Application form

Assessment of clinical research involving gene therapeutics in the Netherlands

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Viral vectors

September 2017

Application form
Assessment of clinical study involving genetically modified viruses

Part A: Bio-safety aspects

- A1. General information (Public section)
- A2. Construction and composition (Public section)
- A3. Production (Public section)
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If you have any questions, please get in touch with the Gene Therapy Office (E-mail: rik.bleijs@rivm.nl, phone: +31-30-2747569).

September 2017

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Part A. Bio-safety aspects

This part of the application form provides the information needed for the Ministry of Infrastructure and Environment (IenM) to grant the necessary licenses.

All information provided in this form and the accompanying documentation constitutes part of the decision to be made and for this reason is in principle open to inspection by the public; the information will also be available for such public inspection during the procedure.

The applicant may ask for parts of the information provided to be kept confidential. In that case, the applicant must give reasons why the information is of a confidential nature as well as a convincing explanation that the lifting of confidentiality will adversely affect the applicant's competitive position. A publicly available summary of confidential information must be given, containing enough information for a clear general understanding of the application and in order to assess the risk analysis as described in the application and the decision. Confidential information must be included in a separate attachment marked 'confidential'.

An application does not need to be limited to the specific clinical protocol that the applicant wishes to perform. If there are no consequences for the risk analysis, the application can be drawn up with a wider scope, such as for a larger number of patients.

The aim is to draw up the final decision in such a way that several clinical protocols can be performed under it, using the information described in this application. Naturally these activities must be covered by the description of the experiment and the risk analysis provided. Before submitting such a broader application, you are advised to contact the GMO office for an informal discussion of the options.

The term 'test subjects' as used in this form means patients or volunteers taking part in the study.

Specific personal details of the contact person and the environmental safety officer are to be submitted by entering the required information under Section A7, General (personal) information. The submitted data in this appendix will be kept confidential and will thus not be made publicly available in accordance with the Personal Data Protection Act.

This Notification Form may contain some questions that are not relevant to your case. We would ask you NOT to answer in your notification any questions that are irrelevant to the activities for which you are applying.

Specific issues:

- Literature that is referred to has to be sent in together with the application form.
- Confidential information has to be marked as such and has to be sent in separately.
- A SNIF B (other GMO) form has to be completed and to be sent in as an electronic file in Word format.

A1. General application details

General information

A1.1. Application title:

Please give a descriptive title that provides sufficient information on the objectives and aspects relating to the genetic modification. The title should state the type of GMO(s) and insertion(s) used and the nature of the application(s).

Open-label, single-dose, multi-centre trial investigating an adeno-associated viral vector containing a codon-optimized Padua derivative of human factor IX gene (AAV5-hFIXco-Padua, AMT-061) administered to adult patients with severe or moderately-severe hemophilia B.

A1.2. The objective of the study for which the application is submitted:

Provide a short description of the study's objective. For example: 'The objective of this Phase I clinical trial is to study the safety of the oncolytic adenovirus for the purpose of developing a new therapy to treat skin cancer.'

The general purpose of the work is to further establish an AAV-based, liver directed gene therapy approach for treatment of haemophilia B. In general terms the primary objective will be to assess safety and efficacy of a single intravenous infusion of AMT-061 in adult patients with severe (Factor IX (FIX) activity ≤1% of normal) or moderately severe (1<FIX activity ≤2% of normal) haemophilia B.

A1.3. Describe briefly the contents of the application, the objective of the study being aimed for and the intended application of the results

Provide a short description – of no more than half a page – of the genetically modified organisms (GMOs) applied, or of the GMOs that may thus be created, such as via recombination of genetic information between organisms or by integration of genetic material into a genome. Describe the expected action of the gene products of the transgenes and give an explanation of the biological mechanism. Also describe the scientific and public importance of the study, and state how the results of the study may be used in this context.
The information provided will be used as the basis for a brief description of the study in the decision.

The investigational medicinal product is indicated to treat adults aged 18 years or older with severe or moderately-severe hemophilia B with a FIX activity ≤ 2%.
By establishing the continuous endogenous expression of an stable FIX protein levels, the treatment is aimed at modifying the disease phenotype from severe or moderately-severe to moderate or mild or even complete amelioration, thus decreasing annualized bleeding rate (ABR) total FIX usage, including the discontinuation of continuous routine prophylaxis with FIX products.
Congenital hemophilia B is an inherited bleeding disorder characterized by an increased bleeding tendency due to either a partial or complete deficiency of the essential blood coagulation FIX.
Hemophilia care is based on treatment of active bleeding with clotting factor concentrates as needed (on-demand use) or prevention of bleeding with routine prophylactic use of clotting factor concentrates (prophylaxis).
Hemophilia B has been recognized as a target for gene therapy since the early 1990's and a single delivery of the FIX gene to the target organ has the potential to result in sustained therapeutic FIX plasma levels (i.e., ≥ 1% of normal levels) for several years (Nathwani 2011b; Nathwani 2014).

AMT-060 has been evaluated in a Phase I/II clinical trial (IM 05-001/00, IM-MV 14-006/00, IM-MV 14-007/00, IM-MV 14-008/00, IM-MV 15-001/00) to investigate its safety and efficacy in adults with severe or moderately-severe hemophilia B (FIX ≤ 2%).

In an attempt to achieve higher circulating FIX activity levels with the goal of alleviating the need for all exogenous therapy, the Sponsor introduced a design modification by modifying the wild type FIX coding sequence within the AMT-060 vector genome to encode the naturally occurring Padua FIX gain-of-function variant, resulting in AAV5-hFIXco-Padua (AMT-061). The FIX-Padua protein differs from the 'wild type' human FIX protein by a single amino acid; where Arginine is replaced by Leucine at position 338 (R338L) of the mature protein.

This enhanced vector would mediate higher circulating FIX activity levels at the same dose, with the same safety profile whereas the same AAV5 vector is deployed resulting in comparable FIX protein levels, as compared to its predecessor AMT-060.

The Sponsor is introducing this design modification in the form of AMT-061 into the planned further development program for hemophilia B..

A1.4. Describe briefly the intended work.

State in chronological order which types of procedures will be carried out, and for which a license is being applied for (e.g. production, transport, storage and administration of the vector, observation of patients, sampling, transport, storage and processing of samples, waste treatment). Where applicable, also indicate for which part of the study a Contained Use (IG) permit applies, and provide the number of the GMO permit concerned.

The following activities pertaining to clinical evaluation of AMT-061 are within the scope of the GMO application:

- Receipt and storage
- Investigational Medicinal Product preparation
- Transportation within the hospital
- Administration and observation of patients
- Patient sampling; sample processing, analysis, and storage
- Waste management and disposal of unused materials

The scope of the application excludes manufacturing of AMT-061, transport of AMT-061 to and from the hospital, and transport of biological samples from the hospital to an external central laboratory.

The activities to be carried out under the scope of the GMO application for AMT-061 are described below.

Receipt and Storage

The genetically modified organism (GMO), AMT-061, is manufactured by uniQure Inc. in compliance with current Good Manufacturing Practices. Manufacturing facilities are in Lexington USA. The manufacturing is not within the scope of the GMO application. The hospital pharmacy, or equivalent, will receive the product. Storage will be in a facility with restricted access using equipment that is labeled in accordance with GMO Regulation (annex 9.3.3.2).

Investigational Medicinal Product preparation

The dosage form is a solution for intravenous administration. The drug product is formulated as a sterile solution at a concentration of $1x10^{13}$ vector genome copies per mL (gc/mL). In the hospital pharmacy, or equivalent, AMT-061 will be pulled from the supplied 10 mL vials into an infusion bag in a Class II Biological Safety Cabinet.

Transportation within the hospital

The AMT-061 infusion bag will be transported to the treatment room in accordance with GMO Regulation (annex 1).

Administration and Observation of patients

Treatment of patients will occur in a hospital environment without any additional precautionary or containment measures. Administration and monitoring of the patient occur in a patient treatment room.
AMT-061 will be administered to the patient as an intravenous infusion. Observation of the patients is done by means of post-administration surveillance. Note that the post administration surveillance is not related to any anticipated environmental risk. Subjects will be followed with respect to safety and efficacy parameters for at least 5 years.

Patient sampling; sample processing, analysis and storage

Biosampling will be performed regularly for study purposes, and as needed for routine patient care purposes.
Sampling and transportation of the samples, as well as processing and/or storage of samples will be performed within the hospital in accordance with GMO Regulation (annex 1 and 9.3.3.2). Samples that are not analysed at the hospital will be transported to the external central laboratory in accordance with the GMO Regulation (annex 1). Transportation of patient samples to, and analysis of patient samples at the external central laboratory are not within the scope of the current application.

Waste treatment and disposal of unused material

Following handling and administration of AMT-061, the unused product remaining in the opened vials and all the materials that have been in contact with the GMO (such as gloves, syringes, needles, tubing and infusion bag) will be disposed of in accordance with GMO Regulation (annex 9.3.3.2). Unused (non-opened) vials of AMT-061 will be returned to uniQure or disposed per study procedures, in accordance with GMO Regulation (annex 1 for transport; annex 9.3.3.2 for waste).

A1.5. Intended start and end date:

The decision must state a time period within which the procedures will be carried out, and so an end date must be given. The chosen end date will be included in the decision. It is possible to obtain an extension to the decision; please note, however, that any extension procedure must be completed before the decision end date has passed.

First Patient Dosed (estimated): Q4-2018
Last Patient Last Visit (estimated): Q4-2030

A.1.6. Do you want to keep other information confidential? If so, please specify in concrete terms how the release of the information would harm your competitive position.

Unless marked "Confidential", all the information contained in the notification and its appendices may enter the public domain when the notification is publicly processed and the decision is published.
For the sections marked "Confidential", you are requested to give a publishable summary that contains enough information to ensure a good general understanding of the notification. Furthermore, give a reason why certain information is marked "Confidential".

Specific confidential details on these sections are included in A.7 marked confidential.

The information in A.7 as well as the indicated pre-clinical reports must remain confidential as it includes or is based on uniQure proprietary know-how (i.e. tangible and intangible techniques, methods, knowledge, results and other information) onto which the company's R&D capabilities and more specifically manufacturing expertise are built.

These sections include specific details about methodology used that has been carefully developed in-house over the past decade, as well as the description of key assays and study data that could potentially be exploited by other commercial organizations working in the same field. Since our manufacturing platform comprises a modular system that is applied to all uniQure development products and was the first gene therapy that received approval by the European Medicines Agency (EMA) (EMA/CHMP/474664/2012), there may also be wider negative implications for the company's competitive position in the area of AAV gene therapy.

Furthermore, we intend that some of these results described are published in a scientific journal as original research and therefore disclosing them would invalidate that.

Details of applicant

Only the legal entity that has final responsibility for the work to be carried out may act as the applicant. This means that the applicant will normally be the Board (management) of the hospital (institution) where the treatment will be given. The license holder must be able to enforce compliance with the license regulations when carrying out the work. In order to do so, it is necessary for the employees involved in the clinical procedures to come under the authority of the license holder. For this reason, employees must be directly employed by the license holder. In those cases where an employee does not come under the authority of the license holder, such as where a treating doctor is part of a partnership that is independent of the license holder, an employment contract must be arranged for carrying out work under the license, such as through a zero-hours contract with the license holder. A contract must be concluded with the party or parties carrying out this work for non-clinical procedures that are not carried out in the institution in question, in such a way that final responsibility continues to rest with the license holder.

A1.7. Name of legal entity:
Erasmus MC

A1.8. Chamber of Commerce (KvK) number:
24485070

A1.9. Visiting address of legal entity:
Wytemaweg 80

A1.10. Postal code and town/city of location of legal entity:
3015 CN Rotterdam, The Netherlands

A1.11. At which locations will the intended work take place?
Since the work applied for may only be carried out under the direct control of the license holder, it is only possible to carry out work at several locations if the license holder has full control of the way in which the work being applied for is carried out at all locations, in such a way that the license conditions are complied with. In that case you must state for each location what work will be carried out at which address and in which building. To clarify: you must state for all activities at which location they will be carried out. Apart from the location for the clinical activities with the GMO, you must also state the location or locations of laboratories in which activities with the GMO are carried out under the terms of this

license application, such as procedures with patient samples.
In cases where central control is not possible, such as with a multi-center study, a separate application must be submitted for each location.

The intended work will be carried out in the Erasmus MC, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.

- Receipt, storage, and preparation of the GMO will be carried out at the hospital pharmacy, Erasmus MC Apotheek.
- Administration of the GMO will be carried out in a patient treatment room at the Erasmus MC.
- Patient samples will be processed and/or stored at Erasmus MC or in an external central laboratory.

A2. Construction and composition of the GMO

Virus from which the genetically modified vector was derived

A2.1. Which virus was used as the original virus in the construction of the GMO?

Where applicable, supply the full scientific name, a trivial name (e.g. the commercial name), the strain and the isolate used.

The parental virus is Adeno-Associated Virus (AAV) and consists of a single-stranded DNA vector genome (derived from AAV2) which is encapsidated by an icosahedral protein capsid (derived from AAV5). Both parental AAV's, AAV-2 and AAV-5, are AAV serotypes that naturally occur in the human population. The vector genome used contains two DNA sequences (inverted terminal repeats: ITRs) that are derived from the viral genome of AAV serotype 2 (AAV-2). The capsid proteins are derived from AAV serotype 5 (AAV-5).

