreen-H/XEGF, a recombinant measles virus expressing GFP and a H/EGF hybrid protein, efficiently entered CD46-negative rodent cells expressing the human EGF receptor, causing syncytium formation and cell death (205). A different strategy was used in the measles virus vector MVT7 that contains the high affinity T-cell receptor that was able to fuse murine cells expressing an MHC–peptide complex (206).
MV depends on the ubiquitous intracellular protease furin to process and activate its envelope fusion (F) protein that mediates virus to cell and cell to cell fusion. Recombinant MV expressing an altered F-protein that is activated by tumour-secreted matrix metalloproteinases (MMP) specifically fused MMP-expressing cells. This alteration may be combined with other modifications adding an extra level of selectivity (207).

Tropism modification with (partial) ablation of native binding

To develop a targeted HAdV-5 vector that is systemically more effective the natural CAR tropism can be ablated by fiber (knob) replacement. An additional advantage of this strategy is the concomitant reduction in non-target organ transduction and sustained bloodstream persistence because the HAdV-5 fiber (in a CAR independent manner) is responsible for viral uptake by, and toxicity to the liver (186, 189, 208). In fiber chimeric vectors the knob domain of the HAdV-5 fiber protein is replaced by the knob domain of for instance HAdV-B serotype viruses (like HAdV-3) that bind to a different cellular receptor (189). The fiber chimeric vector Ad5/3-Δ24, an HAdV-5 CRAd pseudotyped with the HAdV-3 fiber knob and therefore deficient in CAR binding,

was superior to AdΔ24-RGD in killing ovarian cancer cells *in vitro* and *in vivo* (209, 210). The infectivity of fiber chimeric viruses can be further enhanced by incorporating the RGD motif in the fiber protein allowing more effective binding to integrins that are overexpressed in many cancer types (211). A strategy that has been recently developed for replication deficient HAdVs and that has been proposed for future incorporation into CRAds is to replace the entire fiber protein with a fusion molecule comprising the virion-anchoring domain of the fiber and the oligomerization domain of the reovirus attachment protein S1 (212).
Fully retargeted GFP expressing recombinant measles viruses were generated by displaying C-terminal antibodies on mutant H-proteins in which CD46 and SLAM native binding is almost completely ablated. Ablated viruses containing α-CD38 or α-EGFR single chain antibodies remain some residual infectivity on normal monkey vero cells without inducing cytopathic effects. The viruses efficiently infected hamster cells or human tumour cells and demonstrated specific anti-tumour activity against $EGFR^+$ ovarian cancer xenografts and $CD38^+$ lymphoma cell line xenografts. Compared to viruses containing only the mutated H-protein these viruses showed reduced infectivity and replication in 'non-permissive' human cells *in vitro* and *in vivo* suggesting that the single chain antibodies ablate residual CD46 tropism (213, 214). Oncolytic measles virus strains retargeted to a mutated EGFRvIII receptor had comparable therapeutic efficacy compared to MV-GFP against EGFRvIII-expressing glioma lines and intracranial xenografts. Absence of nucleocapsid protein expression and complete protection of normal fibroblasts and astrocytes was observed after infection with the retargeted strains as opposed to MV-GFP at high viral doses, improving the therapeutic index (215).

A.4.8 Genetic shielding of RCVVs

PEG or polymers can be conjugated to the HAdV capsid to conceal HAdV vectors from pre-existing antibodies and to decrease systemic clearance. The drawback of this method is that shielding is lost during viral replication. Recently, a genetically based shielding method was developed. Large proteins like TK are incorporated in the virion capsid by fusion to the minor capsid protein pIX leading to reduced recognition by HAdV antibodies. A shielded version of AdΔ24-RGD, AdΔ24S-RGD is being developed at this moment (188).

A.4.9 Transcomplementing vectors systems

To increase the safety of use of AdV vectors several transcomplementing strategies have been developed (216, 217). An example is the co-administration of an E1-deleted non-replicating HAdV vector expressing the TK gene (AV.C2.TK) and Ad5.dl1014, an E4-deleted/E4orf4-only expressing HAdV in order to allow full replication competence in co-infected tumour cells (217). Binary systems also represent a flexible platform for screening of multiple gene products and the enhanced insert capacity of replication-deficient vectors compared to replication-competent vectors allows for the cloning of larger inserts. For instance co-infection of Ad Flk1-F, a replication deficient vector with a VEGF therapeutic transgene and the replication competent vector dl922-947 resulted in repackaging of the replication deficient vector leading to tumour selective replication, increased transgene expression and increased *in vivo* anti-tumour activity in mice (218). Similarly expression of fusogenic glycoproteins, which promote cell fusion and increased dispersion of viruses throughout tumours, was increased by co-infection of a replication deficient HAdV with replication competent helper virus (219). Transcomplementing systems have also been developed for the HSV-1 and MLV viruses (4). Given the already substantial complexities of clinical development of single viral agents, clinical development of binary systems seems very unlikely.

A.4.10 Complex RCVVs containing multiple modifications

Treatment of aggressive disseminated cancers asks for the generation of vectors that can be administered systemically, that are specific and highly infectious for tumour cells and that have profound tumour-specific cytotoxic effects. Several of the abovementioned modifications have to be combined in order to construct vectors with such qualities. Many of the previously discussed vectors already contain several modifications (e.g. type 1 and 2 CRAds armed with therapeutic transgenes). Recently, adenoviral vectors containing even more complex modifications have been constructed.

Ad.MCDIRESE1.71Hsp3 is an example of a vector combining several types of modifications. First, it contains the E1B-55kD deletion. Second, the E1A and CD genes (linked by an IRES) are driven by a tyrosinase enhancer element inserted in the E1 region, to make the replication of this vector and the induction of CD gene expression specific for melanoma cells. Third, this vector contains a CMV-Hsp70 expression cassette in the (deleted) E3 region allowing overexpression of Hsp70 that stimulates several innate immune responses in infected cells. Last, an RGD peptide is inserted in the fiber protein to increase the infection efficiency of this vector. This multimodal vector specifically replicated in melanoma cell lines (as opposed to Hela cells) and had an increased melanoma-specific cytotoxic effect in the presence of 5-fluorocytosine *in vivo* compared to a control vector without the Hsp70 insertion (220). The OV1195 vector is another example of a vector containing several modifications. The viral E1A is driven by the E2F-1 promoter, the human GM-CSF gene is inserted instead of the E3-gp19kD gene and the vector contains a chimeric HAdV-3/5 fiber with an RGD insertion. Although the cytotoxicity and replication of this and other fiber chimeric viruses was attenuated in normal cells compared to wildtype HAdV-5, the chimeric viruses were more cytotoxic to head and neck tumours, but also to normal cells than an oncolytic virus with the normal HAdV-5 fiber (220, 221).

Increasing complexity is also observed in the development of HSV and VV vectors to increase their efficacy while attempting to maintain their relative safety (4).

Complex vectors are still in the pre-clinical stage but may be expected to enter clinical trials in the future once the safety of vectors containing each of the individual modifications is proven. In this respect it is important to note that recently the first tropism-modified adenoviral vectors has been applied in a clinical trial (4).

A.4.11 Chimeric viruses

To improve the oncolytic efficacy of viral vectors chimeric viruses can be constructed, containing single or multiple complete viral genes from one species in a different viral backbone.

MV-eGFP-Pwt is a chimeric oncolytic measles virus derived from MV-eGFP and the wild-type MV IC-B strain that was generated to reduce the induction of interferon by MV-eGFP in human myeloma and ovarian cancer cells. Insertion of the N and P gene of wild-type measles virus completely impaired IFN induction leading to increased anti-tumour efficacy *in vitro* and *in vivo* (222). Oncolytic HSV-1 vectors containing the γ34.5-deletion have an attenuated neurovirulence but are limited in late viral protein synthesis. Late proteins have a function in PKR mediated host evasion. To restore late protein functions without restoring the neurovirulence, human cytomegalovirus (HCMV) / HSV-1 chimeric viruses were generated containing HCMV PKR evasion genes. These viruses showed improved replication in malignant gliomas, resulting in enhanced tumour reduction and prolonged survival (223).

A.5 Other strategies to optimize the use of replication competent viral vectors

A.5.1 RCVV backbones based on different viral strains

Vector backbones based on viral strains from the same or different viral species belonging to the same genus or a different genus are being investigated as an alternative for the currently used species, as they may have properties that make them attractive to be used against certain types of tumours. For instance serotype species belonging to the HAdV-B species are being investigated as an alternative for HAdV-2/5 vectors because the immune prevalence for these viruses in the human population is lower and they bind to the CD46 receptor that is overexpressed on many tumour cells (188, 189).

Likewise oncolytic vectors based on HSV-2 have been developed as an alternative for HSV-1 based vectors for targeting of cancer cells with an activated Ras pathway (224) and newly isolated more oncolytic HSV-1 strains have been used for genetic engineering. Oncovex$^{GM\text{-}CSF}$ that is in several phase I clinical trials (102) was constructed by deleting the genes encoding ICP34.5 and ICP47 and inserting the gene encoding the cytokine GM-CSF in the freshly isolated, more oncolytic, HSV-1 strain JS1 (225). Novel more oncolytic vaccinia strains are based on the strain Western Reserve (226).

Recently, the first non-human CRAd based on the canine AdV CAV-2 was generated. This vector was developed to be able to study the effects of immuno-suppression in a host system that is totally permissive for AdV. In the canine vector OC-CAVE1 the E1A gene was driven by the osteocalcin promoter, causing efficient replication and oncolysis of dog osteosarcoma cells *in vitro* and in a mouse xenograft model (227). This group has also generated fiber chimeric replication deficient HAdV-5 vectors that contain the CAV-2 knob and a polylysin insertion to improve the infectivity of dog osteosarcoma cells and are planning to generate infectivity enhanced canine CRAds. HAdV vectors with the canine knob were more effective and yielded more efficient gene transfer than vectors with the HAdV-5 knob in canine cells and human cells. This may be linked to the fact that CAV-2 has the ability to transduce CAR-deficient cells suggesting that CAV-2 is using a second cellular receptor. This is potentially interesting for treatment of certain human cancers since the improvement of infectivity observed with HAdV-5/3 fiber chimeric vectors in human epithelial-derived neoplasms, was not observed for mesenchymal neoplasms, like sarcomas (228).

