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KENMERK	CGM/190905-01
ONDERWERP	Advice 'Generic environmental risk assessment of clinical trials with AAV vectors'

Dear Mrs Van Veldhoven,

In the past COGEM has issued many advisory reports on gene therapy studies with AAV vectors. Armed with this knowledge, COGEM has drawn up a generic risk assessment for clinical applications of these vectors with the aim of streamlining the authorisation procedure for such trials.

Summary:
Hundreds of clinical trials have been carried out worldwide in which use was made of viral vectors derived from adeno-associated viruses (AAV). In recent years COGEM has published a large number of advisory reports on such trials, and in all these trials the risks to human health and the environment proved to be negligible. Drawing on these findings, COGEM has prepared a generic environmental risk assessment for clinical applications of AAV vectors. This generic environmental risk assessment can simplify and streamline the authorisation process.
AAVs are non-pathogenic viruses that can only replicate in the presence of a helper virus. AAV vectors are stripped of all viral genes except the viral sequences at the ends of the genome, the inverted terminal repeats (ITRs). As a result, the vectors are unable to replicate even in the presence of a helper virus. COGEM therefore concludes that, given the characteristics of the AAV vectors used, the risks to human health and the environment posed by clinical trials with these vectors are negligible.
The Commission notes that as AAV vectors used in clinical trials cannot spread further in the environment, there are no environmental risk grounds for refusing applications for these clinical trials if they are conducted under contained use conditions. The attached report contains COGEM's advice on this topic and a discussion of the underlying reasoning.

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Sincerely yours,

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Voorzitter COGEM

c.c. Dr. J. Westra, Head of the GMO Office
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Generic environmental risk assessment of clinical trials with AAV vectors

COGEM advice CGM/190905-01

1. Introduction

Clinical gene therapy studies make much use of vectors derived from adeno-associated virus (AAV). Among the advantages of these vectors compared with other viral vectors is that AAV is not a pathogen, the vectors contain no viral genes and do not replicate in the cell, and it is possible to produce vectors with different tissue tropisms.[1] The first genetically modified AAV vector to be used in a gene therapy study dates from the mid-1990s.[2] Since then, more than 200 clinical trials have been carried out worldwide with AAV vectors.[3] Two gene therapeutics based on AAV have been authorised for placing on the market in the EU.[4,5]

Generic environmental risk assessment

In recent years, COGEM has published a large number of advisory reports on clinical trials with AAV vectors. The therapies and the vectors used display many similarities, and in all cases the risks to human health and the environment were found to be negligible. Drawing on these findings, in this advisory report COGEM presents a generic environmental risk assessment for clinical applications of AAV vectors. The aspects and information relevant to the environmental risk assessment are discussed below.

2. Adeno-associated virus

2.1 Taxonomy of AAV

Adeno-associated viruses belong to the genus *Dependoparvovirus*, which is part of the *Parvovirinae* subfamily within the *Parvoviridae* family. To date, more than 100 AAVs have been isolated from various hosts. According to the current taxonomic classification, more than 20 AAVs have been assigned to the genus *Dependoparvovirus.*[6] They are distributed among six species: *Adeno-associated dependoparvovirus A* (includes, among others, Adeno-associated virus 1, 2, 3, 8 and 9), *Adeno-associated dependoparvovirus B* (includes, among others, Adeno-associated virus 5), *Avian dependoparvovirus 1* (includes the Avian adeno-associated virus), *Chiropteran dependoparvovirus 1* (includes the Bat adeno-associated virus), *Pinniped dependoparvovirus 1* (includes the California sealion adeno-associated virus) and *Squamate dependoparvovirus 1* (includes the Serpentine adeno-associated virus).[6] The other AAVs have not yet been classified.