Adeno associated viruses are small (approximately 25 nm in diameter), non-enveloped, icosahedral, non-pathogenic parvoviruses. AAV infect cells through a receptor mediated process, after which the viral DNA is transported to the nucleus. AAV needs a helper virus, such as adenovirus or herpes virus, to replicate.

A2.2. Describe how the identity of the original virus was determined.

The GMO to be administered is often based on a strain that was derived, sometimes by genetic modification, from the original virus. The properties of the original organism form the basis for the environmental risk assessment, which is why confirmation of the identity is important. Provide a description of the characteristics that determine the identity of the original virus. This may be based on bio-information analyses, such as sequence analysis, alignments or phylogenetic analysis. Information on the origins of the original virus, such as a micro-organism originating from the American Type Culture Collection (ATCC), may also be submitted.

Wild-type AAV have a linear single-stranded DNA genome approximately 4.7 kilobase (kb) long. The genome consists of two coding elements: the replicase (rep) gene (encoding rep78, rep68, rep52 and rep40) required for AAV replication and packaging, and the capsid (cap) gene encoding the capsid proteins (VP1, VP2, and VP3). These elements are flanked by two inverted terminal repeats (ITRs), which serve as substrates for the Rep proteins during replication and packaging of the vector genome. The vector genome may consist of a positive (+) or a negative (-) strand.

The GMO is not constructed from a parental virus or vector in the classical sense. Rather, AMT-061 is assembled from individual molecular components which are expressed using an insect cell-based Baculovirus Expression Vector System (BEVS), see Section A2.8 Molecular characterization for more details.

For molecular characterization see section A2.12 and confidential description.

A2.3. What is the host range of the original virus?

Describe the hosts in which the original virus naturally occurs, also including hosts that serve as a reservoir. For each possible host, indicate the tissue and cell tropism.

Dependoviruses with similarity to AAV have been identified from other species; the AAV serotypes found in humans seem to be restricted to primates (Arbetman 2005). Both humans and primates may show pre-existing (neutralising) antibody titres against AAV. Infections with AAV occur frequently, and are world-wide. The prevalence of neutralising antibodies against AAV is found in the adult European population to be for AAV5 (3.2%) followed by AAV8 (19%) and is highest for AAV2 (59%)

and AAV1 (50.5%) (Boutin 2010; Calcedo 2013). AAV infections are non-pathogenic, i.e. not associated with disease manifestations.

Table 1 Classification of Adeno-Associated Virus

Baltimore classification	Group II
Family	Parvoviridae
Genus	Dependovirus
Species	Adeno-Associated Virus
Common name	AAV5

Tissue/cell tropism is determined by serotype, i.e. the capsid moiety. In the case of AMT-061, the capsid is AAV-5 derived. Preclinical studies in non-human primates (including uniQure' s own studies in non-human primates) have shown that, following administration, AAV-5 displays strong liver-directed tropism, with vector DNA sequence also detected in the spleen and adrenal glands (Nathwani 2011).
The vector genome is packaged into capsids composed of the three viral proteins, VP1, VP2, and VP3. Each capsid consists of 60 VP proteins in total, which are arranged in icosahedral symmetry.

A2.4. Provide relevant data on pathogenicity and possible attenuation and biological restrictions of the original virus.

What is the class of pathogenicity of the original virus? If this concerns an attenuated virus, the basis for attenuation must be described. If the organism is biologically restricted in another way, the grounds for the biological restriction must be substantiated.

Wild type AAV-2 and AAV-5, elements of which form the basis of the GMO, are non-pathogenic. They have been classified accordingly (pathogeniciteitsklasse 2, COGEM).

Regarding containment: it should be noted that these parental strains are not in fact used to generate the GMO. They are merely the origin, from which molecular components have been isolated and used to ultimately generate the GMO. As actual handling of wild type AAV strains does not take place, further (physical) containment is therefore not applicable.

Regarding attenuation: wild type AAV per se is dependent on the presence of a helper virus (adenovirus or herpes virus) to allow successful replication (Berns K & Parrish CR 2007). Wild type AAV can infect host cells and release its genome to the host cell nucleus. However, in the absence of a helper virus, it remains dormant.

It should be noted that the GMO AMT-061 itself is an attenuated (recombinant) AAV: by design it does not contain any of the AAV genes that are essential for replication of viral DNA, the formation of viral particles, or the packaging of viral DNA into these particles.

A2.5. What are the pathogenic properties of the original virus and what are the available treatment methods?

Name the symptoms that are known or assumed to be caused by the original virus. Also indicate the treatment methods that are available to treat such an infection and provide an indication of the effectiveness of these treatments.

Infection with wild type AAV is asymptomatic and AAV is not known to cause any noticeable pathology (Berns 2005). Treatment methods are therefore not considered relevant.

A2.6. What are the transmission routes of the original virus?

State all the observed and assumed transmission routes of the virus, and indicate the effectiveness of the transmission.

Wild type AAV likely spreads via the respiratory or gastro-intestinal route (Berns K & Parrish CR 2007).

As noted above, infections with wild type AAV occur frequently, and are world-wide. For example, up to 59% of the human population is seropositive for AAV-2 (Boutin 2010).

Wild type AAV is dependent on a helper virus for replication, e.g. adenovirus or herpes virus. During an active helper virus infection, newly formed wild type AAV particles can be spread together with the helper virus. The spread of wild type AAV therefore will depend on whether a helper virus is present, and whether it is present in the same compartment, i.e. at the site of active infection (Berns 2005).

A2.7. How could the original virus survive outside the host?

State all survival options and the survival time of the original virus under optimal environmental conditions, and describe the factors that may be of influence.

Wild type AAV is a small, non-enveloped virus with a very stable capsid. Little is known about the stability of wild type AAV. Exposure to heat, UV radiation, or extreme pH can inactivate (recombinant) AAV (non-published data).

The genetically modified viral vector

A2.8. Describe the 'original vector or vectors'.

Describe the 'original' vector, and indicate – with or without the help of bio-information analysis – in what way the original vector deviates from the original virus. For the application of a viral system (e.g. lentiviral particles), in addition to the viral (transfer) vector, you must also describe for example packaging and pseudotyping plasmids. Provide a description of the characteristics that determine the identity of these original vectors. This can be done on the basis of bio-information analysis, such as sequence analysis, alignments or phylogenetic analysis.

The GMO is not constructed from a parental virus or vector in the classical sense. Rather, AMT-061 is assembled from individual molecular components which are expressed using an insect cell-based Baculovirus Expression Vector System (BEVS).

The Baculovirus Vector Expression System (BEVS) for recombinant AAV is based on the fact that the three elements required for the generation of functional AAV particles, being the Rep protein(s), the Cap protein(s) and the vector genome, can be provided *in trans*. In practice this means that each can be provided by a different Baculovirus vector. Intracellular expression of Rep and Cap, in the presence of the AAV vector genome, results in (1) replication of the AAV vector genome by Rep protein, (2) assembly of capsid proteins to capsid particles, and (3) packaging of vector genome DNA into the capsid particles by Rep, in this case resulting in AMT-061. The three recombinant baculoviruses used for the production of AMT-061 are 1) Bac-Rep, 2) Bac-Cap and 3) Bac-hFIXco-Padua).

The system to generate these recombinant baculoviruses entails two elements, being (1) the Baculovirus genome ('backbone DNA') and (2) the pPSC10 donor plasmid. The Baculovirus DNA is linearized, using Bsu36I. This restriction enzyme cuts the Baculovirus DNA at two sites; one flanking the ORF 603 open reading frame and one within the essential ORF1629 open reading frame. This digestion excises the polyhedrin gene (Figure 1A). The pPSC10 donor plasmid is a plasmid that can be conventionally propagated in bacteria. It contains a cloning site that is flanked by sequences

homologous to the remaining ORF603- and ORF1629 sequences in the Baculovirus backbone, and can be conventionally engineered to contain an insert of choice, e.g. the Rep gene, the Cap gene or the vector genome (Figure 1B).

When Sf9 insect cells are transduced with linearized Baculovirus DNA and with pPSC10 containing the insert, homologous recombination occurs between the ORF sequences in the linearized Baculovirus DNA and in the ORF sequences present in the pPSC10 plasmid. Recombination results in exchange of the insert and restoration of ORF603 and ORF1629, generating circular, recombinant Baculovirus DNA (Figure 1C). The recombinant Baculovirus DNA is polyhedrin-deleted but otherwise replication competent, hence it propagates in Sf9 cells and positive clones can be selected by plaque forming assay. Clones are selected for genomic integrity and stability.

To survive outside the host, wild type Baculoviruses organize into so called occlusion bodies, to which the Polyhedrin protein is an essential component (Rohrmann 2013). Due to the absence of the Polyhedrin gene, and the resultant absence of polyhedron protein, recombinant Baculovirus cannot form occlusion bodies and are not stable outside of the culturing system.

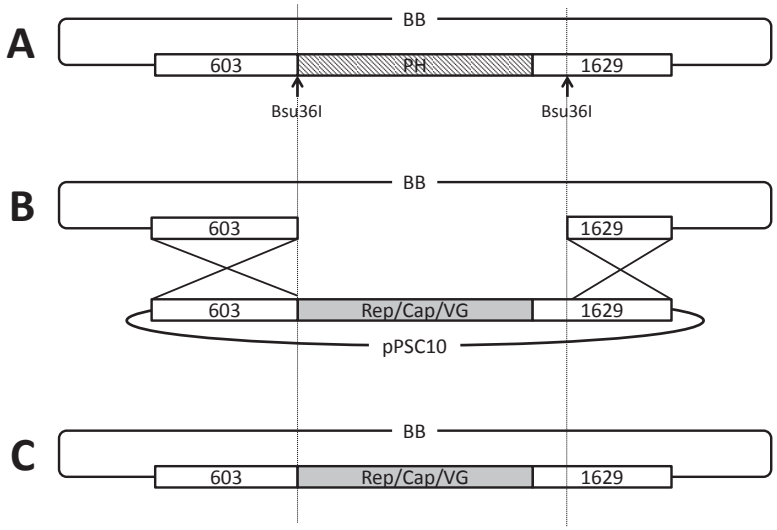

Figure 1 Principle of recombinant Baculovirus generation.

A: The Baculovirus backbone is generated from circular Baculovirus DNA by means of a digest with Bsu36I. Resultantly, the entire polyhedrin gene and part of the flanking essential ORF1629 sequence are excised. B: Upon co-transfection of Sf9 cells with linearized Baculovirus backbone and pPSC10 containing the insert of choice recombination takes place, resulting in exchange of the insert and restoration of ORF1629. C: As a result of recombination, circular recombinant Baculovirus DNA is generated. This Baculovirus can be propagated in insect cell culture but does not form occlusion bodies due to absence of the polyhedrin gene. Legend: BB; Backbone, PH; Polyhedrin, VG; Vector genome

Generation of the baculovirus constructs

To manufacture the source baculovirus vectors used for the production of AMT-061 three transfer plasmids containing the different expression cassettes are constructed i.e. the hFIXco-PADUA cDNA (plasmid vector 1), AAV2 *rep* gene (plasmid vector 2) and the AAV5 *cap* gene (plasmid vector 3). These transfer plasmids are used to construct the three required baculovirus constructs by recombination with a linearized baculovirus DNA (Bac-PSC1).

Plasmid vector / baculovirus 1

The wild type hFIXco expression cassette was subcloned into pPSC10. The Factor IX Padua mutation was introduced in the FIX coding sequence through site directed mutagenesis. The resulting plasmid containing the hFIXco-PADUA transgene is identified as plasmid vector 1. Plasmid vector 1 was co-

transfected with baculovirus backbone (Figure 1) into Sf9 cells to generate the recombinant baculovirus 1.

Plasmid vector / baculovirus 2
Plasmid vector 2 contains the expression cassette for AAV Rep (encoding replicase proteins Rep78 and Rep52), essential for AAV DNA replication and packaging. The plasmid vector 2 was co-transfected with the baculovirus backbone into Sf9 cells to generate the recombinant baculovirus 2. This baculovirus 2 was also used as a source baculovirus in the production of AMT-060.

Plasmid vector / baculovirus 3
Plasmid vector 3 contains the expression cassette for AAV5 Cap (encoding AAV capsid proteins VP1, VP2 and VP3), essential for the formation of AAV5 capsids. Plasmid vector 3 was co-transfected with the baculovirus backbone into Sf9 cells to generate the recombinant baculovirus 3. This baculovirus 3 was also used as a source baculovirus in the production of AMT-060.

For molecular characterization see section A2.12 and confidential description.

A2.9. Regarding the pathogenicity of the original virus, have certain properties of the 'original vector' been altered that would determine the pathogenicity of the original vector?

Here, an elaboration could be given of the modifications made to the 'original vector', such as replication deficiency, which cause attenuation of the pathogenic properties.

Baculovirus vector considerations
Baculoviruses are incapable of replication in mammalian cells. They have a limited range of hosts, and are typically restricted to a range of closely related insect species. Regarding pathogenicity, baculoviruses are not harmful to humans.

The parental (baculovirus) vectors used in the manufacture of the GMO AMT-061 can infect and replicate in the insect cell line used for the production (production cells: derived from the insect *S. frugiperda*.
It should be noted that a number of steps in the down-stream production process of AMT-061 are designed to clear the baculovirus vectors from the crude GMO AMT-061 bulk by means of inactivation or physical removal. The final GMO AMT-061 preparation is tested for residual infectious baculovirus to confirm such inactivation/removal. The test entails a bioassay with a detection limit of 6.8 infectious units (i.e. infectious particles) per mL. To date, no residual infectious baculovirus has ever been detected in any lot of final product.