A.5.2 Random selection of RCVVs

Random *in vitro* selection is another strategy for optimizing viral vector efficacy. Random mutagenesis has been applied to the replication competent HSV vectors G207 and Baco-1 to select vectors with fusogenic properties (168-170, 229). Isolation of novel more potent HSV variants (GM and wildtype) may also be possible by using serial passaging (36).

A.5.3 Combination strategies that may influence RCVV replication

All strategies described in the previous paragraphs make use of properties of the viral vector backbone and the alteration of this backbone by genetic modification or random mutagenesis to generate replicating vectors with increased oncolytic potential. Several other strategies are able to temporally influence viral replication in the host. Based on current mathematical models of tumour oncolysis it is predicted that if uninfected tumour cells propagate at a faster rate than the virus can spread and induce cytotoxic effects, the therapeutic outcome will be poor. Therefore tumour eradication, even with multi-armed viruses, will probably require potent suppression of

innate immune clearance mechanisms, combination with other treatments or concomitant therapeutic gene expression with cytotoxic or immunostimulatory bystander effects (230, 231). We will shortly discuss the most important strategies that have been proposed to be applied in clinical studies. As these strategies may influence viral specificity and replication they should, when applied, be evaluated in the ERA of RCVVs. While this overview is certainly not exhaustive, it illustrates the wide variety of clinical protocol elements that may interact with the vector in the patient and that should be taken into account in the ERA.

Suppression of innate and acquired immune responses

Cyclophosphamide (CPA) is an immune suppressing agent that is able to inhibit both innate and neutralizing antibody responses. CPA has been shown to limit the effects of the host response against oncolytic AdΔ24CMV-Luc and oncolytic HSV in *in vivo* immunocompetent glioma models allowing prolonged viral replication and transgene expression (232-234). The effects of CPA on replication of other viruses are also being tested. MV-NIS, a recombinant measles virus expressing the humane sodium iodine symporter, is being applied intravenously in presence or absence of CPA with subsequent I^{123} imaging in a clinical trial in patients with multiple myeloma (118, 235).
Another substance that is able to enhance viral replication is rapamycin. Vesicular stomatitis virus (VSV) infection induces the expression of GADD34, an intracellular protein that is induced by a variety of stress signals. GADD34 suppresses viral replication by interacting with mTOR, a regulator of the cellular translation machinery. Rapamycin is an inhibitor of mTOR (236) that prolonged survival of immunocompetent rats bearing brain tumours by enhancing viral replication of intratumourally administered myxoma virus and intravenously administered VSVM51 (237, 238).

Shielding

A strategy to circumvent the neutralization of adenovirus vectors by antibodies is to mask their surface by covalent attachment of the polymer polyethylene glycol (PEG). PEG-modified adenovirus was protected from neutralization in mice with high antibody titers to adenovirus, suggesting that PEGylation will improve the ability to administer HAdV vectors on a repeated basis (188). An alternative for shielding of RCVVs is the administration of cells that are pre-infected with the RCVV (239). VV-DD-GFP is a double mutated (VGF^-/TK^-) and GFP expressing recombinant VV that was generated to replicate with increased selectivity in actively dividing cells (240). Administration of cytokine-induced killer (CIK) cells preinfected with VV-DD-GFP resulted in increased regression of human ovarian cancer and murine breast cancer tumours in both immuno-deficient and immuno-competent mouse models showing both the effectiveness of viral shielding and of combining virotherapy with immunotherapy (241). Repeated intravenous administration of VSV in carrier cells may improve the therapeutic efficacy compared with viral particle injection by circumventing the antiviral immunity (239). Carrier cells have also been applied to optimize the systemic use of measles virus (242).

Chemotherapy and radiotherapy

Chemotherapy and radiotherapy in general may non-specifically influence viral replication by means of effects on the immune system, i.e. immuno-suppression. Furthermore these therapies may enhance the cytolytic effects of oncolytic viruses by non-specific synergistic effects on tumour cells (19). Chemotherapy and radiotherapy have already been safely applied in combination with the first generations of oncolytic viruses (see tables IV and V). Also examples

of specific interactions of chemotherapeutic agents with oncolytic viruses are known. Temozolomide (Tm), a chemotherapeutic agent, enhanced replication of the HSV-1 vector G207 thereby increasing oncolysis in glioma cells that had become temozolomide resistant. The increased replication was proven to be due to the increased expression of GADD34 (a homologue of the γ34.5 gene deleted in G207) in Tm resistant glioma cells (243, 244).

Kuppfer cell depletion

Kupffer cells in the liver are involved in adenoviral clearance. Depletion of Kupffer cells by intravenous administration of multilamellar liposomes containing dichloromethylene-bisphosphonate can delay clearance of adenovirus in mice after intravenous administration (245). Hepatectomy (partial removal of the liver) supported viral replication of the HSV-1 viruses G207 and NV1020 for 7 days (246).

Heat-shock

Local hyperthermia can be applied to augment oncolysis by replication competent HAdV but the exact mechanism is unclear. Proposed mechanisms are apoptotic effects of the treatment itself or induction of heat shock proteins like Hsp70 that may influence viral replication via diverse mechanisms (167). In case of the HAdV vector Onyx-015, heat shock may stimulate viral replication by promoting late viral RNA transport. A way to enhance viral replication in patients by ‘heat shock’ may be to not treat treatment-related fevers (247, 248).

Appendix B: Overview of replication competent viral vectors applied in clinical trials

B.1 Introduction

From the middle of the 19th century onward there have been several reports of tumour regression coinciding with natural virus infections, but it was not until 1949 that the first serious clinical trial with an oncolytic virus was performed. In this study serum or tissue extracts containing hepatitis virus were administered to Hodgkin disease patients. During the 50's-70's various other wildtype viruses, including adenovirus, Egypt 101/WNV and mumps virus were tested in clinical trials. The limited success and the apparently unethical circumstances under which some of these trials were performed were held responsible for many researchers abandoning the field in the 70's and 80's (28, 117). The possibilities of genetic engineering and the availability of better pre-clinical models resulted in a renewed interest in the 90's leading to several clinical trials with both genetically engineered (e.g. HAdV, HSV and VV) and wildtype viruses (e.g. NDV, reovirus).
This chapter summarizes the main findings from clinical trials with RCVVs performed since the 1990s and provides an overview of available clinical safety data for these vectors. Besides engineered viruses also published trials with non-engineered viruses are included in this overview since it is in most cases likely (see also Appendix A and table I) that genetically modified variants of these viruses will be used in the future. Pre-clinical data that may be of use for the ERA of RCVVs vectors will be briefly discussed. The supplementary document (4) gives a more extensive overview of preclinical and clinical data concerning RCVVs vectors currently in clinical trials and of those vectors that are currently under (RAC) review.

B.2 Safety data and regulatory approval

Acquirement of safety data is important for the assessment of the safety for the patient as well as for assessment of the environmental safety. Again it should be emphasized that according to Annex II of EC Directive 2001/18 safety for the patient is not part of the ERA. However, since safety data may be used for extrapolation of risks to public health, most preclinical and clinical safety data will be useful for the ERA.
Data from pre-clinical safety tests will most often be required to get regulatory approval for clinical trials involving GM vectors in Europe (see chapter 2). In the USA, where most clinical trials involving RCVVs have taken place, the trial can be initiated after the approval of the Institutional Review Board (IRB) and Institutional Biosafety Committee (IBC) at the clinical site and the approval of an investigational new drug (IND) application by the FDA. NIH funded trials need to get approval from the Office of Biotechnology Activities (OBA) and have to submit their protocol to the recombinant DNA advisory committee (RAC) that may select protocols for public review and that gives recommendations. RAC review is however not necessary in all cases for the initiation of clinical trials, since RAC approval is totally voluntary for non-NIH funded trials. Pre-clinical testing may be advised by the OBA/RAC and may be requested by the FDA. Most of these tests will however be associated with the safety for the patient and not with environmental safety. Institutions that have a role in biosafety in the USA are the IBC and the FDA. The FDA only looks at environmental safety under extraordinary circumstances and the ERA is usually waived. Thus, the responsibility mainly resides at the local IBC at the clinical trial site. Besides preclinical safety data, safety data from vaccination studies and previous clinical trials, with the same vector or related vectors containing similar insertions, may be of aid in the approval of clinical protocols. An example is vaccinia tumour vaccines that since their initial use are gradually

containing more immunomodulary insertions in the same backbone. For these vectors there are usually no specific recommendations by the RAC suggesting that no novel risks are associated with the use of more complex vectors compared to the vectors used in previous trials.

B.3 Preclinical safety data

Most of the experiments described in pre-clinical publications are performed with the aim to prove the activity of the GMO and to assess the safety for the patient. These safety data may of course also be used to assess the safety for public health in general. In the supplementary document, for each of the specific vectors that have been applied in clinical trials, an overview of specific pre-clinical data that have lead to the initiation of the clinical trial is provided (4). Here we will merely mention and shortly comment on the general pre-clinical strategies that have been applied to assess safety.

The main strategies include:

1. *In vitro* assessment of tumour selective replication.

Viral replication in tumour cells is usually compared with replication in a panel of non-dividing and dividing human cells. Dividing normal cells may especially be included in case of viruses that have a tropism for dividing cells (e.g. retrovirus).

2. *In vivo* assessment of tumour selective replication and biodistribution in (disease) models that can be extrapolated to the human situation.