The various AAV serotypes are represented by a letter/number code (e.g. AAV1, AAV2, AAV5, AAV8, AAV9, AAVrh10). Serotypes are identified by hypervariable structures on the outer surface of the protein coat (capsid) surrounding the virus particle. These structures have antigenic properties and are key contributory factors to host specificity and tissue tropism.[7,8,9]

2.2 Characteristics of AAVs

AAVs can infect almost all vertebrate animals, including humans.[10] Infections with AAVs are frequent and occur worldwide, but as far as is known they are not associated with disease symptoms.[11,12,13] About 95% of the human population have been exposed to AAV2 at one time or another, and 80% are seropositive for this strain.[14] The virus is probably transmitted via the respiratory or gastrointestinal route.[8]

AAVs are small, single-stranded DNA viruses with a genome consisting of about 5 kilobases (kb). The genome is enclosed within a capsid and contains two genes: *rep* and *cap/AAP*.[8] The DNA contains three promoters for the transcription into mRNA. After splicing, the mRNAs are transcribed and various proteins are formed.[8] The *rep* (replication) gene encodes four replicase proteins (Rep78, Rep68, Rep52 and Rep40), which are involved in virus replication, expression of the structural proteins and integration of the viral genome into the host genome. The *cap* (capsid) gene encodes three capsid proteins (VP1, VP2 and VP3), which make up the capsid.[8] 'Nested' within the *cap* gene is an alternative open reading frame (ORF) which encodes the assembly activating protein (AAP).[14,15] AAP is presumed to play a role in the assembly of the capsid. The *rep* and *cap/AAP* genes are flanked by two inverted terminal repeats (ITRs). These contain the origins of DNA replication and serve as the packaging signal, and are involved in the integration of the viral DNA into the host chromosome.[10]

AAVs differ from the other viruses in the genus *Dependoparvovirus* in that they are non-autonomous (replication-deficient) and depend for replication on the assistance of a helper virus, such as adenoviruses or herpesviruses.[10,16,17] Without the presence of a helper virus the viral genome will not replicate, resulting in a persistent 'infection' in which the AAV genome remains latent in the cell nucleus as an intrachromosomal or extrachromosomal episome.[10,14] Only about 0.1% of AAV2 virus particles are integrated into the genome of the host (humans and great apes).[18,19] Integration of the AAV2 genome occurs specifically at the AAVS1 site in the long (q) arm of chromosome 19.[20] *Dependoparvovirus* species that lack the ability to self-replicate (non-autonomous) can be identified by their names, because they include the term 'adeno-associated virus'. *Anseriform dependoparvovirus 1* and *Squamate dependoparvovirus 2* are the only virus species within the genus *Dependoparvorvirus* that can replicate independently and do not need the assistance of a helper virus.[10,16] The names of viruses within these species do not contain 'adeno-associated virus', but the term 'parvovirus'. *Anseriform dependoparvovirus 1* contains the bird viruses Duck parvovirus, Goose parvovirus and Goose parvovirus-PT. *Squamate dependoparvovirus 2* contains the lizard virus Bearded dragon parvovirus.[6]

3. AAV vectors

3.1 Characteristics of AAV vectors

AAV vectors are made by removing the *rep* and *cap/AAP* genes and replacing them with a transgene expression cassette composed of the target gene and regulatory sequences, such as promoters and terminators. The expression cassette is inserted between the ITRs. During the production of the vector

the AAV *rep* and *cap/AAP* genes are delivered *in trans* and the vector genome is enclosed within the capsid proteins expressed *in trans*.

AAV vectors used in gene therapies are usually derived from AAV2; in other words, they possess the AAV2 ITRs.[21] The capsid proteins may be from another AAV serotype, which is indicated by AAV2/n, where n stands for the AAV serotype of origin.[22] Modifications can be made to the capsid proteins, or capsids can be modified by exchanging parts from different serotypes, in order to influence cellular tropism and intracellular expression of the transgene or to resist neutralising antibodies.[22,23,24] Some AAV vectors (self-complementary (sc-) AAV vectors) contain mutations in the 'left-hand' ITR sequence. In the production cell line hairpin forming and self-annealing lead to the generation of a double-stranded (ds) DNA molecule, which is packaged within the vector particle. In the cells of the patient this ds-DNA facilitates the transcription of the transgene.[25] AAV vectors have a limited cloning capacity; the maximum packaging size within the AAV capsid is 5.2 kilobases.[26] Dual vector systems have been developed to expand the cloning capacity of AAV vectors.[27,28]