AAV vector considerations
Wild type AAV can only replicate in the presence of a helper virus. The wild type AAV genome carries only two genes; the Cap gene, which encodes the proteins forming the capsid, and the Rep gene, which encodes for proteins that replicate the viral genome and package it into the capsids.

The AMT-061 vector genome can be considered a gutted wild type genome, as it does not contain the Rep and Cap genes. Thus, AMT-061 is replication-defective, even in the presence of a helper virus.

As a result, AMT-061 is expected to harbor even lower pathogenicity as compared to the parental AAV2 and AAV5 viruses that were used for its construction, where wild type AAV are already considered to be non-pathogenic. The pathogenic potential of AMT-061 in relation to the parental viruses is therefore considered negligible.

A2.10. Describe the method of production of the clinical viral vector from the 'original vector or vectors'.

Answer this question preferably by using a diagram that describes the various production steps.

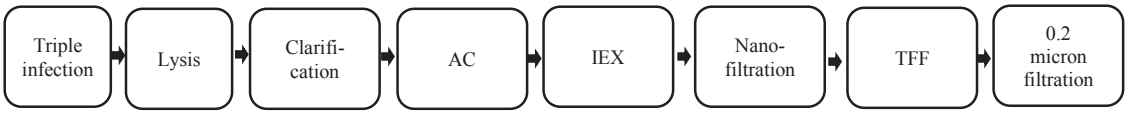

Figure 2 AMT-060 and AMT-061 Manufacturing Platform

The upstream production process consists of infection of the insect cells with these three baculoviruses, followed by fermentation and lysis of the (remaining) insect cells, to release the newly formed AMT-061 vector particles into solution. The downstream production process consists of a series of chromatography steps and a nano-filtration step, serving to specifically isolate the AMT-061 particles and to clear live baculovirus and other impurities. The isolated AMT-061 particles are then dia-filtered against the formulation solution.

A2.11. Describe the coding genes and the regulatory sequences present in the vector and in the DNA inserted into the vector.

A full description must be provided of the inserted or deleted genetic material, also discussing the functions of the sequences, for example:

- *regulatory sequences, such as promoter, terminator, and enhancer sequences;*
- *structural genes;*
- *in case of insertion of a transgene: the function of the coded proteins in the donor organism (the donor organism is the organism from which the gene originally was isolated or in which it occurs naturally) and the expected function in the test subject;*
- *in case of deletion: the function of the deleted genetic material in the original organism;*
- *in case of point mutation(s): the effect of the point mutation or mutations on the function of the modified genetic material in the original organism and the expected function in the test subject;*
- *whether the vector or the DNA inserted into the vector contains elements of which the origin or function is unknown.*

Coding genes and the regulatory sequences present in the vector

The following relates to the resulting GMO, the AMT-061 vector, produced from the parental baculoviral vectors described above.

The 5' and 3' terminal regions of the vector genome are known as Inverted Terminal Repeats (ITRs). These sequences, cloned from the viral genome of wild type AAV-2 (Samulski 1987), contain self-complementary sequences forming hairpin structures. During production of the GMO, the ITRs are required for packaging of the viral genome into the particles. After transduction of the target cells, the ITRs are required for stabilization of the viral genome. The ITRs initiate complementation of the (unstable) single stranded genome DNA into (stable) double stranded DNA by host cell polymerases. Alternatively, because the ITRs are palindromic, multiple viral genomes can assemble ITR to ITR to form larger double stranded DNA structures known as concatemers. These concatemers remain transcriptionally active and stable episomal structures (Schnepp 2005). The ITRs do not contain any open reading frames for protein expression.

The ITRs directly flank the expression cassette, consisting of the LP1 enhancer/promoter, the SV40 intron, the codon optimized hFIX-Padua therapeutic transgene and a polyA sequence.

The LP1 enhancer/promoter consists of consecutive segments of the human apolipoprotein hepatic control region (HCR) and the human alpha-1-antitrypsin (hAAT) gene promoter. The enhancer/promoter is followed by a modified SV40 small t antigen intron, to enhance expression (Nathwani 2006). The LP1 enhancer/promoter mediates robust and liver-specific expression of the therapeutic transgene (Nathwani 2011).

The codon optimized hFIX-Padua sequence encodes for the normal human clotting factor IX protein. The nucleotide sequence was codon optimized to enhance protein expression. The codon-optimized hFIX-Padua sequence was designed based on a naturally occurring FIX-Padua variant.

Codon optimization was performed by replacing the codons in the natural FIX-Padua sequence which are less frequently found in highly expressed human genes with the (synonymous) codons that are most frequently found in highly expressed eukaryotic genes, using a previously described algorithm (Haas 1996). The optimized sequence was synthesized as oligonucleotides, and subsequently assembled by ligation of these oligonucleotides. This strategy of codon optimization does not affect the amino acid sequence of the protein. The protein translated from codon optimized messenger RNA is therefore the same as the naturally occurring protein. By consequence it does not have any effect on the normal functioning of the protein and the immune response.

Expression of this transgene in liver cells yields functional human clotting factor IX-Padua which is secreted into the circulation. Hence, the Padua Factor IX transgene is the therapeutic payload of AMT-061.

The SV40 polyA sequence serves to stabilize the messenger RNA.

The nucleotide sequences between the abovementioned functional sequences mainly consist of sequences that enable(d) molecular engineering of the construct (e.g. multiple cloning sites). The vector genome contains 5 cloning/joining sites. These sites are extremely short in length, at 18, 4, 12, 5, and 23 nucleotides. Because of this, the possibility that coding or functional sequences in the cloning/joining sites can reasonably be excluded.

A2.12. Provide a molecular characterization of the genetically modified viral vector.

A sequence analysis must be supplied for all inserted or deleted sequences, so that the precise location of the modification and, in case of insertion, the number of copies present and the orientation of the insertion cassette can be determined. The sequence to be submitted must contain the region of the intended modification as well as the recombination sequences used, also including the flanking sequences of the genome of the original organism. In the case of biological unrestricted GMOs, newly created fusion ORFs must be described and subjected to a bio-information analysis.
The complete genome of the viral vector must be characterized on a molecular level. This may be done according to a sequence analysis or a Southern blot analysis. During the production of a GMO, naturally occurring processes may lead to unintended modifications in the genome. These modifications may affect the fitness of the ultimate GMO and, thus, the results of the environmental risk assessment. Observed anomalies in the genome compared with the expected sequences, such as unexpected deletions, mutations or recombinations, must be described and interpreted. Phenotypic data may be used to support the data on the molecular characterization.
The sequence of the complete genome does not need to be submitted. A bio-information analysis must be carried out for the sequences and the annotated results must be presented. For more information, please refer to the COGEM advisory report CGM/130227-05.

Molecular characterisation of the Baculovirus constructs
As outlined above to produce AMT-061 in the BEVS, three recombinant baculoviruses are offered to (baculovirus-susceptible) insect cells. These three recombinant baculoviruses are the parental vectors used to manufacture the GMO (AMT-061).

The three baculoviruses consist of the same baculovirus backbone, but each contains a specific insert. One baculovirus contains two AAV rep genes (both AAV-2 derived and under an insect-cell specific promoter), one contains the AAV cap gene (AAV-5 derived and under an insect-cell specific promoter), and one contains the AAV vector genome (consisting of the therapeutic transgene expression cassette which is flanked by two AAV2-derived Inverted Terminal Repeats, or ITR's). Expression of Rep and Cap proteins by the insect cells results in the vector genome being replicated and packaged into newly formed capsid particles. The result of this process is a recombinant and fully replication-defective AMT-061 (AMT-061) particle that contains only the transgene expression cassette, flanked by the two ITR's. A detailed description of the three baculoviral vectors is provided below (see also section A2.8 and confidential description).

Baculovirus vector 1 (Bac-hFIX-Padua) provides the vector genome to the producer cells. It comprises a codon optimized sequence the human Padua Factor IX gene under control of the promoter-enhancer, and is flanked by AAV2-derived inverted terminal repeats (ITRs). The vector was generated through homologous recombination between the linearized Baculovirus backbone and a donor plasmid containing the insert. After successfully generating recombinant Baculovirus clone the complete insert was sequenced using massively parallel sequencing methodology. The Baculovirus backbone as compared to the reference sequence. The sequence of the FIX-Padua expression cassette present in the recombinant Baculovirus was fully identical to the reference (i.e. intended) sequence.

Baculovirus vector 2 (Bac-Rep) encodes two AAV2-derived Rep proteins. This vector was generated through homologous recombination between a linearized Baculovirus backbone and a donor plasmid containing the insert. After successfully generating the recombinant Baculovirus clone the complete insert present in the recombinant Baculovirus was sequenced using Sanger sequencing methodology. The returned sequence was fully identical to the reference sequence (i.e. the sequence as present in the donor plasmid) confirming correct sequence identity of the vector.

Baculovirus vector 3 (Bac-Cap) provides the Cap gene to the producer cells. It comprises the wild type AAV5 Cap gene (i.e. as it is present in wild type AAV5), with a minor modification to the promoter sequence as compared to the wild type gene in order to facilitate translation of the Cap transcript in insect cells. This vector was generated as described above from the linearized Baculovirus backbone and a donor plasmid containing the insert. After successfully generating recombinant Baculovirus clone the insert was partially sequenced using Sanger sequencing methodology, focusing on the region around the start codon. The returned sequence was fully identical to the reference sequence (i.e. the sequence reported to be present in the donor plasmid). Mass spectrometry analysis of AMT-061 only returned peptides that corresponded to the AAV5 capsid protein, confirming correct sequence identity of the parental vector.

Molecular characterization and confirmation of the identity of AAV5-hFIXco-Padua
The rep sequences used to produce AMT-061 was cloned from the vector genome of wild type AAV-2.

The cap gene used to produce AMT-061 was cloned from the vector genome of wild type AAV-5. The resulting baculovirus construct was designed so that it encodes all three Cap proteins of the wild type AAV-5 serotype. These proteins, originating from overlapping reading frames, together form the dense icosahedral capsids of AAV-5.

The ITRs that are present in the vector genome of the GMO AMT-061 were cloned from the viral genome of wild type AAV-2 (Samulski 1987). The Baculovirus Expression Vector System exploits the fact that for the production of recombinant AAV particles, rep and cap can be provided *in trans*, i.e. the vector genome does not need to contain these sequences as long as the proteins they encode are provided alongside it. As such, the genome of a recombinant AAV-based vector can be gutted to

contain little more than the transgene expression cassette encoding the therapeutic protein of interest. The resulting baculovirus construct thus contains the two AAV-2-derived ITR's, which flank the transgene expression cassette. The gutting of the vector genome renders the resulting recombinant AAV-based vector completely replication defective, i.e. even in the presence of a helper virus, because the rep and cap genes are missing.

In conclusion, the three components necessary for production of AMT-061 in insect cells are offered in the form of three recombinant baculoviruses, containing a rep gene, a cap gene, and the AAV vector genome (ITRs + transgene expression cassette). These three sequences were each derived (cloned) from wild type (parental) AAV. The DNA sequences were generated in vitro using molecular biological techniques. As such, the DNA sequences used to produce AMT-061 are synthetically derived and they do not have a true physical origin in AAV.

An (early) batch of AMT-061 was assigned to serve as primary reference standard. The primary reference standard was subjected to massively parallel sequencing. Full coverage was returned and alignment to the reference standard was demonstrated (see also confidential information). These results demonstrated correct sequence identity of the vector.

AMT-061 batches other than the reference standard are not routinely sequenced, as they are produced using the same Baculovirus seed lots as the primary reference standard. Nonetheless, a subset of the QC tests are indicative of correct DNA sequence identity, being (1) Vector DNA identity, (2) Vector DNA composition, (3) Potency, (4) Infectious vector titer, and (5) Genome copy concentration. Although none of the individual tests provide full sequence confirmation, together they return a complementary set of unrelated parameters all of which depend on vector genome identity and/or -integrity. These assays entail reference standards that were bridged with Phase I/II and AMT-060 reference standard. In brief, vector DNA identity is assessed by PCR from the amplification of an 90 base pair amplicon located within the codon optimized FIX-Padua sequence, vector DNA composition is assessed from the expected DNA banding pattern on agarose gel, potency is assessed using a bioassay followed by parallel line analysis against a reference standard, infectious vector particles are detected by bio-assay followed by sequence specific readout by QPCR, and genome copy concentration is assessed by QPCR using sequence specific readout by QPCR.
Altogether, the confirmed sequence identity and integrity of the parental Baculovirus seed lots, the confirmed sequence identity/integrity of the AMT-061 reference standard, and the panel of orthogonal test methods that is in place for routine QC testing of AMT-061 batches safeguards correct sequence identity/integrity of AMT-061 batches.