The assessment should imitate the situation for the proposed use in humans as much as possible (e.g. similar route of administration, equal dose per kg bodyweight, similar combination treatment). Immunocompetent and/or immunodeficient models are often applied. Although ideally animal species / strains should be used that are equally or more permissive to the virus compared to humans and in which the virus displays a similar tissue tropism, this will rarely be the case.

3. *In vivo* assessment of safety in rodents (e.g. cotton rats/hamster for HAdV or non-human primates and owl monkeys for HSV virus) that can be extrapolated to the human situation.

Similar and higher equivalent doses per kg bodyweight compared to the proposed dose for use in humans are injected systemically or directly in permissive normal organs and may be compared with for instance the fully virulent wildtype virus or other GMO strains. Ideally animal species or strains are used that are equally or more permissive to the virus compared to humans and in which the virus displays a similar tissue tropism.

Various parameters can be assessed under strategy 2 and 3 including:
- pathogenicity to normal cells (histology, IH);
- toxicity (LD50, liver enzymes, scoring of disease symptoms);
- systemic distribution (PCR, plaque assay on blood);
- biodistribution in different organs (PCR, plaque assay, IH, ISH, EM);
- viral shedding in saliva, tears, vagina, stool, urine (PCR, plaque assay);
- viral genome stability (e.g. in case of RNA viruses);
- reactivation of latent virus (e.g. in the case of HSV);
- recombination.

B.4 Clinical trials with RCVVs and safety data from clinical trials

B.4.1 Types of vectors applied in clinical trials

Tables I, IV and V provide a survey of the clinical trials that have been performed with RCVVs abroad and of published clinical safety data. A more exhaustive overview of the specific vectors can be found in the supplementary document (4).
In table I the viral vector backbones and types of modifications that have entered the clinic or that are currently under review are highlighted. As shown in table I clinical trials with wildtype viruses have used VV, NDV, CVA and Reovirus. GM viruses that have been applied in clinical trials are based on the HAdV, HSV-1, VV and MV backbones.
The VV and MV backbones are based on vaccine strains. Numerous studies have been conducted with GM vaccinia virus containing single or multiple tumour antigens and immunostimulatory insertions in the low pathogenic vaccine strain Wyeth for the specific use as a tumour vaccine. The measles virus vectors that are currently being applied are based on the naturally attenuated Edmonston vaccine strain containing marker insertions.
Most of the applied GM vectors in oncolytic virotherapy (at least the HAdV, HSV-1, VV based viruses) are however conditionally replicating vectors containing deletions of viral genes (the adenoviral vectors Onyx-015 and H101, the HSV-1 vectors HSV1716, G207 and NV1020 and the VV vector JX-594). In case of adenovirus also conditionally replicating transcriptionally targeted type 2 CRAds (e.g. CV706, CV787) have been applied in clinical trials. Besides vectors without therapeutic insertions a number of conditionally replicating vectors containing donor insertions have entered clinical trials. These include vectors containing suicide genes (Ad5-CD/TKrep), marker inserts (G207) and vectors expressing immunostimulatory cytokines like GM-CSF (the HSV-1 vector Oncovex$^{GM\text{-}CSF}$ and the VV vector JX-594). Currently trials are being conducted in China and Korea with Type 1 CRAds containing respectively the heat shock protein Hsp70 and a relaxin insert encoding a protein that promotes viral tissue penetration (4). Last year the first trial with a tropism modified HAdV vector was initiated in the USA. This vector, AdΔ24-RGD, is used to treat patients with ovarium cancer.

B.4.2 Efficacy and safety of RCVVs so far used in clinical trials

Safety data from clinical trials, when published, may include (see tables IV and V), (4):

- information about occurrence of adverse events including toxicities;
- establishment of MTD and DLT;
- systemic distribution data (PCR, plaque assay on blood);
- shedding data from e.g. injection site, urine, stool, sputum (PCR, plaque assay);
- presence of virus (PCR, EM, plaque assay) in normal versus tumour tissue;
- data about ongoing viral replication (PCR, plaque assay at different time points).

In the next paragraphs we will summarize the most important safety data from published clinical trials. For this purpose vectors have been grouped in E1B deleted (type 1 CRAd) HAdV vectors, type 2 CRAds, conditionally replicating HSV vectors, VV vectors (wildtype plus TK deleted vectors), and wildtype NDV. For other viruses insufficient published data are available. More detailed information can be found in tables IV and V and the accompanying supplementary document (4). Regulatory organizations like the FDA may have additional safety data (for instance data about shedding), but these data are not publicly available (249).

B.4.2.1 E1B deleted vectors; Onyx-015, H101, Ad5CD/TKrep

Toxicity

In a staged clinical research approach, systemic exposure to Onyx-015 was sequentially increased from intratumoural injection via intraperitoneal injection to intra-arterial and intravenous administration. $2x10^{8}$-$2x10^{13}$ viral particles have been administered under various regimes for treatment of several types of tumours. No maximally tolerated dose could be established and symptoms were usually mild. In two cases a DLT was observed that was possibly related to the treatment with Onyx-015 (65, 77). Treatment with E1B deleted vectors also was found to be safe for the patient in combination with chemotherapy, radiotherapy or prodrug administration (19, 87, 250).

Responses

Clinical responses were variable. In most cases <50% of the patients showed a partial response. This percentage appears to improve upon application of combination therapies (table IV)(4).

Biodistribution

In one clinical trial post-mortem tissue samples from a patient were studied 56 hours after the third intravenous dose of Onyx-015; this study showed that the highest levels of Onyx-015 were detected in normal liver and spleen and not in the tumour tissue (90). These sparse data suggest that after systemic administration Onyx-015 may have a widespread tissue distribution.

Replication

Most reports indicate that viral replication is restricted to tumour tissue (61-64). However some studies show evidence that surrounding normal tissue also supports viral replication but there is controversy about whether this surrounding tissue is 'normal' tissue (68, 78-80). Proof for replicative cycles has been shown in a number of studies both after IT and systemic administration (see below).

Systemic virus

Data from various clinical trials indicate that Onyx-015 is rapidly cleared post-infusion, both after intratumour and systemic administration. Systemic reappearance of Onyx-015 was observed in some (63, 67, 84, 86, 87, 89, 90) but not all trials (61, 65, 66, 77). Furthermore these studies indicate that viral replication is short-lived (maximum of 14 days after the last injection) and decreases further at later treatment cycles. No data about the viability of virus shed in the circulation have been presented in any study. Higher levels of detectable circulating viral DNA were observed when Onyx-015 was applied in combination with enbrel (a therapeutic agent that suppresses signalling of the cytokine TNF) in the first cycle of treatment, compared to the second cycle without enbrel treatment, emphasizing the potential influence of combination treatments on viral replication (91).

Shedding data

Shedding data were only presented in few of the studies. In one study urine, bile, ascites and stool shedding data were presented (after intraperitoneal or intra-tumour administration). In this case no shedding of live virus was observed in urine. Viral genomes were detected for up to 5 and 9 days in ascites and bile respectively, but no cythopathic effect (CPE) assay was performed and it is unknown whether later time points were analyzed (78). In a second study, after intra-tumour administration, swabs from the oropharynx and the injection site remained negative at days 0, 8, 15, 22 and 29 post-treatment (61). In a Chinese trial (intratumour administration of H101) no adenoviral DNA was detected by PCR in plasma, urine and swabs from the oropharynx and the injection site (time points unknown) (24, 71).

Data from trials with Ad5-CD/TKrep indicate that viral genomes may be present for prolonged periods in the circulation after intratumour administration. Most patients are negative after two weeks but in some patients genomes are detected at later time points (in one patient for up to 76 days). However, no live virus was recovered at any time point from blood or urine (24, 74, 75).

Transmission
No data reported.
Risk management
In general patients with acute adenoviral infections are excluded. In one trial it was reported that patients were allowed to go home 2 hrs after administration or were kept overnight to guard bleeding from the liver capsule puncture site (82).

B.4.2.2 Type 2 CRAds

Toxicity
Doses up to $1x10^{13}$ vp of CV706 (IT) or $6x10^{12}$ vp of CV787 (IT, IV) were well tolerated. Toxicities were mostly mild and/or temporal (92, 93).
Responses
For both vectors partial responses were observed in some patients.
Biodistribution
In prostatic needle biopsies taken at day 4, 22 and 3 months after CV706 treatment, adenovirus was detected by EM and IHC. Viral hexon staining was confined to prostate epithelial cells only.
Replication
Secondary peaks of circulating genomes indicated ongoing viral replication in both trials.
Systemic virus
After IT administration of CV706, circulating virus was near baseline 12-24h after administration. A second peak of detectable CV706 occurred in most patients 2-8 days after treatment. By day 15, all blood samples were negative. After a single intravenous injection of CV787 circulating genomes were found up to day 29 in 3/23 patients. Later time points were not assessed. Secondary peaks, indicative for viral replication, occurred in 70% of all patients between day 2 and day 8.
Recombination
In plaque assays using cells that are permissive to wildtype HAdV but not to CV706 no plaques were formed, indicating that no wild-type recombinant viruses were present.
Shedding data
After intra-prostate administration of CV706 urine of some patients was positive for live virus (plaque formation), 2 and 8 days post-treatment. By day 15 and 29 all urine samples were negative. The presence of live virus in the urine is likely to be associated with the direct administration in the prostate. After IV administration of CV706 urine remained negative up till 29 days post-treatment (plaque assay). However, saliva samples were positive for live virus in three patients (2/5 and 1/18 receiving $>10^{12}$ and 10^{12} viral particles, respectively) on day 4 or day 8, but were negative on day 15 and 29 (92). Shedding of live adenovirus following systemic exposure (that may also occur following intratumour administration) may take place via the urine, stool and saliva for up to 14 days post-treatment, especially at higher viral doses. In case of intra-prostate administration shedding of live virus from the urine is likely to occur.
Transmission
No data reported.
Risk management
In general patients with acute adenoviral infections are excluded.