3.2 Production systems for clinical applications

The production of AAV vectors requires the assistance of a helper virus, but most production systems do not make use of fully virulent helper virus strains. The most commonly used systems consist of specific cell lines transfected with the plasmid containing the vector genome (the vector plasmid) and one, or more usually, multiple helper plasmids. They contain the AAV *rep* and *cap/AAP* genes and the required adenovirus helper genes.[22,29] Some production systems make use of a replication-deficient adenovirus in combination with HEK293 or HeLa cell lines. In these systems the relevant adenoviral genes are integrated into the chromosomes of the cells. There are other systems as well, such as those that make use of recombinant herpesviruses or baculoviruses.[30] Baculoviruses infect insect cells, but cannot infect mammals.

3.3 Vector batches

During the production of an AAV vector, heterologous sequences (e.g. from cellular or plasmid DNA) may be packaged. It is known that in vector batches 1:1000 to 1:100 vector particles contain heterologous sequences (unintended and not related to the vector).[31,32,33,34] It is also possible that recombination between the vector plasmid and helper plasmids during the production process can lead to the formation of replication-competent AAV (rcAAV)[1] or pseudo-wild-type AAV (virus particles containing fragments of the AAV genome).[35,36] Production systems have been developed that prevent this from occurring.[31,33,36,37,38] Vector batches also contain some 'empty' virus particles.[22,32,33,34]
After production, the virus batches are purified to remove unpackaged nucleic acids (vector genome, plasmid DNA, cellular DNA, RNA) and empty capsids.[29,30,39] If a fully virulent virus is used in the production process, it is inactivated or removed and the batch is checked to ensure no virulent virus is present.[29,33,40,41,42]

[1] The term 'replication-competent AAV' is used to indicate that it is similar to wild-type AAV. However, AAV is unable to replicate on its own.

4. Previous COGEM advisory reports

In 2018, COGEM advised to assign all AAVs belonging to the species *Adeno-associated dependoparvovirus A* and *Adeno-associated dependoparvovirus B*, and AAVrh10 and AAVpo1 to the lowest pathogenicity class (pathogenicity class 1), as they are apathogenic.[43] AAVrh10 and AAVpo1 were mentioned separately because these viruses have not yet been taxonomically classified by the ICTV.[6]

Since 2005 COGEM has issued nine advisory reports on the possible risks to human health and the environment resulting from clinical trials with AAV vectors for the treatment of metabolic diseases,[44,45,46] heart failure,[47] haemophilia,[48,49,50] rheumatism[51] and spinal muscular atrophy.[52] Different production systems were used to make the vectors; an HEK293 cell line in combination with a vector plasmid and one or two helper plasmids,[44,45,46,51,44] an HeLa S3 cell line in combination with a vector/packaging plasmid and wild-type adenovirus (HAdV5),[48,50] and insect cell line Sf9 in combination with three different recombinant baculoviruses.[48,49] The vector genomes all contained AAV2 ITRs, however they were packaged in different capsids (from AAV1, AAV5, AAV8, AAV9 and AAVhu37). The doses varied from $1x10^{11}$ to $1.1x10^{14}$ vector genome copies per kg body weight (vg/kg).[44,44] The vector was parenterally administered into muscle tissue, joints or blood vessels.