Clinical trials with AMT-060 were performed previously. The current AMT-061 is identical except for a two-nucleotide substitution resulting in a single codon change (AGG to CTG) in the coding sequence for FIX, corresponding to an Arginine to Leucine substitution in the transgenic protein.
The R338L substitution represents a gain-of-function FIX variant, which results in a FIX activity-to-antigen ratio of up to 8.6 (Simioni 2009). This augmentation is thought to be largely caused by increased affinity of the activated protein to activated clotting factor VIII (FVIIIa; Kao 2013). A number of preclinical studies in haemophilic mice and dogs demonstrated the potential utility of this variant for hemophilia B gene therapy, as it enables higher FIX activity levels without the need for a higher administered vector dose to increase transgene expression (Crudele 2015; Cantore 2012; Finn 2012; Suwanmanee 2014; Monahan 2015; Kao 2013). Therefore, the design modification to AMT-061 would enable achievement of higher circulating FIX activity, without altering any of the previously established *in vivo* correlations between vector dose, circulating FIX protein levels, and safety.
As expected, the modified transgenic protein was shown to display increased activity *in vitro* as well as in non-human primates.
As compared to AMT-060, manufacture of AMT-061 involves the exact same Rep- and Cap baculoviruses, and a transgene-containing Baculovirus that differs by only two nucleotides. On the product level, the critical quality attributes of both vector preparations are therefore expected to be comparable, which will be confirmed through further comparability/bridging studies. Initial production run for AMT-061, as used for testing in non-human primates were analysed and results were compared with results for batches of AMT-060 used for clinical Phase I/II supply.

As expected, because the key starting materials for manufacture (insect cells used for production, and baculovirus seed stocks) and the manufacturing process steps are essentially the same, protein composition for AMT-061 (capsid proteins content for VP1, VP2, and VP3) as well as DNA composition was similar for AMT-061 and AMT-060. In line with a similar AAV particle composition, the infectivity or ability to transduce target cells (expressed as genome copy to infectious vector titer ratio) was also similar for AMT-061 and AMT-060.
For similar reasons, the impurity profiles of AMT-061 and AMT-060 are also expected to be the same, i.e., for quantitative assays being within similar range and for limit tests being below similar limits.

Clinical grade vector preparations are routinely (QC-) tested for a panel of critical quality attributes including the dose-defining parameter of (vector) genome copy concentration. Key critical quality attributes, i.e. attributes that directly reflect the quality of the AAV particles which are in these preparations, include the ratio of particles to genome copies, the ratio of genome copies to infectious particles, and potency. Analysis of research-grade AMT-061 confirmed that genome copy concentration in the drug substance was within the expected range, as were the ratio of particles to genome copies and the ratio of genome copies to infectious particles Table 2).

Table 2 Analytical comparison of AMT-060 and AMT-061

	AMT-060		**AMT-061**
Parameter	Historical range*	Specification	Result
Genome copy concentration (gc/mL)	1.1 - 3.3 x10^{13}	≥ 1.0x10^{13}	1.4 x10^{13} – 2.9 x10^{13}
Ratio of total particles to genome copies (tp/gc)	9-16	≤20	7 - 11
Ratio of genome copies to infectious particles (gc/ip)	16-54	To be reported	25 - 67

*Range across 11 clinical grade batches

Potency of AMT-060 and early development AMT-061is assessed by an *in vitro* bio-assay (Table 3). This assay comprises human hepatocarcinoma cells as the target cells for transduction, and a commercially available (chromogenic) assay to measure resultant response of the cell supernatant. To confirm that AMT-061 mediated higher potency in vitro, AMT-060 and AMT-061 were compared head-to-head in vitro. For this, equal amounts of genome copies of each were plated onto the hepatic cells under appropriate conditions. After 48 hours, both supernatants were assessed for FIX activity by chromogenic assay and FIX protein levels by ELISA.

As shown in Table 3, AMT-060 and AMT-061 mediated comparable FIX protein levels, confirming similar vector characteristics as suggested in Table 2 (above). The FIX Padua protein, having an significant intrinsic increase in clotting activity as compared to wildtype FIX, translated in an increase of FIX activity levels of about 6 to 7 -fold for AMT-061 as compared to AMT-060. Taken together, these results confirm that AMT-060 and AMT-061 are comparable except for the increased activity of the FIX-Padua transgene.

Table 3 Comparison of the biological activities of AMT-060 and AMT-061.

Product*	FIX protein concentration†	FIX protein activity‡
AAV5-hFIXco (AMT-060 Drug Product)	0.5 µg/mL	0.24 IU/mL
AAV5-hFIXco (AMT-060 Drug Substance)	0.5 µg/mL	0.25 IU/mL
AAV5-hFIXco-Padua (AMT-061 Drug Substance)	0.5 µg/mL	1.41 IU/mL

*Plated onto human hepatocarcinoma cells at equal amounts of genome copies. †As assessed by ELISA. ‡As assessed by chromogenic substrate assay.

A2.13. Describe the origins of the cells/cell lines in which the original viral vector is cultivated. Also indicate which of the genetic components of the cell could possibly cause complementation or recombination.

When answering this question, elaborate on the characteristics of all cell lines to be used. Describe which cell types this concerns as well as their origins (e.g. human kidney epithelial cells).Also discuss the possibility of the genetic material in the cells/cell lines causing a certain interaction with the original vector, such as by complementation or recombination.

As outlined, AMT-061 is produced in an insect cell-based production platform in which the three sequences required to produce AMT-061 particles are offered by three different recombinant baculoviruses.

The platform utilises a proprietary cell line (expresSF+), which is derived from the insect *S. frugiperda*. The baculovirus backbones used to generate the recombinant baculoviruses are part of the same proprietary system.

Regarding potential for interactions between cellular DNA and parental (baculovirus) vectors: high throughput sequencing data of the GMO AMT-061 vector preparations have shown that more than 99% of the DNA present in AMT-061 represents the anticipated vector genome. Small amounts (0.2% of total) of baculovirus derived DNA and traces of insect DNA were detected. These DNA impurities are controlled for during quality testing. For previous vectors produced using the Baculovirus Expression system it was shown that these fragments do not harbor coding sequences. (for further information see confidential information).

The traces of insect DNA detected in AMT-061 represented short sequences randomly scattered across the insect genome. These results strongly suggest that the baculovirus- and insect-derived DNA impurities present in AMT-061 are not the result of recombination events but due to promiscuity of the Rep packaging protein.

AMT-061 does not contain the rep- or cap sequences necessary for replication of wild type AAV. However, these sequences are provided *in trans* during the production process. A quality control assay for the presence of replication-competent AAV particles in AMT-061 preparations is in place. To date, no replication-competent particles have been observed in any of the GMO AMT-061 preparations tested, confirming that the formation of replication competent particles is a hypothetical risk and not a commonly occurring phenomenon.

A2.14. Summarize the data in a diagram ('map') of the genetically modified organism. Also indicate any relevant helper sequences that may possibly be present.

Present clear maps of the genetically modified organism, such as plasmid maps, showing all the constituent parts of the vector. In this schematic depiction, the construction of the GMO must be clearly indicated.

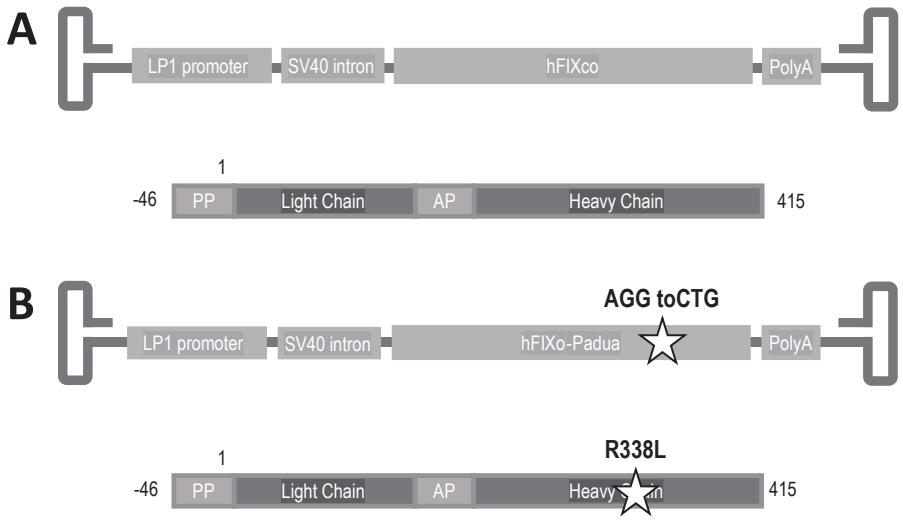

Figure 3 Structure of Vector Genomes and Corresponding Expressed FIX Protein, for AMT-060 (A) and AMT-061 (B).

A: Structure of AMT-060 (AAV5-hFIXco) vector genome and derived wild type human FIX protein. The hFIXco expression cassette is flanked by two ITRs (hairpin structures) and consists of the LP1 promoter, SV40 intron, hFIXco coding sequence, and polyA signal, in that order. Below the vector genome a schematic representation of the translated protein is provided (PP: pre-pro-peptide, AP: activation peptide).

B: Structure of AMT-061 (AAV5-hFIXco-Padua) vector genome and derived human FIX-Padua protein. The AMT-061 vector genome is identical to the AMT-060 vector genome except for a two-nucleotide substitution (AGG to CTG as indicated). This substitution results in an Arginine to Leucine substitution in the translated protein, at position 338 (R338L).

A2.15. Indicate the degree to which the host range of the genetically modified viral vector has been or may be altered, relative to the original virus.

When answering this question, provide an argument that elaborates on the host range, host specificity and the tissue and cell tropism of the genetically modified viral vector, relative to the original virus. Also consider any modifications that were made in order to create the original vector.

The host range, tissue specificity, and tropism of AAV particles are determined by the capsid. The capsid of AMT-061 is composed of the exact same proteins that make up wild type AAV-5. The host range and tropism of AMT-061 and wild type AAV-5 are therefore not different.

A2.16. What physiological (including pathogenic) effects may be caused by the genetically modified viral vector; and what are the available treatments?

Indicate which physiological processes may occur following the application of the GMO in the host.

A comparison must be made between its possible pathogenic properties and those of the original virus. In particular, the pathogenic properties that may be created specifically by the GMO should be considered.

Infection with wild type AAV is asymptomatic; AAV is not known to cause any noticeable pathology. Given that the structure and thus the potential to infect will not have changed, it is expected that the safety profile of the parental wild type AAV and the GMO AMT-061 are similar. In addition, it should

be considered that AMT-061 lacks the Rep- and Cap genes present in wild type AAV2 or wild type AAV5 (i.e. the parental strains). Due to the lack of these two genes, the vector is replication-defective. Even in the presence of helper virus, the vector genome of AMT-061 will therefore not be replicated, nor will capsids be formed. As such, two processes associated with wtAAV infection (i.e. replication and capsid formation) will not take place after infection with AMT-061. In a strict sense, the safety profile of AMT-061 is therefore theoretically more favorable as compared to the safety profile of wtAAV.

Following administration in humans, the GMO AMT-061 is expected to home to the liver, where it will infect and transduce liver tissue. Such GMO administration will elicit an immune response very similar to natural infection with (wild type) AAV.

Following liver transduction delivery of the GMO-derived DNA to the nucleus, the therapeutic transgene is transcribed and translated, and human Factor IX protein is produced by the liver cells. The liver is a natural source of Factor IX protein, i.e. the transgene is expressed in a natural environment. Local responses to GMO-mediated Factor IX expression are therefore not expected. The GMO-derived Factor IX protein is a human protein to which eligible patients have extensively been exposed in the form of protein replacement therapy, therefore immunological responses against this Factor IX protein are not expected to occur. The codon optimized Padua-FIX sequence resembles a naturally occurring FIX protein with normal function of the protein although a significantly higher specific activity.

Altogether as infection with wtAAV already does not result in any noticeable pathology and Factor IX is expressed in its natural environment (the liver), in practice, the safety profiles of AMT-061 and the parental strains will be similar, in that neither will mediate any noticeable pathological effects.

Infection with wild type AAV is asymptomatic and AAV is not known to cause any noticeable pathology. Thus, treatment methods against the GMO are therefore not considered relevant.

A2.17. Indicate the possible transmission routes of the genetically modified viral vector.

Provide all the observed and assumed routes of transmission. Indicate the degree to which the transmission of the GMO could be made easier by helper functions, or by the presence of replication-competent virus. Furthermore, indicate the degree of impact on transmission due to the modification and the cells in which the GMO was cultivated or those infected with the GMO. Make a comparison with the original virus.

GMO derived DNA is expected to shed through blood, urine, saliva, faeces and semen. The GMO-derived vector DNA which is known to be shed in body fluids or excrements is assumed not to represent infectious GMO.

The GMO AMT-061 is replication-deficient even in the presence of a helper virus infection. Replication-competent AAV (rcAAV) can theoretically be formed during production of the GMO. Presence of rcAAV is monitored as part of routine quality control for the GMO. To date, no rcAAV has ever been detected (detection limit: 10 rcAAV per 2×10^{10} gc of GMO). In the unlikely event that rcAAV levels would have increased in a specific batch, this batch would fail rcAAV acceptance criteria and consequently this batch would not be administered to a patient. Altogether this scenario is considered unlikely to occur.

Ultimately, all the above scenarios are likely without consequence: in the remote chance that wild-type/replication-competent AAV is formed, this does not result in pathology/disease symptoms as infection with AAV is asymptomatic. Therefore, the GMO or sequence derived from the GMO, are not likely to replicate within the recipient following administration and therefore the absolute amount of GMO that could theoretically be spread cannot exceed the dose administered. Spread of the GMO is

not facilitated through helper functions, presence of rcAAV, or recombination events between the GMO and wild type AAV.

A3. Production of the GMO

A3.1. State under whose responsibility the production of the GMO is carried out.

Answer:

O Production will be under the responsibility of the applicant and forms part of this license application.