B.4.2.3 HSV-1 vectors

Toxicity

Treatment with selectively replicating HSV-1 vectors (G207, NV1020, HSV1716) was found to be safe for the patient after IT administration or injection in the rim of resected tumours (up to $3x10^9$ pfu). Intra-arterial administration of up to 10^8 pfu of NV1020 was well tolerated also in combination with chemotherapy. Toxicities may be higher in HSV-1 seronegative compared to seropositive patients (24, 94-101). In studies with the Oncovex^{GM-CSF} vector a DLT (10^7 pfu IT) was reached upon first treatment in seronegative patients. However in seropositive patients 10^8 pfu was well tolerated (102).

Responses

In all trials in which efficacy was measured partial responses were observed in <50% of patients.

Biodistribution / persistence

Biopsy material indicates that HSV1716 staining was confined to tumour tissue. PCR data indicate that HSV-1 (HSV1716 and G207) may persist for a prolonged period in tumour tissue. Genomes have been recovered up to 251 days post-injection. Biopsies taken 2.5y post-injection in the HSV1716 trial were negative by PCR (95-99).

Replication

Replication of HSV1716 was demonstrated since virus was recovered at a higher titer than the input dose from tumours 5-9 days after injection (95-97).

Systemic virus.

PCR data indicate that after IT administration viral genomes may be present for up to a week (100, 102). However while genomes were detected at day 4 and 6 in case of NV1020, CPE assays were negative indicating that live virus may be rapidly cleared from the circulation (100).

Recombination

In none of the trials reactivation of endogenous HSV was observed or reported in patients that were seropositive at baseline.

Shedding data

The available data suggest that shedding of current HSV-1 vectors may be very limited. No shedding of G207 in saliva was observed 4 days to 1 year after inoculation. Samples of saliva, urine, conjunctival and vaginal secretions were tested by PCR and culture through the course of the NV1020 study. One patient was positive by PCR in one saliva sample taken at day 3. However no live virus could be recovered (99). In the Oncovex^{GM-CSF} study viral DNA was detected by PCR in urine in 2/13 patients between 8 hours and 1 week after injection. Live virus was detected at the tumour surface by plaque assay for up to two weeks indicating that shedding may take place from the tumour surface. No virus could however be detected outside of the occlusive dressing. In a patient with a history of cold sores, lesions of the lip and cheek that developed 48h after injection tested negative for HSV (102).

Transmission

No data reported.

Risk management

In the Oncovex^{GM-CSF} trial injection sites were protected with an occlusive dressing. Patients were discharged if they felt well and if the 24h swab of the dressing and the injected lesion was negative (102).

B.4.2.4 Vaccinia vectors

Toxicity
No significant toxicity was observed in trials in which wildtype Wyeth/NYCBOH strain (103) and TK deleted vectors derived from this strain (VV-IL2) were injected intratumourally (up to 10^8 pfu) (104). IT administration (up to $8x10^7$ pfu after revaccination) of the GM-CSF and lacZ encoding vector JX-594 which is based on the NYCBOH strain (and TK deleted) was also well tolerated (105). Also in several tumour vaccination studies no significant toxicities were reported with GM tumour vaccine vectors based on the Wyeth strain (13, 251).

Responses
Responses were variable ranging from none (VV-IL2) (104) to the good response of patients following treatment with wildtype Wyeth strain against bladder cancer and JX-594 in melanoma (103, 105). Responses in tumour vaccination studies in general have been limited ranging from no response to partial responses in a minority of patients.

Biodistribution / persistence
VV-IL2 mRNA was detected in 4/6 tumour biopsies; expression was highest 1-3 days after injection and detected for up to three weeks. 3/3 patients were positive for GM-CSF by PCR on tumour biopsies taken 18 hours after the first injection of JX-594 and after subsequent injections given in week 5 or week 31 (104). According to a review from Kaufman vaccinia virus is generally cleared from patients within 5–7 days, especially following repeated exposure (252).

Replication
Second waves of viremia have been reported for JX-594 following IT administration (8, 106).

Systemic virus
Systemic presence of virus has been reported for JX-594 following IT administration (8, 106).

Recombination
No data reported.

Shedding data
Culture and PCR on sputum, urine and blood samples indicated that VV-IL2 was not excreted (104). In trials with a live recombinant vaccinia strain (TA-HPV) encoding HPV E6 and E7 oncoproteins as a vaccine for treatment of anogenital neoplasias live virus could be recovered from the vaccination site and the scab and dressings in immediate contact with the vaccination site, but not from the outside of the dressing. No virus was recovered from the throat or genital swabs, suggesting no systemic spreading. In one of these trials, the final scab (4 weeks after vaccination) was found to be positive for live virus in 5/12 patients (107-109). No replication or shedding data were presented in any other report with VV tumour vaccines.

Transmission
Patients and contacts of patients did not show symptoms of VV infection or an increase in VV antibodies in the VV-IL2 study (104). Also in case of the JX-594 trial it was reported that there were no cases of interpersonal transmission but it was not reported how this was established (105).

Risk management
In the JX-594 and TA-HPV trials injection and scarification sites were protected with an occlusive dressing. Moreover in the TA-HPV study, patients in household contact with at risk individuals were excluded from the study.

B.4.2.5 NDV

Toxicity
The MTD of PV701 (IV) was $120x10^9$ pfu. In this trial an initial 'desensitizing dose' of $12x10^9$ pfu was applied. Several grade 3 and 4 events occurred during the first cycles but the adverse events decreased with subsequent dosing. Toxicities are lower upon slow infusion of virus in a two-step desensitization protocol (49, 114).
For the NDV/HUJ strain doses up to 55 billion particles (IV) were well tolerated and no MTD was established (48).
Responses
In the performed trials a partial response was observed in a minority of patients.
Biodistribution
Viral presence in tumours has only been presented by EM (two weeks after administration) or IH (48, 114). No other data are available.
Replication
No data about ongoing replication have been reported.
Systemic virus
Infectious NDV particles were recovered from blood samples during the first dosage cycle and in samples obtained 9 days after the last dosage from the previous cycle (48).
Recombination / Mutation
No data available
Shedding data
Sputum and urine samples have tested positive for live infectious virus. No sputum samples were positive at day 14 of the first cycle or beyond. 3 weeks after the last dose of the first cycle all tested urine samples were negative. Shedding further decreased at later treatment cycles (48, 49, 114).
Transmission
No data available
Risk management
No data available

B.5 Future developments

Genetically modified viruses based on HAdV, HSV, VV, MV and VSV appear to be the most promising candidates for future therapies. Clinical and/or pre-clinical developments with these GM viruses are lying ahead on other viruses. Reovirus is also a promising agent, since incidentally clinical responses were observed in a number of trials in the USA and Canada. Many of the human viruses that are being developed preclinically have a 'safe history of use', but still specific risks may be associated with their application. For instance measles virus and poliovirus are known human pathogens. Although the vaccine strains on which existing MV and polio RCVVs are based on, are considered as safe, risks could still develop because of revertation of the attenuating mutations under certain conditions of use.
Risks may also arise from the genetic modifications that are being introduced. For example in case of large viruses like VV, mutations in genes with an unknown function could lead to variants with an altered tropism. Although it is very unlikely that this may affect the host range of VV (given the strict species specificity of pox viruses) the consequences could be large if such a rare event would occur. Special considerations apply to the use of tropism modified RCVVs. In case of HAdV vectors it is not deemed likely that risks will evolve due to transmission to other species, because of the intracellular blocks for HAdV in non-human cells that are unrelated to extracellular binding. A potential concern may be the tissue specificity of tropism-modified HAdV vectors in

the human host itself. At this moment it is not entirely clear what are the effects of the insertion of the RGD motif on HAdV infectivity (e.g. in endothelial cells) and pathogenicity. The results of the first clinical trial in the USA making use of a tropism modified HAdV vector may clarify this issue soon.
Some of the animal viruses that are being applied have a safe history of use. For instance the application of lentogenic NDV strains appears to be safe since the human population has already been frequently challenged before because of the broad use of these viruses as poultry vaccines. There are however concerns on the use of viruses not known to have been in contact with the human population before. The application of Seneca Valley Virus in a clinical trial may pose a risk since not much experimental data on this virus are available (e.g. the host species is unknown). Before such a virus is injected into human subjects sufficient data on pathogenicity and toxicity from permissive animal models should be available. The application of replication competent retroviruses in clinical trials appears to be unlikely at this moment given the problems associated with their use. These problems include stability problems, the tropism for normal dividing cells and especially the genotoxic effects (leukemia development in immunocompromized persons). Future development of for instance replication competent lentiviral vectors and coronavirus vectors also seems to be unlikely given the negative tags associated with these viruses.
Besides the efficacy and safety of viral vectors many other motives, including commercial ones, play a role in which products will enter future clinical trials. Therefore therapies in which viruses are combined are not expected in the near future. It is more likely that the applied RCVVs will have an increased complexity, but these complex vectors are expected to be tested in humans in a step-by-step approach. It is important to emphasize that more complex vectors may also include many safer vectors.

Appendix C: Current ERA of replication deficient viral vectors in the Netherlands

The first part of the present ERA contains a summary of the data provided by the applicant. These data include data about (A) the parental organism, (B) the genetic modification(s) and a table with an overview of the donor insertions (sequence, organism, location in the vector, function in patient), (C) the (expected) new physiological traits (phenotype) of the GMO, (D) data from previous experiments (clinical and pre-clinical), (E) the intended release or use including its scale, (F) information about containment, control, follow up and treatment of waste and (G) production of the viral vector and information about the batch.

The second part is the environmental risk analysis. For each of the inserted sequences present in the table a separate risk analysis is performed on the influence on characteristics of the vector, taking into account the function of the inserted sequence.
Risks that are evaluated for clinical trials were deducted from Annex II of Directive 2001/18/EC and include points 1-8 of page 18 (point 9 is only important for the ERA of higher species like GM plants). Risks associated with the following mechanisms are evaluated in table format:

-A Persistence and invasiveness (pathogenicity and virulence, infectivity, host range and tropism, replication and transmission);
-B Selective advantages;
-C Transmission to other species;
-D Effects on non-target population (toxicity and allergenicity);
-E Effects on human health;
-F Effects on human health by consumption;
-G Effects on microbial populations;
-H Changes in medical practice.