COGEM was of the opinion that in all these trials the risks to human health and the environment were negligible and advised the use of some additional protocols or approved the protocols proposed by the applicant. In its latest advisory reports, COGEM noted that the risk of germline transmission of AAV vectors is negligible and consequently the use of effective contraception is not necessary.[45,50] The Commission was also of the opinion that the instruction prohibiting the use of the AAV vector when there are clinical indications of an active viral infection, is not necessary from an *environmental risk* perspective.[45,49,50]

5. Considerations regarding the environmental risk assessment

The main concern when carrying out environmental risk assessments of clinical trials is whether people or animals not taking part in the trial ('third parties') can be infected with the AAV vector, or sequences derived from it, and suffer adverse effects as a consequence. The potential environmental risks are related to the pathogenicity of the vector, the distribution of the vector in the environment, and the possibility of the formation of new recombinant viruses. The basis for this advisory report is supported by a more detailed discussion of these aspects below.

5.1 Pathogenicity of an AAV vector

Pathogenicity of wild-type AAV

COGEM has assigned the apathogenic species *Adeno-associated dependoparvovirus A* and *Adeno-associated dependoparvovirus B* to pathogenicity class 1.[43]

Potential adverse effects

Recently a serious effect was reported during a clinical trial with an AAV vector. It concerned the death of a patient (a baby) with spinal muscular atrophy during an ongoing phase 3 trial.[53,54,55,56] Although in the first instance the manufacturer reported that administering the AAV vector may have contributed to the baby's death, research later showed that the death was not caused by the treatment.[57] The gene therapeutic has now been approved by the U.S. Food and Drug Administration (FDA) for use in the United States.[58]

COGEM observes that in trials with AAV vectors the clinical pictures can be complex and that some illnesses are associated with high mortality rates. Serious side-effects from the use of AAV vectors in patients already in a poor state of health therefore cannot be ruled out.

In 2018, a vector with the same transgene expression cassette as in the phase 3 trial mentioned above, but with a slightly different capsid protein, was reported to have caused serious toxic responses in non-human primates and piglets.[59] The study involved tests on a small number of animals (three monkeys and three piglets). No clear picture of a causal connection with the adverse effects has yet emerged in the literature. COGEM notes that the vector batch may have been contaminated. It is also possible that the expressed protein was toxic, because it was a human-specific protein and not an endogenous protein.[59,60]

In a number of clinical trials with AAV vectors, patients have been found to have raised liver enzyme levels in their bloodstream, but without any serious adverse effects.[61,62,63,64,65] Patients with very high levels were treated with corticosteroids (prednisone).

Integration of the vector genome into the genome of the test subject or laboratory animal

Targeted integration of AAV into the host genome depends on the presence of the Rep proteins, especially Rep78/68.[66] Because the viral genes are absent, the probability of integration of the AAV vector into the specific integration site in the host genome is negligible. Incidental integration of recombinant AAV at random sites into the genome may occur, particularly during in vitro experiments with cell lines.[67] It has been reported in the literature that in mice AAV vectors have been successfully integrated at various sites in the genome.[14,22] In two studies it was reported that in newborn mice this resulted in the development of hepatocellular carcinoma.[68,69] The results indicate a correlation with the administered vector dose and the integration site in the mouse genome (*Rian* locus) and a possible relation with the nature of the promoter used in the transgene expression cassette.[22,69] COGEM notes that AAV vectors do not integrate efficiently into the genome, but mainly persist in the cell nucleus as episomes, such as concatemers.[10,70,71,72] As a result they do not persist in actively dividing cells, which reduces the chance of integration.

More than 200 clinical trials have been carried out worldwide with AAV vectors.[3] Much research has also been done in large mammals (non-human primates, dogs).[22] Of all these studies, just one mentions the integration of the vector genome into the DNA of a patient, who was being treated for lipoprotein

lipase deficiency. The vector genome was randomly integrated into the nuclear and mitochondrial DNA.[73] To COGEM's knowledge, no reports have been made of the administration of AAV vectors in large mammals or humans leading to tumour formation.

5.2 Replication, shedding and distribution

Replication
Wild-type AAVs are dependent on a helper virus for replication.[8,10] However, even in the presence of a helper virus, the absence of the Rep and Cap proteins will prevent an AAV vector from replicating. Therefore, AAV vectors are biologically contained.