O Production will be by and under the responsibility of the applicant but does not form part of this license application:

- O A separate application for production will be submitted for contained use
- O Reference is made for the production to an existing license for contained use: (State here the number of the relevant GMO license)
- X Production will be under the responsibility of third parties. If production is in the Netherlands, please state the number of the relevant GMO license. Please state if production is outside the Netherlands.

AMT-061 will be produced by uniQure Inc., Lexington, United States of America using a similar baculovirus based platform as was applied for manufacture of AMT060 which was used in Phase I/II studies.

A3.2. During which steps of the production process does quality control take place, which test methods are used and how are the tests carried out?

Give an overview of the production process of the GMO and describe the points in the production process at which quality control takes place. State which controls are carried out, the sensitivity of the tests and which methods are used for the controls.

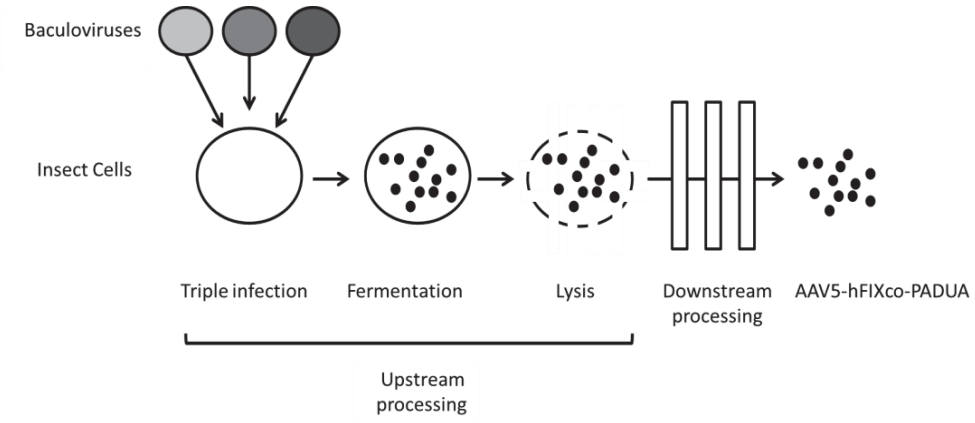

Figure 4 Schematic overview of the production process

Producer (insect) cells:
The Sf+ insect cells are stored in banks which are controlled for bacterial and fungal contamination including mycoplasmas, mycobacteria and spiroplasmas, adventitious agents, and identity, in line with ICH Q5A and European Pharmacopeia (Ph. Eur.) monograph 5.2.3.

Recombinant baculoviruses:
The baculovirus expression system is characterized by its excellent safety characteristics. First, baculoviruses are insect-specific viruses that are not capable of replicating in vertebrates (Airenne

2013). Second, they can easily be cleared from recombinant AAV preparations, because in contrast to AAV they are large enveloped viruses which are susceptible to inactivation by surfactants and to removal by the downstream chromatography steps and nano-filtration.

The recombinant baculoviruses are stored in banks which are controlled for sterility, bacterial endotoxins, adventitious agents, specific viral agents (of bovine, porcine, or insect origin), myco- and spiroplasma, mycobacteria, and identity, in line with ICH Q5A and European Pharmacopeia (Ph. Eur.) monograph 2.6.16. The genomic stability of these baculoviruses during the upstream process is confirmed.

Baculovirus genomic stability testing:
Recombinant Baculoviruses containing the different inserts (Rep, Cap or the human PADUA FIX cDNA) are generated by means of homologous recombination between the modified Baculovirus acceptor backbone and the donor plasmid containing the insert. Clones are selected through plaque assay. Baculovirus clones are subsequently amplified in vitro (see confidential information for further details).

Genomic stability of each Baculovirus is assessed by passaging it beyond the maximum number of passages that are used to produce AMT-061. For genomic stability assessment, each passage is analysed for (1) the relative frequency of specific regions spaced across the recombinant Baculoviral genome (i.e. the insert versus Baculoviral genomic regions) and by (2) assessing the integrity of the entire expression cassette. The first is generally assessed using QPCR-based methodology, the latter by end-point PCR or Western blotting). For each of the three Baculoviruses used for the production of AMT-061, both were confirmed, demonstrating that the Baculoviruses are genetically stable within the number of virus passages that is relevant for the production process.

Vector intermediates and final product:
In-process control and product release takes place at several stages of the upstream- and downstream production process, by testing process intermediates for microbiological contamination and vector particle content. The purified vector preparations are controlled by testing various parameters related to content, purity, biological activity, and identity, as well as for a range of known and hypothetical impurities (see confidential information for further details).

The quality tests relevant for environmental risk analysis are summarized in Table 4. Test parameters were relevant for environmental risk assessment when they (1) were representative of a GMO other than the intended vector, or (2) were representative of a nucleic acid sequence derived of such a GMO. The tests are discussed in detail below. All other quality testing is performed on test parameters not relevant for environmental risk assessment such as concentration or purity of the AMT-061 vector itself, or chemical or other non-GMO related impurities.

Table 4 Quality tests assessing environmental risk-related parameters

Test	Method	Method Sensitivity
Replication competent-AAV (rcAAV)	Bio-assay	10 rcAAV per 2 $\times 10^{10}$ gc[1] (LOD [2])
Residual infectious baculovirus	Bio-assay	6.8 iu/mL (LOD)
Residual baculovirus DNA	Q-PCR	$5.0x10^{-10}$ geq/mL (LOQ [3])
Rep full-length sequences	Q-PCR	2.2×10^{7} copies/mL (LOQ)

[1](AAV5-hFIXco-Padua) genome copies. [2]Limit of Detection. [3]Limit of Quantitation.

Replication competent AAV (rcAAV) is defined as any AAV which is capable of replicating in the presence of helper virus (as stated elsewhere, AMT-061 is not able to replicate, even in the presence of helper virus). Generation of rcAAV during manufacturing with the Baculovirus system and insect cell-based production platform is a theoretical possibility with an extremely low likelihood. AAV5-hFIXco-Padua is controlled for the presence of rcAAV by a qualified bio-assay able to detect 10 rcAAV amidst 2 x 10^{10} genome-containing AAV5-hFIXco-Padua particles. To date, no rcAAV has been found in any of the batches produced in the Baculovirus production system. In contrast, replication-competent AAV are frequently detected in AAV preparations that were produced using conventional, mammalian cell-based production platforms; as an example, Allay et al reported approximately 1 rcAAV per million particles (Allay 2011). The reason for the increased safety profile of Baculovirus system-produced AAV resides in the fact that the Rep and Cap genes are under control of an insect promoter and hence, in humans, do not mediate functional expression of these genes.

The production of AAV5-hFIXco-Padua entails large amounts of infectious Baculovirus. The process incorporates several steps to clear Baculovirus through neutralization (by detergents) or physical removal (by chromatography steps and filtration). Nonetheless, AAV5-hFIXco-Padua is tested for the presence of residual infectious Baculovirus by bio-assay. The test was qualified for use, and has a detection limit of 6.8 infectious Baculovirus units (i.e. particles) per mL. To date, no residual infectious Baculovirus has been found in any of the batches produced.

Because the production process of AMT-061 entails large amounts of Baculovirus, residual Baculovirus-derived DNA is a known process-related impurity. AMT-061 is assessed for residual Baculovirus DNA by means of a QPCR-based method. The associated drug substance release criteria is a ratio of Residual Baculovirus DNA relative to Genome Copies. The test was qualified for use, with an upper specification limit well above the levels detected in AMT-061.

AMT-061 is assessed for full length Rep levels by means of long-amplicon QPCR. The test was qualified for use. The associated drug substance release criteria is a ratio of Rep full length sequences relative to Genome Copies which is well above the levels detected in AMT-061.

A3.3. Which criteria are imposed on a batch of the GMO before it is released for the application in question?

State which criteria are used to reject a batch.

An overview of the tests relevant in the context of the environmental risk assessment are included in Table 4.

Of the process- and product-related impurities, residual infectious baculovirus and replication competent AAV are the most relevant for environmental risk assessment. These impurities are controlled for by sensitive tests, (Table 4). Related acceptance criteria are provided as confidential information (see quality tests assessing environmental risk-related parameters).

A4. Description of the research

Administration

A4.1. How many test subjects will take part in the study?

Here, the maximum number of test subjects to be treated should be indicated. This number can be higher than the intended number of test subjects. Please be aware of the fact that the number you enter limits the permit. For example, if you indicate that there will be 50 test subjects, then no more than 50 can be included in the study.

Maximum number of test subjects is 40.

A4.2. Describe how the batch containing the GMO will be transported and prepared for administering to the test subjects.

Describe how, after delivery, the GMO preparation will be transported to the hospital pharmacy for example. Also describe the way in which the GMO preparation will be handled, as well as the conditions under which they will be handled, in order to get the preparation ready to be administered. If this handling will be carried out under an existing permit for Contained Use (IG), the number of the GMO permit concerned must be stated. In addition, describe the way in which the GMO preparation will be transported to the test subject.

The hospital pharmacy, or equivalent, will receive and store AMT-061 in accordance with the requirements laid down in the GMO Regulation (annex 9.3.3.2).

In the hospital pharmacy, or equivalent, AMT-061 will be pulled from the supplied 10 mL glass vials into an infusion bag in a Class II Biological Safety Cabinet.

The AMT-061 infusion bag will be transported to the treatment room in accordance with GMO Regulation (annex 1).

A4.3. How will the GMO preparation be administered to the test subject?

State via which route and how the GMO is administered. Also state what aspects may affect the safety of human beings and the environment.

Treatment of patients will occur in a hospital environment without any additional precautionary or containment measures. Administration and monitoring of the patient occurs in a patient treatment room.

The vector will be administered to the patient as an intravenous infusion.

Connecting the infusion bag to the main infusion tubing in the clinic, may create an increased likelihood of spillage and/or aerosol formation. For AMT-061 administration the possibility of spillage and /or aerosol formation is strongly reduced as the hospital pharmacy, or equivalent, will prepare and provide the infusion bag connected to a pre-filled tubing containing a sterile saline solution. In the exceptional case, spillage and/or aerosol formation occur, this will be only diluent without any vector material.

The pre-filled tubing of the infusion bag will be connected to the main infusion tubing, which will have been primed with sterile saline. When removing the intravenous catheter after completion of the infusion, there is an increased likelihood of spillage and/or aerosol formation. However, by flushing the infusion administration line with sterile saline before removing the intravenous catheter, the likelihood of spillage and/or aerosol formation that may result in an environmental exposure of AMT-061 is reduced.

In the exceptional situation of spillage the spill and the surrounding of the spill will be disinfected with chlorine solution.

The catheter, tubing, infusion bag and any other ancillary items used are all disposables. All these items that have been in contact with the GMO and all used vials are directly disposed of as GMO waste according to the GMO Regulation (annex 9.3.3.2).

A4.4. Which doses will be administered and at what time points during the study will they be administered?

Indicate which doses of the GMO will be administered. Also provide an administration diagram, showing at what times they will be administered.

Patients will be treated with a single intravenous infusion in a range of 5×10^{12} to 1×10^{14} gc/kg AMT-061.

A4.5. What other medication will be administered to the test subject that may possibly affect the GMO to be administered?

Any medication that would affect the GMO and possibly also the environmental risk assessment should be named here. If, for example, a vaccination study is conducted, this may also include challenge with the wild-type virus.

If the endogenous FIX activity is below 2% in at least 2 consecutive central lab measurements, FIX prophylaxis may be restarted. Re-initiation of FIX continuous prophylaxis may also be considered if the endogenous FIX activity is between 2 and 5%, based on clinical judgment and patient preference.

Treatment with prednisolone at a starting dose of 60 mg/day is recommended for ALT level increments of at least 2-fold from the baseline.

The medication described above is not expected to affect the GMO distribution or clearance.

Sampling

A4.6. Describe which of the samples taken from the test subject may contain GMOs.

Provide an overview of the samples and indicate whether GMO material is expected to be present in them.

The clinical results to date with regards to AAV5-hFIX in hemophilia B (AMT-060) demonstrate that GMO material may be present in urine, saliva, faeces, nasal secretions, semen, and blood. However, following the risk assessment as described in this document for AMT-061, the likelihood that infectious vector particles are present in biosamples is extremely low.

A4.7. Describe the method of sampling and how the samples will be subsequently processed.

In answering this question, also indicate how transmission of the GMO during sampling and testing will be prevented. For the subsequent processing, indicate what physical restrictions apply. In case the work concerned will be carried out in the Netherlands but is not part of the current permit application, then refer to the permit for work under Contained Use and provide the number of the GMO permit this concerns.

Biosampling will be performed regularly for study purposes, as needed for standard-of-care purposes, and/or on medical indication.

Blood samples for FIX activity measurements, FIX inhibitors, and ALT/AST will be taken in duplicate. One duplicate will be analysed at the local laboratory, and the other duplicate will be send to an external central laboratory for analyses.

All other samples taken for study purposes will be sent to an external central laboratory which is outside the scope of the current application (no analyses at the local laboratory involved).

The test subject may be called in for additional visits, at the discretion of the investigator. The subject may also contact the clinical trial site for an additional visit. An extra visit may include additional assessments, as deemed necessary by the investigator; these additional assessments could include biosampling.

Any sampling performed as needed for standard-of-care purposes and/or as medically indicated will be analysed at the local laboratory only.