In general steps F and G are not relevant and therefore not evaluated, for gene therapy products in clinical trials.

The text in the ERA states that the effects on the viral life cycle (A, C and D) are only important if spreading and exposure to the GMO can not be excluded. Indeed this notion has been applied in most of the gene therapy applications received by the GMO Office up to date.

Finally, in part 3 the overall risk of the vector is evaluated compared to the risk of the wildtype vector taking into account the identified risks. Permission of the activities is granted if the overall outcome of the analysis means that the environmental risk is low to negligible.

Appendix D: ERA of the HAdV vector Onyx-015[4]

A) GENERAL INFORMATION ON THE WILDTYPE VIRUS, THE GM REPLICATION COMPETENT VIRUS AND THE INTENDED USE.

1. Information about wildtype virus

1a. General information

Wildtype human adenovirus (HAdV) serotypes 2 and 5 both belong to the HAdV-C species (Mastadenovirus, Adenoviridae). Adenoviruses have a double stranded DNA genome of approximately 36 kb. Most adenoviral gene therapy vectors are based on HAdV-5 or HAdV-2 (34, 253, 254).

1b. Host-range

The host range of HAdV-2 and HAdV-5 is restricted to humans and chimpanzees (34, 60, 253, 254).

1c. Pathogenicity

HAdV-2 and HAdV-5 are human adenoviruses of pathogenicity class 2 (255). HAdV infections are mostly asymptomatic but may cause diseases of the respiratory, ocular and gastro-intestinal system, especially in children. The incubation period for getting disease is 1-10 days. Persistent shedding of adenovirus can take place from lymphoid organs. In young children undergoing bone marrow transfer the consequences of adenoviral persistence can be quite dramatic (60, 256). Most of the human population is sero-positive for adenovirus, and HAdV-C is a widespread species (34, 253, 254).

1d. Biodistribution and persistence

After oral administration in humans HAdV-4, HAdV-7 and HAdV-21 vaccines caused asymptomatic infections in the gut and shedding in the stool was observed for 2-3 weeks. HAdV-2 and HAdV-5 live vaccines administered to humans via the enteric route produced active infection in the gut and pharynx. Live HAdV-4, HAdV-5 and HAdV-7 vaccines administered intranasally/orally to chimpanzees were detected by CPE in the stool for up to 50 days, demonstrating spreading to and replication in the enteric system. *In vivo* experiments in swine indicate that after intravenous injection, wildtype HAdV-5 has a widespread tissue distribution, with high levels found in lung, liver (with ongoing replication) and kidney and low levels in heart, skeletal muscle, brain and gonads (34, 257, 258).

1e. Tissue tropism

The broad tissue tropism of wildtype HAdV-5 observed in tissue culture is mainly determined by mechanisms that determine viral binding and uptake into cells. Entry is mediated by binding of the adenoviral fiber protein to the cellular coxsackievirus and adenovirus receptor (CAR) receptor. CAR is expressed in variable degrees in many human tissues including heart, prostate, pancreas, brain, kidney, liver, and lung. In mice, CAR expression in the liver is more prominent, but otherwise CAR expression is comparable to humans. Internalization and uptake of HAdV-5 is mediated by interaction of the RGD motif of the penton-base protein with cellular integrins and by lysine residues in the fiber shaft that bind to cellular heparin sulphate glycosaminoglycans. Recent

[4] In some European countries, e.g. the U.K., Onyx-015 is not considered to be a GMO. In the Netherlands Onyx-015 is considered to be a GMO since it was generated applying recombinant DNA techniques.

data show that *in vivo* HAdV-5 binds by unknown mechanisms to platelets and plasma proteins resulting in targeting to and clearance by Kupffer cells in the liver. For this uptake process CAR expression is dispensable. To reduce the native *in vivo* tropism of adenovirus it may be necessary to disrupt several adenoviral binding mechanisms (60, 187, 189, 208, 259-261).

1f. Cell lysis and lateral spreading

Adenoviruses cause lysis of cells late during infection, resulting in the release of non-enveloped particles. Several proteins (ADP, E1B-19kD and Ad L3) are involved in this poorly understood process (262).

1g. Horizontal transmission

Adenovirus enters its host via the respiratory tract or the eye through aerosols generated by an infected individual (coughing or sneezing). Adenoviral transmission can also take place by contact with saliva, or via the oral-faecal route. According to the public health agency of Canada the lower limit for infection by inhalation is 150 plaque forming units. A literature review states that 'just a few' viral particles are necessary for adenoviral infection by inhalation. Nonetheless adenoviral infections are usually self-limiting. Studies with live HAdV vaccines have shown that after enteric administration transmission may take place, presumably via the oral-faecal route, and that transmission requires intimate physical contact. Infection of casual contacts after enteric administration is unlikely, even with fully virulent adenoviruses. Besides humans and chimpanzees there is no effective infection of other hosts (34, 253, 254).

1h. Vertical transmission

Adenoviruses are able to integrate in the host cell DNA but no adverse effects are known. Despite considerable efforts scientist have not been able to create transgenic mice by means of direct injection of adenoviruses in the testis (123).

1i. Physical stability

Adenovirus is quite stable; resistance to chemical and physical agents allows for prolonged survival outside of the host. Adenovirus type 3 and type 2 survived at room temperature 10 days on paper and 3-8 weeks on environmental surfaces, respectively (254).

1j. Genetic stability

Genetically modified HAdV-5 can be genetically stable as long as the size does not exceed 105% of the normal HAdV-5 genome. Larger vectors grow poorly and undergo rapid rearrangement, resulting in loss of non-essential DNA sequences, usually the insert (263). Recombination between adenoviruses is thought to play a major role in the evolution of new strains with intermediate or unique immunogenic and tropic properties. There is evidence that recombination can generate hybrids in immunocompromized patients (264).

1k. Availability of anti-viral treatment

The symptoms of HAdV infection can be treated but no specific anti-viral treatment is available (254).

2. Information about the RCVV

2a. General information about the RCVV

Onyx-015 is an HAdV-2/HAdV-5 chimera containing a deletion between nucleotides 2496 and 3323 in the E1B region and a stop codon at position 2022, eliminating E1B-55kD protein expression. Furthermore, Onyx-015 contains the *dl309* mutation, replacing the E3B region genes

10.4, 14.5 and 14.7 with a piece of inert salmon sperm DNA. The insertion is the 3' region of the prolactine II gene. The insertion does not lead to the expression of a functional protein (265, 266).

2b. Role of deleted or mutated genes / sequences

2b1 Role of the first deleted gene: E1B-55kD

The E1B-55kD protein promotes viral replication in normal cells by shut-off of host cell protein synthesis, stimulation of the export of late viral RNAs and prevention of apoptosis by inactivation of p53. Because of the deletion of the E1B-55kD gene replication of Onyx-015 is supposed to be attenuated in normal cells (128). However *in vivo* studies in permissive cotton rats show that dl110, an E1B-55kD deleted HAdV-5 vector, replicated as efficient during the first five days of infection as wildtype HAdV-5 virus in normal lung tissue, but with reduced inflammation of the lungs (267).

2b2 Role of the second deleted gene: E3B genes

Proteins encoded by the E3B region (the 10.4, 14.5 and 14.7kD proteins) prevent infected cells from being killed by the action of apoptosis inducing and immunostimulatory cytokines. E3B encoded proteins mediate the degradation of cell surface receptors including the receptors Fas, Tr1 and Tr2 and the epidermal growth factor receptor (EGFR). Signalling through these receptors activates specific death pathways within the cell. Furthermore, the E3B-RID complex (consisting of the 10.4 and 14.7kD proteins) blocks TNF mediated signalling at various levels preventing TNF mediated inflammation and apoptosis (23, 268, 269). Deletion of E3B genes resulted in accelerated viral clearance in mouse models that was associated with increased TNF and interferon expression and macrophage infiltration (141). In line with this finding E3 deleted vaccines were detected for a shorter time span in chimpanzees (but still for 50 days) and at lower titers compared to vaccines in which the E3 region was present (258). It is important to note that the accelerated clearance of E3 mutants may go concomitant with increased immunologic and pathogenic effects (141, 270). However, deletion of E3B genes, in contrast to deletion of the E3 gp-19kD gene or the entire E3 region, did not result in increased pneumonia compared to wildtype HAdV-5 in cotton rats (270).

2c. Role of inserted transgenes / sequences

The insertion of the 3' region of the salmon prolactine II gene does not lead to the expression of a functional protein (265, 266).

2d. Replication of the RCVV in normal cells

Onyx-015 is able to replicate in tumour cells because of (genetic) defects that result in disruption of the pathways that are counteracted by E1B-55 kD. *In vitro*, in primary epithelial cells and hepatocytes, Onyx-015 replication was attenuated compared to wildtype virus but data indicated a slight replication over time (128). Most reports indicate that *in vivo* viral replication is restricted to tumour tissue (61-64). However some studies show evidence that surrounding normal tissue also supports viral replication but there is controversy about whether this surrounding tissue is 'normal' tissue (68, 79, 80).

2e. Availability of preclinical models for monitoring effectivity, toxicity or biodistribution

Mouse and rat tissues do not support efficient replication of human adenoviruses, but are applied as hosts in tumour xenograft models. Cotton rats, Syrian hamsters and swine are recently published semi-permissive immunocompetent pre-clinical models that may be used to study the effect, toxicity and biodistribution of adenoviruses (126, 257, 271).