Shedding and distribution
After a vector is administered, it will spread to various tissues and organs in the body and will be shed over time. AAV vectors can be shed via blood, faeces, semen and urine.[74,75] The amount of vector that is shed is dose-dependent and declines gradually over time, partly because the patient develops neutralising antibodies against the capsid.[61,62,76,77] In a clinical trial with a vector based on AAV2/5 ($6x10^{13}$ vg/kg body weight) the vector was shed via semen, saliva and faeces until week 52. However, COGEM notes that when vector DNA is found in body fluids or tissues, for example by PCR analysis, this does not necessarily mean that the vector is infectious. AAV vectors shed to the environment are biologically contained and will not spread further. COGEM therefore considers that the environmental risks resulting from AAV vector shedding are negligible.

With respect to the shedding of vectors via semen, COGEM notes that AAV vectors found in the semen of the two patients in week 52 (see above)[64] were not present within sperm cells. In a clinical trial with an AAV2 vector, further study of the motile fraction of the semen showed that it did not contain any vector sequences.[78] This finding is supported by an in vivo study on rabbits in which vector sequences were found mainly in the seminal fluid and not in the cellular fraction.[79] This study also showed that after four days no infectious vector particles were detectable in semen and that over time no vector genomes could be detected in newly formed sperm cells. Studies in which AAV vectors with different capsids were administered have shown that no germline transmission takes place.[80,81] COGEM points out that wild-type AAV persists in latent form in certain lymphocytes and therefore may be present in the cellular fraction of semen.[82,83]

Based on the above information, COGEM concludes that the presence of (the genome of) AAV vectors can be demonstrated in semen for several weeks to several months. This is a temporary phenomenon. Moreover, the vector genome is present in the seminal fluid but not in sperm cells. COGEM therefore considers the chance of germline transmission as a result of treatment with an AAV vector to be negligible.

5.3 Recombination and complementation

A viral vector could possibly be mobilised, either during production or after administration to the patient. By complementation or recombination replication-competent viruses could also be formed.

AAV vector batches may contain low levels of plasmid DNA or host cell DNA sequences in the end-product (see section 3.3). COGEM is of the opinion that these heterologous sequences do not lead to risks to human health and the environment, because they do not provide a selective advantage and cannot replicate or spread further.

Should, during the production process, recombination occur between sequences of the *rep* and *cap/AAP* containing helper plasmids and vector sequences of the vector plasmid to form rcAAV, COGEM is of the opinion that the risks to human health and the environment would be negligible, because the recombination would not lead to a rcAAV containing transgenes. In this case, the rcAAV virus would be similar to the apathogenic wild-type AAV.

After administering the AAV vector to the patient, it is theoretically possible that in the presence of wild-type AAV and a helper virus, homologous recombination or complementation could occur. However, the probability of the vector, a wild-type AAV and a helper virus all being present in the same cell is very small to negligible.

Complementation in the test subject or patient could lead to the formation of new vector particles, because the vector genome would be replicated and packaged within the proteins of the wild-type AAV. As the enclosed vector would not have the *rep* and *cap/AAP* genes, it would still be biologically contained. In vivo mobilisation of AAV vectors through superinfection by wild-type AAV and adenovirus has been shown to be possible, to a certain extent, under laboratory conditions,[84] but to COGEM's knowledge, neither mobilisation of the vector nor recombination of AAV vectors has ever been reported in medical or veterinary applications. In the light of the above, COGEM is of the opinion that the environmental risk resulting from complementation is negligible.

In the theoretical case that recombination does occur between vector ITRs and wild-type AAV, the *rep* and *cap/AAP* sequences of the wild-type AAV will be exchanged with the vector expression cassette. The resulting viruses would not be new, but, again, would be the vector or apathogenic wild-type AAV. COGEM therefore considers that the risks resulting from recombination with wild-type AAV are negligible.

It is also theoretically possible that the AAV vector genome could recombine with the wild-type AAV genome to create a hybrid genome. COGEM points out that the packing capacity of AAV vectors is limited – a virus particle can contain no more than 5.2 kb[26] – and therefore considers that the probability of a virus with a hybrid gene being created is negligible.