Sampling and transportation of these samples, as well as processing and/or storage of these samples within the hospital will not require additional precautionary of containment measures. For all human samples routine precautions will be taken, designed to contain pathogens in blood and to protect the hospital staff from pathogens in blood. These precautions include the use of gloves and a laboratory coat, gown or uniform. No further physical restrictions will be applied in addition to these routine precautions taken per local hospital procedures for handling human samples (designed to contain pathogens in blood and to protect the hospital staff from pathogens in blood). After sampling the waste will be processed as hospital specific waste (SZA).

A4.8. How is the GMO preparation detected after being administered?

State, if applicable, when GMO components or are detected during or after administration and why detection at that particular moment of the test is regarded as important. Describe the nature of the samples that are tested, the method used and the detection limit that can be achieved.

The monitoring of the GMO has been designed as per the EMA ICH considerations on General Principles to Address Virus and Vector Shedding from June 2009 (ICH Considerations General principles to address virus and vector shedding June 2009). The sampling scheme is based on the shedding profile observed with AMT-060 in previous clinical studies.

After AMT-061 administration, sampling of the following types of matrix will be performed to determine vector DNA levels at the selected time points: Blood and semen. These matrixes are justified based on the observations in previous clinical studies with AMT-060. Particularly blood samples have shown the longest persistence for AMT-060 related sequences.

Sampling should continue for the individual subject and for a specific matrix until 3 consecutive negative samples have been detected for the subject for that particular type of matrix, as this will be taken as evidence of complete and permanent clearance.

The samples will be analysed for the presence of vector DNA using a validated QPCR-based method. This technique is the most sensitive technique available to date for this specific purpose.

Waste management

A4.9. Give an overview of the nature and quantity of the waste produced and describe how the waste will be disposed of.

State which waste flows can be distinguished. State which waste flows may potentially contain the GMO and how the GMO is prevented from being released into the environment via the waste flows.

The nature of the waste generated includes (partially) used vials, ancillary components used for preparation and administration (e.g. tubing, needles, syringes, infusion bag, personal protective equipment, etc.) and components used for collecting body fluids samples after administration. Following handling and administration of AMT-061 the unused vector remaining in the vials, and ancillary materials, will be disposed of per GMO Regulation (annex 9.3.3.2).

A5. Environmental risk analysis

Environment-related information originating from earlier experiments

A5.1. Describe the results originating from earlier (pre-) clinical studies with the GMO and which are important for the environmental risk assessment.

In answering this question, you should elaborate on results that were achieved using an identical or similar GMO, if these are relevant for the environmental risk assessment of the present application. Important data include data on shedding, duration of latent presence of the vector/GMO, transmission of the vector/GMO, and possible interaction with other micro-organisms (including viruses). For each of these results, describe the trial set up (e.g. doses used, method of administration, detection test and detection limit) and the relevance to the work in the present permit application.

In order to achieve greater FIX activity levels with the goal of alleviating the need for all exogenous therapy, uniQure has modified AMT-060 (used in Phase I/II clinical trials) by replacing the wild type FIX gene (present in AMT-060) with a sequence that encodes the naturally occurring hFIX PADUA variant present in AMT-061. The FIX gene constructs present in AMT-060 and AMT-061 are identical except for a two-nucleotide substitution (AGG to CTG) in the coding sequence, resulting in an Arginine to Leucine substitution in the transgenic protein which results in higher FIX specific activity of the AMT-061 product.
AMT-060 was evaluated in a Phase I/II clinical trial to investigate its safety and efficacy in adults with severe or moderately-severe hemophilia B. 9/10 hemophilia B patients treated with AMT-060 shifted from severe to the moderate/mild phenotype, with continuous (transgenic) circulating FIX activity levels ranging from 3% to 12%.

AMT-060
The Sponsor completed enrollment and dosing in a first-in-human Phase I/II study (CT-AMT-060-01) with a planned 5 year follow up. CT-AMT-060-01 was designed to investigate the safety and efficacy of two dose levels of AMT-060 in adults with severe or moderately-severe hemophilia B. Comparable dose levels of the AAV5 vector had previously been tested in a patient population with Acute Intermittent Porphyria, which were cross-referenced and informed the dose rationale for the CT-AMT-060-01 study. Safety assessments included treatment-related adverse events, as well as immunological assessments, including T-cell activation assays (ELISpot) against AAV5 capsid antigens and assessment of total antibody and inhibitory antibody titers against the viral capsid and FIX, respectively; vector/DNA shedding was also assessed from blood, saliva, nasal secretions, urine, faeces and semen. Efficacy assessments included endogenous FIX activity, total exogenous FIX usage and spontaneous annualized bleeding rates (sABR).
To date, all 10 subjects received a single IV dose of AMT-060 in one of two escalating dose cohorts; 5×10^{12} gc/kg (N=5) or 2×10^{13} gc/kg (N=5). Seventy-eight (78) week follow-up from all 5 subjects in Cohort 1 and 52 week follow-up from all 5 subjects in Cohort 2 are currently available. The durability of FIX activity maintained by FIX protein encoded by the codon-optimized human coagulation factor IX cDNA obtained at this point in time is considered robust and sustainable.
A summary of the preliminary results are provided.

CT-AMT-060-01 Preliminary Summary of Results
Safety:

1. The single IV infusion of AMT-060 was safe and well-tolerated in both the low- and high-dose cohorts
2. No capsid-specific T-cell responses were observed across both cohorts
3. Three out of 10 patients (2 out of 5 patients in the high dose cohort) experienced mild, asymptomatic elevations of ALT (Alanine Aminotransferase) which were treated with a tapering course of corticosteroids.

4. ALT elevations were not associated with a detectable activation of capsid-specific T-cell response and not correlated with a loss of endogenous FIX activity
5. The following SAEs were reported
    1. two elevations of ALT
    2. one short febrile episode that prolonged hospital stay
    3. myelopathy that required hospitalization
6. No patients developed inhibitory antibodies against FIX

Efficacy:

1. Clinically relevant endogenous FIX activity (≥ 3%) was established in 9 out of 10 patients across both cohorts (5 out of 5 in the high dose cohort)
2. Continuous, regular prophylaxis was discontinued in 8 out of 9 patients previously dependent on prophylaxis (4 out of 4 in the high dose cohort)
3. FIX expression levels remain stable and durable for up to 1.5 years in the low dose and up to 1 year in the high dose cohort without indications of loss of expression
4. Shift to mild hemophilia B phenotype (> 5%) was achieved in
    1. two out of five patients in the low dose
    2. four out of five patients in the high dose cohort
5. A reduction/elimination in the use of FIX replacement therapy and a lowering of the annualized bleeding rate was demonstrated in both cohorts

As part of the clinical assessments vector shedding through various bodily fluids and other excreta is measured. Samples of whole blood, saliva, nasal secretions, urine, semen and faeces are being collected on days 1 and 2 (semen and faeces only on day 1), weekly from week 1 through 12 (semen and faeces only on weeks 1, 3, 6, 9 and 12), every second week from week 12 through 26, every 13 weeks from week 26 through 156, and every 26 weeks from week 156 through 234. As of day 1, sampling continued for the individual subject and for a specific matrix until 3 consecutive negative samples have been detected for the subject for that matrix. All samples were analysed for the presence of vector DNA by QPCR, more details are listed in section A7 Confidential information.

Table 5 Shedding - Number of weeks to negative: Cohort 1

Excreta	First week negative (range) (N=5)
Blood	27-27 (n=1)
Urine	3-11 (n=5)
Saliva	6-20 (n=5)
Nasal	5-18 (n=5)
Faeces	6-16 (n=5)
Semen	9-48 (n=3)

Time in weeks until first 0 or <LOD in three consecutives
Program: T_shedd.sas, Run: 23JUN2017 12:30:53

Table 6 Shedding - Number of weeks to negative: Cohort 2

Excreta	First week negative (range) (N=5)
Blood	All positive until last assessment
Urine	8-22 (n=5)

Excreta	First week negative (range) (N=5)
Saliva	9-16 (n=3)
Nasal	7-12 (n=3)
Faeces	16-20 (n=3)
Semen	12-22 (n=3)

Time in weeks until first 0 or <LOD in three consecutives
Program: T_shedd.sas, Run: 23JUN2017 12:30:53

All samples were analysed for the presence of vector DNA by QPCR. In summary, shedding of vector DNA was found to be higher in the high dose cohort with the highest concentration observed in the whole blood where it peaked the day after administration and subsequently rapidly declined. Vector DNA disappeared from whole blood at week 27 for 1 patient in cohort 1, however remained to be detectable for all other cohort 1 patients as well as all patients in cohort 2 until last assessment (Cohort 1, 78 weeks; Cohort 2, 52 weeks). The peak vector DNA concentration in serum (in gc/mL) was in general higher than the peak concentration in the other tissues: in comparison to nasal mucus by approximately 300-fold, faeces by approximately 1,000-fold, saliva by approximately 200-fold, urine by approximately 10,000-fold and semen by approximately 7 times. Vector DNA disappeared from faeces between week 6-16 in Cohort 1 and week 16-20 in cohort 2 (2 patients still positive at last assessment at week 52), from nasal secretions between week 5-18 in Cohort 1 and week 7-12 in Cohort 2 (2 patients still positive at last assessment at week 52), from saliva between week 6-20 in Cohort 1 and between week 9-16 in Cohort 2 (2 patients still positive at last assessment at week 52), from semen between week 9-48 in Cohort 1 (1 patient still positive at last assessment at week 78) and in week 12-22 in Cohort 2 (3 patients still positive at last assessment at week 52).

As already mentioned, detection of a copy of vector DNA does not imply detection of an infectious vector particle. More likely, it would represent DNA from a degraded vector particle, a particle that has been taken up by a cell, or a cell which has been transduced by the vector (e.g. leukocytes or epithelial cells of the bladder). In preclinical studies on recombinant AAV, it has been shown that urine containing AAV vector DNA does not contain infectious particles (Favre 2001), and that infectious vector is restricted to the plasma compartment and cleared from circulation within 48 to 72 hours after infusion.

Other studies
The vector components of AAV5-hFIX (The AAV5 capsid and the wildtype hFIX gene cassette) have been used separately in previous clinical trials (in patients with acute intermittent porphyria and haemophilia B patients (uniQure AMT-060 study, Nathwani 2011), respectively). The principle of using AAV vector approaches for treatment of haemophilia B has already been established in academic studies (Cancio 2013). AAV2 and AAV8, containing human FIX expression cassettes have been used in previous trials of haemophilia B. AAV-based gene therapy vectors have been used in more than 100 patients with various diseases (Ginn 2013), and recently the first AAV based gene therapy product (Glybera; uniQure) has been approved for treating patients with lipoprotein lipase deficiency (LPLD) in Europe (EMA/CHMP/474664/2012 and EMEA/H/C/002145). The clinical experience with liver directed AAV gene therapy includes more than 35 patients with severe or moderately severe haemophilia B. Several clinical trials in haemophilia B patients using liver directed AAV gene therapy approaches are registered on www.clinicaltrials.gov and are known to be actively recruiting patients.

Clinical studies in humans do not include in the study design assessment of biodistribution of the gene therapy in the various organs as it would require taking biopsies. However, biodistribution is frequently studied by measuring the presence of vector sequences in various bodily fluids such as plasma, semen, saliva, nasal mucus and urine, and in faeces.

The clinical results to date with regards to AAV5-hFIX in hemophilia B demonstrate that shedding occurs in (1) urine (2), saliva, faeces, nasal secretions (3) semen, and (4) blood, after administration of the GMO AAV5-hFIX to the test subject. The potential infectivity of rAAV could be assessed in cell cultures but biological fluids are not suitable for (technically amenable to) such testing. As already mentioned, however, studies in non-human primates have shown that rAAV from biological fluids is not infectious (Favre 2001). Transmission and spreading of infectious AAV vector because of the proposed clinical trial is therefore considered to be highly unlikely.

Risk analysis

This is the most important aspect of the whole application!

Give a detailed assessment of the expected effects of the GMO on human health and the environment on the basis of the answers to the above questions and in accordance with Appendix II of EU Directive No. 2001/18/EC and the corresponding guidance notes of the European Commission (2002/623/EC). Please take into account any direct, indirect, immediate and delayed effects of the GMO on human health and the environment.

A risk analysis should be carried out for each GMO included in this notification, as well as for combinations of the GMOs, if any. The risk analysis must cover the effects of the GMOs that are due to interactions between the GMOs and the environment(s) where they are introduced or where they may end up under the present activities. The effects in question are those which are relevant to safety to human health and the environment. Section A6 of this form describes those aspects which must at least be taken into consideration.

The risk analysis should include at least the aspects mentioned in Annex 1 of this form. The risk analysis includes the following sections, which should be given in the same order as shown below (see questions A5.2 – A5.5):

1. *List of the likely adverse effects;*
2. *Estimate of the likelihood of these effects actually taking place;*
3. *Evaluation of the risks and an estimate of the severity of the effects, based on Items 1 and 2 above. The severity can be estimated by comparing it with the severity assigned to similar risks, such as for example the effects that occur with non-GMOs in similar situations ('baseline principle');*
4. *If you have concluded in Point 3 that the risk is high, you are requested to examine what measures can be used to mitigate the risk;*
5. *Final conclusion of the risk analysis, stating the risk management measures that will be employed, and a conclusion as to the acceptability of the risks when these measures are put into operation.*

A5.2. State which potentially harmful effects may be linked to exposure of human beings or the environment to the GMO.