2f. Information about systemic distribution after *in vivo* administration

Onyx-015 was able to spread from injected carcinoma xenografts to contralateral non-injected tumours in nude mice (272). Data from various clinical trials indicate that Onyx-015 is rapidly cleared post-infusion, both after intratumour and systemic administration. Systemic reappearance of Onyx-015 was observed in some (63, 67, 84, 86, 87, 89, 90) but not all trials (61, 65, 66, 77). Furthermore these studies indicate that viral replication is short-lived (maximum of 14 days after the last injection) and decreases further at later treatment cycles.
No data about the viability of virus shed in the circulation have been presented in any study.

2g. Information about the biodistribution after *in vivo* administration

Biodistribution of Onyx-015 has not been extensively studied, neither in pre-clinical models nor in the clinic. When nude mice with subcutaneously implanted tumours were given 10^9 pfu of Onyx-015 intravenously, most virus (90%) was detected in liver tissue 3-6 h after injection. However, after 24 h, the titer in the liver had decreased 1000-fold, and virus was undetectable at 72h. In tumour tissue a 150-fold increase in virus titer was observed between 3 and 72 h post- injection. Spread of virus was shown on tissue sections from tumours, but no evidence of viral replication was found within livers at any time point (272). In one clinical trial post-mortem tissue samples from a patient were studied 56 hours after the third intravenous dose of Onyx-015; this study showed that the highest levels of Onyx-015 were detected in normal liver and spleen and not in the tumour tissue (90). These sparse data suggest that after systemic administration Onyx-015 may have a widespread tissue distribution.

2h. Toxicity

In vitro, in normal human cells the cythopathic effects of Onyx-015 are clearly attenuated compared to wildtype HAdV-5. This is not the case for tumour cells (128, 273, 274). Intravenous injection of $5x10^9$ pfu in nude mice resulted in 50% lethality. Livers from both nude and immuno-competent mice that received this dose exhibited severe hepatic necrosis (272). It was estimated that the lethal dose of Onyx-015 in mice ($2x10^{10}$ vp intravenous) would be the equivalent of $2x10^{13}$ vp in humans. However, in various clinical trials with Onyx-015 no maximum tolerated dose could be established (86). Treatment with Onyx-015 was found to be safe both as single treatment and in combination with chemotherapy in (relatively) immuno-compromised patients. Observed toxicities were mostly grade 1/2 flu-like symptoms, liver toxicity and a drop in white blood cell count. More severe toxicities, when observed, were most often related to disease progression or concomitant other therapies. Also after hepatic artery infusion no severe liver toxicity was observed (24, 34, 250). Recent data show that Onyx-015 replicates poorly in human hepatocytes and that CAR is localized at junctions between hepatocytes in the liver and therefore inaccessible to blood flow (275). Differences in CAR expression between mice and humans are likely to cause the difference in liver toxicity (86). Thus, toxicities related to the use of Onyx-015 in humans are of a mild and transient nature.

2i. Environmental shedding

Onyx-015 and comparable E1B-55kD/E3B deleted vectors

Urine, bile, ascites and stool shedding data were presented in only one study (after intraperitoneal or intra-tumour administration). In this case no shedding of live virus was observed in urine. Viral genomes were detected for up to 5 and 9 days in ascites and bile respectively, but no CPE assay was performed and it is unknown whether later time points were analysed (78). In a second study, after intra-tumour administration, swabs from the oropharynx and the injection site remained negative at days 0, 8, 15, 22 and 29 post-treatment (61). In a Chinese trial (intratumour administration of H101) no adenoviral DNA was detected by PCR in plasma, urine and swabs from the oropharynx and the injection site (time points unknown) (24, 71). Data from studies with

a HAdV-5 vector very similar to Onyx-015 (Ad5-CD/TKrep) indicate that viral genomes may be present for prolonged periods in the circulation after intratumour administration. Most patients are negative after two weeks but in some patients genomes are detected at later time points (in one patient for up to 76 days). However, no live virus was recovered at any time point from blood or urine (74, 75).

Data from other adenoviral vectors
After intra-prostate administration of the E3 deleted type 2 Crad CV706, by day 15, all blood samples were negative. Urine of 11/19 and 2/19 patients was positive for live virus (plaque formation), 2 and 8 days post-treatment respectively. By day 15 and 29 all urine samples were negative. The presence of live virus in the urine is likely to be associated with the direct administration in the prostate (92).
CV787 is a second-generation virus with more specificity for target cells and this vector has an intact E3 region. After a single intravenous injection circulating genomes were found until day 29 in 3/23 patients. Urine remained negative up till 29 days post-treatment (plaque assay). However, saliva samples were positive for live virus in three patients (2/5 and 1/18 receiving $>10^{12}$ and 10^{12} viral particles, respectively) on day 4 or day 8, but were negative on day 15 and 29 (93).

Conclusion
Shedding of live adenovirus following systemic exposure (that may also occur following intratumour administration) may take place via the urine, stool and saliva for up to 14 days post-treatment, especially at higher viral doses. In case of intra-prostate administration shedding of live virus from the urine is likely to occur.

2j. Horizontal transmission
No infection of personnel or spread of virus to thirds has been reported in any of the clinical trials but it is unclear if this has been monitored.

3. Overview of the proposed conditions of use

3a. What is the number of patients, and the dose, route, way and timing of administration?
Onyx-015 has been administered at various doses and using various routes of administration. Systemic administration by direct injection into the circulation of the highest administered doses ($>10^{11}$ pfu) is taken as a starting point for the ERA.

3b. What is the immune status of the subjects?
Cancer patients treated with Onyx-015 are in general, compared to healthy individuals, partially immunocompromized.

3c. Which treatments are applied in combination with the viral vector?
Onyx-015 has been used in various treatment regimens for treatment of various tumour types. In all cases Onyx-015 was found to be safe for the patient also in combination with various other treatments that compromise the health status of the individual (see 2h). For this ERA administration of Onyx-015 as standalone treatment is evaluated.

3d Information about the direct environment of the subjects
In this ERA it is assumed that no particular circumstances apply.

3e. Information about GMO production

In this ERA it is assumed that the batch has been generated using GMP standards and that it has passed the minimal requirements of a generally accepted quality system.

B) IDENTIFICATION OF MECHANISMS THAT MAY DIRECTLY OR INDIRECTLY LEAD TO ADVERSE ENVIRONMENTAL EFFECTS.

4. Recombination and mutation of the GM vector in the subject (human host)

4a. Recombinant GMO

Yes. HAdV-5 and other adenoviruses have humans as their natural host. Even in absence of an active infection, various data indicate that HAdV may persist in humans (1c). Moreover, subjects may acquire an adenoviral infection after treatment with Onyx-015. Recombination between different adenoviral strains has been described in immunocompromized patients (1j). The subjects in the studies applying Onyx-015 are relatively immunocompromized (3b). In the present ERA, a batch of Onyx-015, with no contaminants present, is considered to be applied systemically. This will result in a widespread distribution after administration, and repeated cycles of viral replication and systemic shedding may occur (2d, f, g). Thus, recombinant vectors could arise by homologous recombination with wildtype adenoviruses that are present in the same cell. In such recombinant vectors the deletions in the E1B-55kD and/or E3 genes could be stably repaired. This will result in products that will behave either similar to Onyx-015 or wildtype adenovirus, or in products that behave similar to E3B-only or E1B-55kD-only deleted vectors. Although Onyx-015 appears to be attenuated compared to the wildtype adenovirus (2d), this is not necessarily the case for a vector with just one deleted gene. Therefore these partially complemented vector variants should be considered together with Onyx-015 in the next steps of the ERA.

4b. Complemented GMO.

No. See also 4a. In cells that contain Onyx-015 as well as wildtype HAdV, complementation of missing functions in Onyx-015 can take place, since the E1B-55kD and E3 expression products of wildtype HAdV can be used by Onyx-015, which would facilitate its replication in normal cells. These effects may be temporal and self-limiting, since in the absence of wildtype HAdV the functions are not complemented anymore. Moreover, Onyx-015 will behave similar to wildtype adenovirus present in the cells. Therefore it is not necessary to evaluate a complemented GMO at subsequent steps.

4c. Mutated GMO

No. Onyx-015 contains large deletions of the E1B-55kD gene and E3B genes and a small insertion (2a). The likelihood that Onyx-015 is genetically unstable (1j) is therefore negligible. Mutated variants are not considered in the ERA.

5. Do the modifications in the GMOs affect the viral life cycle by changes in virus structure or by non-structural changes in the context of the subject (human host)?

5a. Changes in binding and entry compared to the parental virus

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

Proteins involved in viral entry (1e) are present and expressed in a normal manner in Onyx-015 or recombinants. The E1B-55kD and E3 genes deleted in Onyx-015 or a recombinant do not play a

role in viral entry (2a-b). The likelihood that the modifications will influence viral entry is negligible.

5b. Changes in transcription and translation compared to the parental virus
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Because of the deletion of the E1B-55kD gene, that is involved in promoting late viral mRNA export, viral translation will be attenuated in normal cells (2b). Moreover, deletion of the other functions of the E1B-55kD gene and of E3 genes (2b) in the GMOs is unlikely to positively influence viral transcription and translation in normal cells. Other viral proteins (e.g. E1A) involved in transcription and/or translation are normally present in Onyx-015 or the recombinants. The likelihood that the modifications in Onyx-015 will result in an increased transcription pace or decreased transcription specificity compared to wildtype adenovirus is negligible.

5c. Changes in assembly compared to the parental virus
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
The role of the E1B-55kD gene and E3 genes has been described (2b). These proteins do not encode structural proteins and do not play a role in packaging. The sequences involved in packaging and the genes encoding structural proteins are normally present and expressed in Onyx-015 or recombinants. The likelihood that both or each of the modifications will result in a more effective assembly is negligible. An influence on viral assembly due to more efficient transcription and translation is also unlikely (5b).