5.4 Conclusions

Based on the above considerations, COGEM comes to the following conclusions:

- AAV vectors are attenuated and biologically contained, and because they are replication-deficient they cannot disperse through the environment; this is true irrespective of the AAV species or serotype from which the vector is derived.
- Occasionally observed incidental adverse effects following administration of AAV vectors are restricted to the patient concerned; these effects do not lead to a higher environmental risk.
- After AAV vectors are administered they can be shed, but because of their biological containment further distribution is not possible.
- The probability of AAV vectors being transmitted via the germline is negligible.
- Should an AAV vector combine with a wild-type AAV, or a wild-type AAV complement an AAV vector, the risks to human health and the environment will be negligible.

6. Other elements of significance for the environmental risk assessment

6.1 Molecular characterisation

In 2013 COGEM issued generic advice on the genetic characterisation of GMOs for clinical applications.[85] Consistent with this advice, COGEM stresses the importance of mapping the whole nucleotide sequence of the vector genome when producing molecular characterisations of AAV vectors and verifying these sequences by comparison with the intended product by means of an alignment. If this requirement is met, COGEM is of the opinion that a conclusive molecular characterisation of the AAV vector will have been obtained.[2]

6.2 Organ or tissue donation

AAV vectors are able to persist for long periods of time in body cells in the form of concatemers.[10,70,71,72] COGEM notes that each cell division dilutes the amount of vector in the cells, but that in the liver, for example, this is a slow process. This persistence means that the vector can be unintentionally transmitted to third parties via blood, tissue or organ donation. COGEM observes that consideration should be given to the advisability of patients being donors. COGEM underlines the importance of organisations involved with the assessment and implementation of clinical trials coming together to discuss how best to deal with the potential risks of this form of transmission, and to discuss which organisations are responsible.

7. Advice

Based on the above arguments, COGEM concludes that it is possible to carry out a generic environmental risk assessment for clinical applications involving AAV vectors. Because of the replication-deficient nature of these vectors and the fact that only AAV ITRs remain present, COGEM

[2] COGEM assumes that all activities involved in producing the vector batch follow Good Manufacturing Practice (GMP) and that requirements such as those in the European Pharmacopoeia to ensure the identity, purity and sterility of the vector batch are met.

is of the opinion that the risks to human health and the environment in clinical trials with AAV vectors are negligible, as long as a number of conditions are met. In such trials COGEM advises complying with the following conditions:

- The ITRs and capsid proteins should be derived from AAVs; in other words, they should be from viruses which are dependent on a helper virus for in vivo replication and belong to the genus *Dependoparvovirus* (such as *Adeno-associated dependoparvovirus A*, *Adeno-associated dependoparvovirus B*, *Avian dependoparvovirus 1*, *Chiropteran dependparvovirus 1*, *Pinniped dependoparvovirus 1* and *Squamate dependoparvovirus 1*).
- If a helper virus is used in the production of the vector, this should be inactivated or removed afterwards.
- The whole nucleotide sequence of the AAV vector should be determined and verified by comparing it with the nucleotide sequence of the intended vector.

8. Observations

This advisory report sets out a generic environmental risk assessment for use in clinical trials with AAV vectors. COGEM notes that AAV vectors are replication-deficient and that once administered to the patient they cannot spread further in the environment.

The written response from the Minister of Infrastructure and Water Management[86] to the contribution by the House of Representatives during the General Consultation on Biotechnology and Plant Breeders' Rights on 25 April 2019, and the accompanying appendix,[87] point out that in the Netherlands consent may be granted for gene therapy under contained use, on the condition that conclusive evidence is provided showing that the GMO used cannot be released into the environment and that therefore no risks to human health and the environment can arise.

COGEM observes that as AAV vectors used in clinical trials cannot spread further in the environment, these trials may be considered for authorisation under contained use. This would mean that the authorisation procedure for such trials could be made considerably shorter.

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