Describe here the effects on human beings and the environment that could occur as a consequence of the use of the characteristics of the GMO described in earlier sections of this application. The aim here is identifying the hazards; the following questions deal with the chance that these hazards will actually occur.

AMT-061 and GMO-related product quality attributes

Recombinant AAV vectors have a good safety profile. Wild type AAV viruses are not disease related and unrelated to any disease in humans. Wild type AAV can only replicate in the presence of helper virus (e.g. adenovirus or herpesvirus).

AMT-061 is manufactured using expresSF+ insect cells from *Spodoptera frugiperda* using three different recombinant baculoviruses. While all three baculoviruses contain a nearly identical baculovirus genome (backbone), the first contains an insert comprising the AAV Rep genes, the second contains an insert comprising the AAV5 Cap genes, and the third contains an insert comprising the therapeutic vector genome which consists of the Padua-FIX expression cassette flanked by two AAV2-derived inverted terminal repeats (ITRs). Expression of Rep and Cap proteins in the insect producer cells results in the assembly of the final GMO (AMT-061).

The baculoviruses used for construction of AMT-061 are unable to replicate in mammalian cells. Baculoviruses are non-pathogenic to humans. Furthermore, baculoviruses are removed from the crude bulk during the Downstream Purification steps. The active substance is tested for residual infectious baculovirus and is rejected if any infectious baculovirus is detected.

Low levels of baculovirus DNA are a known DNA impurity in AMT-061. Short baculovirus DNA sequences, particularly those located close to the vector genome insert, have been detected in baculovirus-produced rAAV. It is assumed that these sequences are co-packed with the vector genome into vector particles due to some degree of promiscuity of the packaging Rep protein. Resultantly, these DNA impurities are co-purified with the vector particles. As AMT-061 is constructed in insect cells, low levels of DNA originating from these cells are a second known DNA impurity of AMT-061. However, these sequences are short and random, and not integrated into the AMT-061 vector genome.

Several AAV gene therapy vectors manufactured in the baculovirus-based manufacturing platform have been administered to patients, i.e. Glybera (approved marketing authorization in 2012) and AMT-060 (applied to patients in Phase I/II clinical trials). For both products no harmful effects that could possibly be related to either of these DNA impurities have ever been observed. This justifies the conclusion that a risk related to residual insect cell DNA and residual baculovirus DNA can be considered negligible.

Since Rep and Cap DNA sequences are abundant during the manufacturing process, formation of replication-competent AAV (rcAAV) during manufacturing may theoretically occur. Replication-competent AAV is defined as being able to replicate in the presence of a helper virus infection. The likelihood of rcAAV formation is however low, as any combined Rep and Cap sequence would exceed the AAV5 capsid packaging capacity (i.e. any functional recombinant would not physically 'fit' into the capsid). Moreover, the Rep and Cap genes used in the baculovirus platform are under control of an insect promoter which would have limited activity in mammalian cells. Regardless, the active substance is routinely tested for the presence of rcAAV. To date, no rcAAV has ever been detected in any baculovirus-produced rAAV lot (detection limit: 10 rcAAV per 2×10^{10} gc of GMO). In the unlikely event that rcAAV is detected, the batch would be rejected.

Potential post-dosing recombination events – AAV sequences
AMT-061 itself is replication deficient. Nonetheless, scenarios in which it would replicate in vivo, or even revert to a replication-competent AAV, are theoretically conceivable. In any case, such scenarios would require Rep and Cap sequences to be present in the same cell, and hence would require co-infection with wtAAV. Presence of wtAAV would however still not circumvent the dependence on a helper virus infection. Hence, replication or revertance of the GMO would require simultaneous infection of one-and-the-same cell with (1) AAV5-hFIX, (2) wtAAV, and (3) a helper virus. This scenario of triple infection is in itself unlikely. Nonetheless, the conceivable scenarios upon triple infection are outlined below.

Without recombination taking place, triple infection will result in replication of the AAV5-hFIX vector genome by the Rep proteins provided by wtAAV. Consequently, the triple-infected cell would in this case produce replication-deficient AAV particles of the wild type serotype containing the AAV5-hFIX vector genome, as well as wtAAV particles. In any case, no replication-competent AAV5-hFIX particles would be formed regardless of the presence or absence of helper virus. Consequently it can be concluded that the presence of helper virus in patients does not impact the risk profile of the product

and its intended use. Therefore, helper virus positive patients can safely be included in the proposed study.

Homologous recombination would only involve the ITRs present in the AAV5-hFIX vector genome and the ITRs present in the wtAAV, as only these sequences may share homology. Essentially, homologous recombination could yield the AAV5-hFIX expression cassette flanked by wtAAV-derived ITRs, and the wtAAV-derived genome flanked by AAV5-hFIX-derived ITRs. The likelihood of homologous recombination would depend on the degree of homology between the AAV5-hFIX-derived ITRs (derived from wtAAV2) and the wtAAV-derived ITRs, which logically depends on the serotype of the wtAAV in question. In the case of triple infection, both recombinants would be subject to intracellular replication by the Rep protein provided by wtAAV. As a result, the triple-infected cell would in this case produce replication-defective AAV particles containing the AAV5-hFIX vector genome, wtAAV particles, and AAV particles containing either of the recombined genomes. Also in this scenario, no replication-competent AAV5-hFIX particles would be formed regardless of the presence or absence of helper virus.

Non-homologous recombination could theoretically produce a hybrid sequence containing wtAAV-derived rep and cap sequences as well as the hFIX expression cassette. The likelihood of such recombination events is intrinsically much smaller than the likelihood of homologous recombination. Depending on its primary structure (i.e. depending on whether it is flanked by ITR sequences or not) such hybrid genomes may or may not be valid substrates for Rep protein. In the case of triple infection, hybrids representing valid Rep substrates would be subject to intracellular replication by the Rep protein provided by wtAAV. Because of their expected size however, such hybrids cannot be packaged into the wtAAV capsids. The maximum packaging capacity of AAV capsids is approximately 5kb, and rep- and cap- and ITR sequences already make up for 4.7 kb (Daya et al. Clin Microbiol Rev (2008):583-593). The triple-infected cell would in this case only produce AAV particles containing the AAV5-hFIX vector genome and particles containing the wtAAV genome, as outlined in the no-recombination scenario described above. Replication-competent AAV5-hFIX particles could however not be formed or released by the infected cell.

Most importantly, in any of the scenarios described above, immunological responses will be mounted against rAAV- and/or wtAAV-derived Cap proteins. Therefore, adaptive immunity would silence any extracellular manifestation that could result from triple infection, just as it would in the case of a naturally occurring co-infection with wtAAV and helper virus. The risk of spread to other cells (which would, also in these cells, require the event of triple infection) is therefore extremely small. It should be noted that cells that are infected with AAV5-hFIX only will not be affected by these immune responses as they do not express AAV-derived proteins. Hence, none of these responses would compromise therapeutic efficacy.

Taken together, the likelihood of recombination is small as it requires concurrence of a constellation of events at a singular moment in time. Replication-competent AAV5-hFIX would not be formed in any case. Recombinant replication-deficient particles could theoretically be formed but none of these would have different characteristics as compared to the vector or wtAAV, respectively. In any case these particles would not have any significant systemic consequences, as they would be neutralized by the immune system as soon as they leave the cellular compartment. None of these events would affect cells transduced with the therapeutic vector alone. The risk associated with recombination is therefore considered to be negligible.

Even in the very unlikely event of recombination, the products of the outlined recombination events would not increase the likelihood of shedding and transmission based on the shedding and transmission scenarios outlined above. It is therefore concluded that the environmental impact will not be affected by any of the theoretical and unlikely recombination events.

Potential post-dosing recombination events – SV40 sequences
The AAV5-hFIX vector contains an SV40 intron and an SV40 polyA sequence. The possible recombination of these viral sequences with wild-type viruses is discussed below.

Recombination of the SV40-derived sequences present in the AAV5-hFIX vector genome with other virus-derived sequences depends on two likelihoods, being (1) the likelihood that homologous sequences are present, and (2) the likelihood that in such case recombination occurs (the likelihood of non-homologous recombination was taken to be negligible).

The simian SV40 virus can infect humans, as approximately 1 in every 5 individuals may be seropositive for this virus (Taronna 2013), (Corallini 2012), (Mazzoni 2014). Infection in any case remains subclinical (Garcea 2003). Persistence of SV40 DNA in the normal population is a matter of debate. A review article summarized that half of the available screening studies suggested SV40 is not present in the normal population, while the other half suggested that it is, at frequencies of 5 to 25% (Paracchini 2006). It is not unthinkable that SV40 sequences are present in patients during AMT-061 administration but the likelihood is low. Using BLAST search engines, no human viruses were found that displayed significant homology with the SV40 sequences present in the AAV5-hFIX vector genome [http://blast.ncbi.nlm.nih.gov], suggesting that, in humans, presence of virus-derived sequences (SV40 or other) with significant homology is not a likely event.

In the hypothetical case that homologous sequences are present, the likelihood of homologous recombination depends on the extent of homology. In a study on this exact relation, the recombination frequency of SV40 DNA in monkey cells sharply dropped when the length of the homologous sequences was less than 200 base pairs (Rubnitz 1984). The SV40 intron in the AAV5-hFIX vector genome is 94 base pairs in length, the SV40 polyA signal sequence is 133 bp in length. As such, even in the presence of homologous sequences, recombination is an unlikely event.

Risks related to the FIX transgene and the Padua modification

In healthy individuals, FIX levels may range from 50% to 200% of the population mean (Khachidze 2006). As a transgene for gene therapy, FIX has a broad therapeutic window. In Hemophiliacs, levels as low as 2% are expected to result in therapeutic benefit. Only extreme overexpression is associated with risk of thrombosis. Extreme overexpression of hFIX as the result of AAV gene transfer has been established in uniQure's pivotal safety study in mice, where infusion of 2.3×10^{14} gc/kg (more than 10 times the high dose of the clinical Phase I/II study) resulted in 70-fold overexpression, i.e. 70 times the level found in the normal human population. No adverse effects were associated with this immense overexpression. The absence of adverse events was not due to impaired or lacking functionality of hFIX, as hFIX expressed in mice displays normal functionality and was shown to revert the clotting deficiency in FIX-deficient mice (Nathwani 2006). These preclinical results suggest that overexpression of hFIX is not associated with adverse effects.

In non-human primates, infusion of AMT-060 at the intended clinical dose resulted in 1% to 10% of normal human levels. This intended clinical dose corresponds to approximately 25 to 100mL of vector preparation per 50kg body weight, infused intravenously to reach the liver.

In uniQure's Phase I/II study on AMT-060, circulating FIX activity levels reached up to 12% of normal human levels, demonstrating that, at the intended doses, the scenario of achieving extreme overexpression was not realistic.

The Padua modification (AMT-061) was introduced to achieve higher levels of circulating FIX activity at the same dose. The modification entails the replacement of two adjacent nucleotides in the wild type FIX coding sequence. The modification results in a non-synonymous codon change which translates to an Arginine to Leucine substitution in the protein, yielding the so called Padua FIX variant. Relative to the wild type FIX protein encoded by AMT-060, the Padua FIX protein encoded by AMT-061 is expected to display a six-to eightfold increased specific activity. Relative to AMT-060, AMT-061 is therefore expected to mediate increased efficacy at the same dose and the same protein expression levels.

The modifications defining AMT-061 are restricted to the FIX coding sequence. Other than potency, all quality attributes of AMT-060 and AMT-061 are expected to return similar, and AMT-061 is expected to mediate identical FIX protein expression levels as compared to AMT-060. The modification is therefore expected to return the same the safety profile as AMT-060. The only risk

associated with the Padua-FIX modification would relate to unintended achievement of supraphysiological levels of circulating FIX activity, either as the result of intended or unintended exposure.

For the case of intended exposure, i.e. in patients, the scenario of reaching extreme levels of circulating FIX activity is unlikely given that Padua FIX will display at most tenfold increased specific activity as compared to wild type FIX, and FIX protein levels will be similar for AMT-061 and AMT-060.

The probability of unintended exposure to significant amounts of AMT-061, in such a way that the vector will be able to transduce hepatocytes and mediate detectable FIX expression is extremely low. It would entail unintended intravenous infusion of 25 mL vector preparation or more. In addition, the probability that such unintended exposure would result in overexpression of FIX expression levels is extremely low, as the target levels for intended exposure are close to 5% of normal. Finally, the probability that overexpression of hFIX would have any clinical consequence for a third party is low, as already in the normal population there is considerable 'over' expression in otherwise healthy individuals, and non-clinical studies suggest that even extreme overexpression holds negligible biological consequence. The overall risk that overexpression of hFIX in third parties due to unintentional exposure will result in observable effects is therefore negligible.

A5.3. State according to which scenario the GMO can disperse from the test subject into the environment.

State in your answer the scenarios in which the GMO may disperse in the environment. Explain the level of risk that spread will actually occur. Also describe in your answer whether the number of test subjects and/or the dosage to be administered affects the risks to be identified.

Overall the exposure of the environment to AMT-061 is considered limited. The most likely groups being exposed to the GMO are:

- Healthcare professionals involved in preparation and administration of the GMO and obtaining clinical samples.
- Laboratory professionals involved in sample preparation and analysis.
- Close contacts of the patients who potentially may be exposed to shed vector.