5d. Changes in replication compared to the parental virus
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Because of the deletion of the E1B-55kD gene and the deletion of E3 genes (2b) replication of Onyx-015 in normal cells is attenuated. In tumour cells these functions are redundant and therefore replication will be less attenuated as compared to replication in normal cells (2d). Since both modifications contribute to the attenuation also recombinants with one deletion will be attenuated compared to wildtype adenovirus. The likelihood that the modifications in Onyx-015 or recombinants will result in an increased replication pace or decreased specificity compared to wildtype adenovirus is negligible.

5e. Changes in release and cell to cell spreading compared to the parental virus
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Several adenoviral proteins are known to be involved in release and spreading (1f). These proteins are normally present in Onyx-015 or recombinants. The function of the deleted E1B-55kD and E3B genes does not include a known direct role in release and spreading (2b). The likelihood that deletion of these genes will enhance virus release and spreading of Onyx-015 or recombinants compared to wildtype HAdV is negligible.

5f: Changes in cell, tissue and host tropism compared to the parental virus
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
The broad tissue tropism of wildtype HAdV-5 is mainly determined by a number of structural proteins (1e). These proteins are normally present in Onyx-015 and recombinants. The deletion of the E1B-55 kD gene in Onyx-015 narrows down the tropism of this vector compared to HAdV-5 for normal cells causing increased tumour-selective replication (2b and 2d). The deleted E3 genes

do not play a role in the determination of tropism (2b). This, combined with the points already addressed before (5a-e), makes the likelihood that the deletion of either one or both genes in Onyx-015 or recombinants broadens the tropism compared to wildtype HAdV-5 negligible.

5g. Changes in route of transmission compared to the parental virus

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

As indicated above all structural proteins that determine the tissue tropism are normally present in Onyx-015. The E1B deletion and/or E3 deletion even restrict replication in normal cells (2b and 2d). The proteins deleted in Onyx-015 do not play a direct role in transmission (2b). Therefore the likelihood that the route of transmission of Onyx-015 or recombinants is altered compared to wildtype HAdV-C viruses (1g) is negligible.

6. What are the effects of the GMO compared to wildtype virus on the subject (human host)?

6a. Changes in immuno-modulation compared to the parental virus

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

Given the known function of the E3B genes and E1B-55kD genes in down modulation of the immune response (2b) deletion of either or both of these genes is unlikely to result in a decreased immune response against Onyx-015 compared to wildtype AdV. Moreover, these GMOs do not contain insertions that may otherwise influence the immune response and all other viral genes involved are normally present. The likelihood that Onyx-015 or recombinants have an increased viral replication (5d), persistence (7) and shedding (8) compared to wildtype HAdV-5 due to immunomodulation is negligible. An increase in immediate pathogenesis (6e) because of an increased immune response to E3B deleted vectors is also unlikely (2a-b).

6b. Changes in induction of cell death (apoptosis and necrosis) compared to the parental virus

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

Both the E1B-55kD gene and E3B genes are involved in preventing apoptosis of the infected cell (2b). Because of the deletion of both or one of these genes, Onyx-015 or recombinants will induce increased apoptosis in normal cells compared to wildtype HAdV-5. Because apoptosis prevents further viral replication this effect is self-limiting. This is less the case in tumour cells which are redundant in the deleted E1B-55 kD function in preventing apoptosis (2d). Onyx-015 or recombinants do not contain other insertions that influence cell death. The likelihood that increased apoptosis will result in increased viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) is negligible.

6c. Changes in growth factor, cyto-/chemokine signalling compared to the parental virus

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

E3B genes are involved in degradation of cell surface receptors including ‘death’ receptors and blocking of TNF mediated signalling (2b). Therefore deletion of E3B genes results in increased apoptosis in normal cells that will self-limit the infection. Although an indirect role of the E1B-55kD protein on growth factor signalling through for instance cross-talk between intracellular and extracellular signalling routes can not be excluded, the E1B-55kD protein does not play a known

direct role in growth factor signalling (2b). Moreover, deletion of the E1B-55 kD gene results in a virus with a reduced fitness. No other inserts are present that may interfere with signalling (2a). The likelihood that the modifications in Onyx-015 or recombinants will result in increased viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) due to changes in growth factor signalling is negligible.

6d. Changes in cell intrinsic signalling and intracellular homeostasis compared to the parental virus

GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
The E1B-55kD and E3 genes have various roles in disrupting apoptotic pathways that make the cell permissive for productive replication (2b and 2d). All other viral genes are normally present and the vector does not contain insertions that influence intrinsic signalling. The deletion of both or either one of these genes in Onyx-015 or in recombinants is unlikely to result in changes in cell intrinsic signalling and intracellular homeostasis that will induce increased viral replication (5d), pathogenesis (6e), persistence (7) and shedding (8) compared to wildtype HAdV-5.

6e. Pathogenesis compared to the parental virus in the subjects

GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Onyx-015 is attenuated by deletion of the E1B and E3B regions and therefore appears to be less pathogenic than wildtype HAdV-5 (1c and 2h). The interactions of the virus with the host (cell) have been considered at steps 6a-d. No alterations that could influence pathogenesis were identified. Various clinical trials have shown that Onyx-015 is safe in immunocompromized cancer patients (2h). Since both the E1B-55kD and E3 genes contribute to the attenuation of Onyx-015, also recombinant vectors with one of the two deletions repaired will be less or similarly pathogenic compared to wildtype HAdV-5 (2b, 2d and 2h). The likelihood that the deletions in Onyx-015 or recombinants will cause increased pathogenesis compared to wildtype adenovirus is negligible.

6f. Other effects in the host

GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Not applicable.

7. Biodistribution and persistence in the proposed clinical setting

7a. Does the GMO spread to the circulation?

GMO (Onyx-015: E1B-55kD and E3 deleted)
Recombinant GMO (4a: E1B-55kD or E3 deleted)
Yes. Onyx-015 is often administered systemically. Also after intratumour administration cycles of systemic spread of Onyx-015 may occur. After systemic administration Onyx-015 is initially rapidly cleared from the circulation the first-hours post-injection, but can be detected again if replication has occurred at distant sites (2d, f). A recombined GMO will behave more closely to wildtype adenovirus. Experiments in swine indicate that after intravenous administration of wildtype adenovirus cycles of systemic spread occur (1d).

7b. What is known about biodistribution after administration of the GMO?

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

The biodistribution of Onyx-015 or single E3B/E1B-55kD deleted vectors has not been extensively studied, neither in pre-clinical models nor in the clinic (2g). It is therefore assumed that the biodistribution will be equivalent to wildtype adenovirus. Experiments in humans, swine and apes suggest that HAdV-5 have a widespread tissue distribution (1d).

7c. What is known about persistence of the GMO?

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

Genomes of Onyx-015 and similar vectors have been detected in patients long after the last treatment, although no live virus could be recovered. Whether live Onyx-015 could be recovered from organs where adenoviruses may persist is unknown (2f, 2g). Persistence of a partly repaired vector is expected to be attenuated compared to the parental virus, but HAdV vaccines (including E3 deleted vaccines) administered to chimpanzees were still detected for up to 50 days in the stool (1d). Moreover, HAdV-5 may persist and/or become latent in tonsils, adenoids and lungs of infected individuals (1c). It can not be excluded that Onyx-015 or a recombinant persists for a prolonged period after administration to the patient.

8. Can shedding from the subject into the environment occur? For how long and via which ways?

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

Yes. There are no long term-data available about replication and shedding of Onyx-015. Extrapolation of data from other studies with comparable AdV vectors suggest that shedding of live adenovirus may take place via the urine, saliva and possibly the stool for up to 14 days post-treatment, especially at higher viral doses. This is the case in relatively immunocompromized patients that may have a slower (adaptive) immune response (2i). Shedding of a partly repaired vector is expected to be increased compared to Onyx-015 and to be attenuated compared to the parental virus. Live AdV vaccines administered to chimpanzees were detected by CPE for up to 50 days post-administration in the stool (1d). Also data from experimental infections with live HAdV vaccines in humans indicate that adenoviruses are shed for 2-3 weeks (1d). In relatively immuno-compromised patients this period may be even longer.

9. What are the effects of the modifications in the GMO on the stability in the environment?

GMO (Onyx-015: E1B-55kD and E3B deleted)

Recombinant GMO (4a: E1B-55kD or E3B deleted)

The modifications in Onyx-015 or recombinants do not influence the structure of the virus (2b, 2c). Repair of either of these modifications will not influence the stability. Environmental stability of Onyx-015 and recombinant or complemented viruses will be equal to wildtype adenovirus. Adenoviruses, including AdV-C viruses can survive for weeks at room temperature (1i).

10. Can transmission of the GMOs to the human non-target population occur and is further replication and transmission possible?

10a. Is transmission possible to thirds?
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Yes. Although no transmission of Onyx-015 to thirds has been reported in clinical trials (2j) shedding (8) can not be excluded. Previous experiences with adenoviral non-attenuated 'live' vaccines, indicate that infection of thirds may take place after intimate physical contact (1g). Physically the Onyx-015 particle is similar to wildtype virus, thus if shedding occurs transmission may take place. Because of the attenuation of Onyx-015 compared to wildtype HAdV-5 the period that transmission can take place will be approximately 14 days (2i). A GMO that is partially complemented by recombination will behave more similar to wildtype adenovirus. The period that transmission can take place will be extended compared to Onyx-015 (1g).

10b. Is replication possible in thirds?
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Yes. Although replication of Onyx-015 is more efficient in tumour tissue, replication may occur in normal non-target tissue (2d). This is also true, in a higher degree, for a partially complemented recombinant GMO. Data indicate that such vectors may replicate as efficient as wildtype adenovirus in some normal tissues (2b).

10c. Is after infection of thirds further spread possible?
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Yes. Replication in normal tissue (e.g. the pharynx) is possible thus replication and further spread can not be totally excluded (2d). On the other hand spread will be slower than wildtype HAdV-5 and usually self-limiting because of the attenuated properties of Onyx-015 or recombinants. Spreading of complemented variants is more likely than spread of Onyx-015 since these variants are less attenuated or decreased replication selective (2b). Spreading of complemented variants may occur but will be at most similar to spreading of wildtype HAdV-5 (1g).

11. Can vertical transmission occur?
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
No. Like plasmid DNA, adenoviruses are able to integrate in the host cell genome. However, no risks of this ability are known: efforts to generate transgenic mice by means of adenoviral infection were not successful (1h). The modifications (deletions) do not alter these characteristics (2b). Vertical transmission of Onyx-015 or recombinant vectors will not occur.

12. Can transmission to animals / cross species transfer occur?
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
No. HAdV-2 and HAdV-5 have a very limited host-range (1b). Although cells from non-human origin can be infected by adenovirus, infections in most non-human tissues are abortive. The introduced modifications in Onyx-015 do not influence the tropism and host-range of the virus (5f). The likelihood that the host-range is broadened is negligible both for Onyx-015 and the partially repaired recombinant vectors.

13. <u>Can transmission to animals / cross species transfer lead to new (adapted) viral variants by recombination, reassortment or random mutation?</u>

GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
No. Man is the exclusive host for HAdV-2 and HAdV-5 (1b). The introduced modifications in Onyx-015 do not influence the tropism and host-range of the virus (5f). Transmission to animals is not possible (12). The likelihood that the application of Onyx-015 will lead to new variants in animals is negligible.

C) INTEGRAL EVALUATION OF THE PREVIOUSLY ADDRESSED EFFECTS ON THE IDENTIFICATION AND EVALUATION OF POTENTIAL HAZARDS OF THE GMO FOR PUBLIC HEALTH AND THE ENVIRONMENT.

14a Do the modifications in the GMO lead to an increased pathogenicity (including toxic and allergenic effects) compared to the parental virus in the human non-target population?

14a1. Hazard:
Man is the natural host for adenoviruses. Adenoviral infections are endemic and most people are seropostive for anti-adenoviral antibodies. Adenoviral infection is mostly without symptoms, self-limiting and restricted to certain permissive tissues (1c). In case of an increased pathogenicity of the GMO vector the consequences for public health could be large, e.g. by resulting in new pathological phenotypes and new disease manifestations in most of the human population.

14a2. Estimation of likelihood:
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Infection of thirds with the GMO or a recombinant vector can not be excluded (10). However, because of the E1B-55 kD deletion and/or E3B deletion, Onyx-015 (or a recombinant vector) is attenuated or comparable to wildtype HAdV-5 with respect to the viral life cycle and the interaction with the host (5 and 6) and Onyx-015 did not induce severe pathogenic, toxic or allergic effects in immunocompromized patients (6e). The viral load to which non-target populations will be exposed is significantly lower than the dose that the patient receives systemically. The likelihood that the single or combined deletions in Onyx-015 or recombinants lead to an increase in pathogenic, toxic or allergenic effects compared to wildtype HAdV-5 is negligible.

14a3. Risk evaluation:
The risks of increased pathogenicity, toxicity or allergenicity of Onyx-015 or a recombinant vector for the human non-target population are negligible.

14b Are changes in medical practice possible due to the deliberate release of the GMO in the environment?

14b1. Hazard:
Adenoviral infection is mostly without symptoms, self-limiting and restricted to a limited number of tissues. Although there is no specific anti-adenoviral treatment and in most cases treatment is not necessary, the symptoms of adenoviral infection can be efficiently treated. If the pathogenicity and virulence of the GMO vector would be increased compared to wildtype adenoviruses the

consequences for medical practice could be large, e.g. if inserts are present that interfere with the current treatment of adenoviral infections or other diseases.

14b2. Estimation of likelihood:
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
Onyx-015 is attenuated compared to wildtype adenovirus and does not lead to severe disease even in immuno-compromised patients (6e). The likelihood that a variant, in which complementation of E1B-55 kD and E3B deleted functions has occurred by recombination with wildtype adenovirus, has an increased pathogenicity or virulence compared to wildtype virus is also negligible (6e, 14a). The vectors do not contain any inserts that may interfere with medical practice. The likelihood that the application of Onyx-015 will lead to changes in medical practice is negligible.

14b3. Risk evaluation:
The risks of changes in medical practice due to the application of Onyx-015 are negligible.

14c Do the modifications lead to increased pathogenic effects including toxic and allergenic effects in animals? Are there possible immediate and/or delayed effects on the feed/food chain resulting from consumption of the GMO and any product derived from it, if it is intended to be used as animal feed?

14c1. Hazard:
Man is the natural host for HAdV-5. No natural animal reservoirs of HAdV-5 are known. If the modifications in Onyx-015 or a recombinant would lead to an increased host range the effects on animal health could be large if the vector could induce new pathological phenotypes and new disease manifestations in animals. If the animals are meant for consumption, inclusion of toxic inserts could have profound effects on the feed/food chain.

14c2. Estimation of likelihood:
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
The modifications in Onyx-015 or a recombinant do not increase the host range compared to HAdV-5 (5f). Transmission to animals is not possible (12). The likelihood that the application of this vector will lead to increased health problems in animals or effects on the feed/food chain is negligible.

14c3. Risk evaluation:
The risks of the application of Onyx-015 for animal health and the feed/food chain are negligible.

14d Are changes in veterinary practice possible?

14d1. Hazard:
No natural animal reservoirs of HAdV-5 are known. If the modifications in Onyx-015 or a recombinant vector would lead to an increased host range the effects on veterinary practice could be large if the vector can induce novel pathogenic effects or disease manifestations in animals or if the vector contains inserts that may interfere with the treatment of other veterinary diseases.

14d2. Estimation of likelihood:
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)

The modifications in Onyx-015 or a recombinant vector do not increase the host range compared to wildtype HAdV-5. Transmission to animals is not possible (12). The vectors do not contain any inserts that may interfere with veterinary practice (2a-c). The likelihood that the application of this vector will lead to problems in veterinary practice is negligible.

14d3. Risk evaluation:
The risks for veterinary practice are negligible.

14e Are there effects on the population dynamics in the natural environment?

14e1. Hazard:
Man is the natural host for HAdV. Adenoviral infections are endemic in the world and most people are seropostive for anti-adenoviral antibodies. Genetic modifications in HAdV may cause an increased infectivity/tropism and pathogenicity and may affect human population dynamics. In case of an increased host-range and pathogenicity for animals or advantages / disadvantages conferred to animal species due to gene transfer the population dynamics in the environment may be affected.

14e2. Estimation of likelihood:
GMO (Onyx-015: E1B-55kD and E3B deleted)
Recombinant GMO (4a: E1B-55kD or E3B deleted)
The vectors are not able to lead to productive infections in other hosts than humans (1, 5f, 10, 12), do not integrate in the host cell genome (11) and are attenuated compared to the parental virus in their life cycle and interaction with the natural host (5, 6, 14a). The likelihood that the application of Onyx-015 will lead to disturbance of the population dynamics in the natural environment is negligible.

14e3. Risk evaluation
The risks of the application of Onyx-015 on population dynamics are negligible.

15. Risk management

No environmental risks of the GMO or recombinants were identified at step 14. Therefore risk management is not necessary. Spreading of the GMO may be unwanted for other (e.g. political, ethical or social reasons). To prevent spreading of the GMO or recombinants in the environment the following measures could be applied:

- Patients with acute HAdV-5 infection or other viral infections are excluded;
- Masks could be worn by personnel, persons in direct contact and subjects up to 14 days after the last treatment, or until the absence of live virus is proven;
- Urine and stool could be disinfected with chloride for 14 days after the last treatment, or until the absence of live virus is proven;
- Absence of viral shedding can be proven by PCR negativity and in case of PCR positivity by absence of CPE on cultured cells.

16. Determination of overall risk after risk management

No public health or environmental risks of the use of Onyx-015 were identified (14) and no risk management is needed (15). The risks after risk management are similar to the risks described at step 14. The findings of the ERA are summarized in the appended table VII.

Table VII. Overview ERA Onyx-015 and recombinants

	Applied vector	*Human recombinant I*	*Human recombinant II*	*Animal recombinant*
	Onyx-015 compared to wt HAdV-5	ΔE1B-55 kD recombinant compared to wt HAdV-5	ΔE3B recombinant compared to wt HAdV-5	N.A.
5. Viral Life cycle				
5a. Entry	-	-	-	
5b. Transcription / Translation	▼	▼	-	
5c. Assembly	-	-	-	
5d. Replication	▼	▼	▼	
5e. Release / Spreading	-	-	-	
5f. Tropism	▼	▼	-	
5g. Transmission route	-	-	-	
6. Interaction with patient / human host				
6a. Immuno-modulation	▼	▼	▼	
6b. Cell death	▲	▲	▲	
6c. Growth factor signalling	-	-	-	
6d. Homeostasis	-	-	-	
6e. Pathogenesis (normal tissue)	▼	▼	-	
7. Biodistribution	-	-	-	
Persistence	▼	▼	▼	
8. Shedding	▼	▼	▼	
9. Stability	-	-	-	
10.Horizontal transmission	▼	▼	▼	
11.Vertical transmission	N.A.	N.A.	N.A.	
12.Transmission to animals	N.A.	N.A.	N.A.	
14. Risks on Human Health				
14a. Pathogenesis / toxicity	▼	▼	-	
14b. Medical practice	-	-	-	
14. Risks on Animal Health				
14c. Pathogenesis / toxicity	N.A.	N.A.	N.A.	
14c. Food feed chain	N.A.	N.A.	N.A.	
14d. Veterinary Practice	N.A.	N.A.	N.A.	
14. Environmental risks				
14e. Population dynamics	-	-	-	
14. Overall Risk	▼	▼	▼	
15. Risk management	N.A.	N.A.	N.A.	
16. Overall risk after RM	▼	▼	▼	

Symbols: (-) = unchanged, (▼) = decreased, (▲) = increased, N.A. = not applicable

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