The likelihood for exposure of these groups will be different and depending on different scenarios. Administration will be performed at the hospital and is restricted to the treatment room. Transport to and from the hospital is carried out under transport conditions applicable for GMOs. Due to the well-controlled administration conditions, the potential contact of the product with healthcare professionals at the administration site environment is extremely limited. The only possibilities for a contact of the administration site environment with the product would be accidental spillage of the product solution to surfaces; accidental self-administration (needle-stick injury), (mucous) skin contact with the product or inhalation of aerosolized product by the administering health care professional. As the vector is replication deficient, the only effect could be a marginal increase in FIX levels if any and an immune response to the AAV5 capsid.
Laboratory professionals working with clinical samples may also be exposed to the GMO. However, the only possibilities for a contact of the laboratory professionals with the GMO would be accidental spillage of the sample to surfaces, a needle-stick injury, (mucous) skin contact with the sample or inhalation of aerosolized sample. The chance that intact vector is present in the sample is very low and it has been described that infectious particles can only be found in blood during the first 3 days after vector infusion (Favre 2001). And as the vector is replication deficient, the only effect could be a very marginal increase in FIX levels if any and an immune response to the AAV5 capsid.
To evaluate the scenarios of exposure of close contacts shedding of the GMO is to be evaluated. Shed vector DNA does not equal infectious vector particles. It has been described that infectious particles can only be found in blood during the first 3 days after vector infusion (Favre 2001). After this period, all infectious particles are likely to have infected test subject cells or to have been rendered non-infectious through other mechanisms (e.g. degradation by test subject effector

mechanisms). The level of risk that spread will occur is therefore dependent of the scenario, i.e. fluid or excrement type.

Because of the above, spread of infectious GMO into the environment through nasal secretions, saliva, urine or faeces is considered negligible. Spread of infectious GMO through blood is conceivable, as blood samples are drawn during the first 3 days after administration of the GMO. However, following the worst-case scenario's described above, even the risk that AMT-061 will spread into the environment through blood and thus leads to a GMO related risk is considered negligible.

It is not known whether recombinant AAV shed in semen is infectious or, like AAV shed in the other fluids, represents non-infectious vector DNA. In either case, the risk of horizontal or vertical transmission cannot be excluded. Although it is not anticipated that this will relate to a risk it is considered an unwanted effect as such any potential risk is addressed by requiring the use of a condom during the trial in the period from administration of the AMT-061 until the AAV5 vector has been cleared from semen, as evidenced by negative analysis results for AAV5 vector for at least three consecutively collected semen samples.

Assessment of vector DNA in semen will be performed using a validated method. The likelihood that the GMO will spread through semen is low and controlled.

Exposure through shedding
Shedding in non-clinical studies was assessed using a QPCR based method. Serum, saliva, urine and faeces were collected at several time points after dosing. Shedding of AMT-060 was assessed in cynomolgous macaque. Clearance curves in saliva and urine mainly followed the clearance from the serum with the vector DNA concentrations bout 2 – 4 logs lower. Serum cleared between weeks 12 and 26. Saliva was cleared between weeks 8 – 12. Vector DNA levels in urine were low and reached the limit of detection around week 8. The shed material will mainly include DNA fragments tested positive using the QPCR method. A high level of shedding of infectious vector particles has not been observed.
Non-clinical biodistribution and shedding studies with AMT-061 confirmed the earlier observations with a highly similar AMT-060 vector and demonstrated distribution and shedding in plasma urine and tissues. For urine AMT-061 vector DNA was not detectable 3 months after administration of the highest dose of 9×10^{13} gc/kg.

Shedding of AMT-060 (which in essence resembles AMT-061), was evaluated in clinical Phase I/II studies. Samples of whole blood, saliva, nasal secretions, urine, semen and faeces were tested. Vector DNA disappeared from whole blood at week 27 for 1 patient in cohort 1, however remained to be detectable for all other cohort 1 patients as well as all patients in cohort 2 until last assessment (Cohort 1, 78 weeks; Cohort 2, 52 weeks). Vector DNA disappeared from faeces between week 6-16 in Cohort 1 and week 16-20 in cohort 2 (2 patients still positive at last assessment at week 52), from nasal secretions between week 5-18 in Cohort 1 and week 7-12 in Cohort 2 (2 patients still positive at last assessment at week 52), from saliva between week 6-20 in Cohort 1 and between week 9-16 in Cohort 2 (2 patients still positive at last assessment at week 52), from semen between week 9-48 in Cohort 1 (1 patient still positive at last assessment at week 78) and in week 12-22 in Cohort 2 (3 patients still positive at last assessment at week 52).

As already mentioned, a detected copy of vector DNA does not necessarily represent presence of an infectious vector particle. More likely, it represents DNA from a degraded vector particle, a particle that has been taken up by a cell, or a cell which has been transduced by the vector (e.g. leukocytes or epithelial cells of the bladder). In preclinical studies on recombinant AAV, it has been shown that urine containing AAV vector DNA does not contain infectious particles (Favre 2001), and that infectious vector is restricted to the plasma compartment and cleared from circulation within 48 to 72 hours after infusion.

In a scenario where infectious AAV is shed into the environment the amount of shed infectious particles is likely to be extremely low. This is based on observations that in most cases only vector DNA material could be observed through PCR, however, infectious particles are restricted to the

plasma compartment and cleared from circulation within 48 to 72 hours after infusion (Favre et al., 2001). In the unlikely event that infectious particles will be shed, these will still be replication-deficient. The amount of shed infectious particles will be extremely low and the material shed will be replication deficient based on the vector design and manufacturing strategy. Shedding of vector material may lead to exposure of third parties which theoretically may result in transmission to these third parties. However, as shedding of infectious vector particles is already considered unlikely, the likelihood of transmission to and infection of third parties should also be considered a highly unlikely event. In conclusion, spreading of the vector through shedding and transmission to third parties is considered a theoretical scenario for dispersion of the AMT-060 and AMT-061 from the test subject into third parties.

Accidental exposure to AMT-061
Accidental exposure of health care professionals to AMT-061 should be treated according to the measures listed in Table 7 below. These are standard measures for which it should be indicated that they are not needed to reduce the already negligible risk.

Table 7 Measures for occupational exposure with AMT-061

Type of exposure	Measure
Needle stick	Encourage bleeding of the wound. Wash injection area well with soap and water. Obtain medical attention.
Eye contact	Immediately flush eyes with water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Obtain medical attention.
Inhalation	When inhaled, move person into fresh air. Obtain medical attention.
Ingestion	Rinse mouth with water. Obtain medical attention.
Skin contact	Wash-off with a gauze soaked in a viricidal disinfectant (not alcohol solution) and subsequently wash with soap and plenty of water. Obtain medical attention.

Safety measure - Spillage
The spill procedures are like those already in place for handling hazard substance such as cytotoxics. The spill kit should be available during all the steps, but minimally during the preparation and administration. The spill kit will contain at least:

- Disinfectant sachets
- Absorbent paper towels
- Disposal forceps
- Biohazard incineration bags
- Emergency contact number
- Copy of the spillage procedure

Influence of the number of test subjects and/or the dosage to be administered on the risks:

Because the negligible risk even when spreading and transmission to third parties would occur the number of test subjects nor the anticipated doses, are expected to influence the conclusion on the negligibility of the environmental risk of gene therapy using AMT-061.

A5.4. Give an estimate of the chance that the adverse effects described in A5.2 could actually occur.

Give a reasoned estimate of the chance (likelihood) of the aspects described in A5.2 and A5.3, also taking account of the number of test subjects and the dosage.

As outlined, shedding of AMT-061 derived DNA is expected but shedding of infectious vector particles is considered highly unlikely. Shedding studies using applicable dose levels demonstrate that the amount of shed vector DNA represents a minute fraction of the dose and corresponds to an even lower amount of infectious vector, if at all.

Thus, the absolute amount of infectious particles that could spread and thus be transmitted is negligible. The chance that the unintended exposure and potential adverse effects could occur is therefore considered to be negligible as well.

A5.5. Describe the risks that could occur as a consequence of the application of the GMO, taking into account the impact of any risk management measures taken.

Describe the risks in such a way that makes clear how the risks can be reduced through risk management. If risk management measures are necessary in order to limit the risks, these should be specified further in the questions below.

There are no significant risks associated with the application of the vector.

The overall risk of the GMO has been evaluated by summing the potential risks and consequences with respect the likelihood, as detailed in Table 8.

Table 8 Overall risks with respect to the likelihood of AMT-061

Adverse effect	Type of exposure*	Magnitude	Likelihood	Risk
Toxic effects to humans	self-inoculation	negligible	low	negligible
	exposure	negligible	negligible	negligible
Pathogenicity to humans	self-inoculation	negligible	low	negligible
	exposure	negligible	negligible	negligible
Tumorigenicity to humans	self-inoculation	negligible	low	negligible
	exposure	negligible	negligible	negligible
Germ-line transmission	self-inoculation	low	negligible	negligible
	exposure	negligible	negligible	negligible
Genome integration in humans	self-inoculation	low	negligible	negligible
	exposure	negligible	negligible	negligible
Disease or any other adverse effect to animals or plants	exposure	negligible	negligible	negligible
Population dynamics and genetic diversity of populations	exposure	negligible	negligible	negligible
Facilitating the dissemination of infectious diseases	exposure	negligible	negligible	negligible
Compromising prophylactic or therapeutic treatment	exposure	negligible	negligible	negligible
Disturbance of environmental biogeochemistry	exposure	negligible	negligible	negligible

* Accidental self-inoculation by a healthcare professional, exposure = due to incidental spillage or shedding.

The overall risk of AMT-061 to people and the environment can be considered negligible.

Therefore, no specific, additional risk management measures are deemed necessary, besides acceptance to use a condom during sexual intercourse in the period from drug administration until AAV5 has been cleared from semen, as evidenced by the central laboratory from negative analysis results for at least three consecutively collected semen samples (this criterion is applicable also for subjects who are surgically sterilized).

Risk management

A5.6. Which inclusion and exclusion criteria are adopted in the selection of test subjects and what is the effect of these criteria on environmental safety?

Give an overview of the inclusion and exclusion criteria that are only necessary for the protection of the environment or which criteria might possibly have an effect on safety for human beings and the environment.

From the risk assessment, it is concluded that there is a negligible risk related to the AMT-061 gene therapy. As such no inclusion or exclusion criteria are applied that relate to the safety of third parties or the environment at large.

A5.7. Describe which measures are provided for in respect of the hospitalization of the test subject.

When answering this question, please emphasize those aspects that are important in preventing spread in the environment of the test subject. Also indicate if, apart from medical reasons, hospitalization is prescribed as a way of protecting against possible effects for humans and the environment.

Hospitalization as means to protect the environment and for humans is not necessary. If a test subject is hospitalized, no additional measures have to be taken compared to other patients in that hospital.

Observation of the patients is done by means of post-administration surveillance of tolerance to AMT-061 administration, duration of post-administration surveillance is several hours up to 24 hours.

A5.8. If the test subject is to be hospitalized, what are the criteria for his or her release from hospital?

Describe the criteria on the basis of which the test subject will be released from hospital.

No discharge criteria have been defined. Patients may leave the hospital as soon as all post-administration assessments have been performed, unless a prolonged stay is medically justified by the investigator.

A5.9. Describe which measures will be taken to prevent the spread of the GMO to third parties (including medical personnel).

For example, give an overview of relevant (hospital hygiene) measures that will be taken. In case existing guidelines will be used, please indicate what they are (such guidelines must be attached to this application). Additional or deviating measures also must be described.

Preparation in hospital pharmacy, or equivalent
The standard precautions that are applied in the hospital pharmacy, or equivalent, setting to control exposure to hazardous substances such as cytotoxic agents are appropriate and will be applied during the preparation of AMT-061. Therefore, no additional measures have been taken. In the hospital pharmacy, the AMT-061 infusion bag will be prepared in a Class II Biological Safety Cabinet. During the preparation of AMT-061 personnel will wear protective clothing and gloves.

Administration in the clinic
The standard precautions that are applied to mitigate spillage and/ or aerosol formation of hazardous substances, such as cytotoxic agents, are appropriate and will be applied during the preparation and administration of AMT-061. Therefore, no additional measures have been taken.

Accidental exposure and/or spilling of AMT-061 are mitigated by the following the standard precautions:

- Personnel administering AMT-061 will wear protective clothing and gloves.
- The hospital pharmacy will provide the infusion bag connected to pre-filled tubing with sterile diluent. Should spillage and/or aerosol formation occur, this will be only 0.9% sodium chloride.
- The pre-filled tubing of the infusion bag will be connected to the main infusion tubing, which has also been primed with sterile 0.9% sodium chloride. When removing the intravenous catheter after completion of the infusion, there is an increased risk of spillage and/or aerosol formation. This risk is mitigated by flushing the infusion tubing with 0.9% sodium chloride, before removing the intravenous catheter.
- The catheter, tubing, infusion bag and other ancillary items used during administration are all disposables and are disposed according to the GMO Regulation (annex 9.3.3.2). Non-disposable materials (tools, devices) are cleaned with a disinfectant with viricidal activity, e.g. a chlorine releasing disinfectant like hypochlorite containing 0.1% available chlorine (1000 ppm) after usage and then autoclaved, if possible. Contact surfaces are disinfected with a similar disinfectant.

Procedure in case of unexpected situations and serious incidences

A5.10. Describe the procedures to be followed if changes in the risk management are required for medical reasons.

This may concern situations in which a test subject needs to be removed from isolation; for example because he or she requires intensive care, or in situations when unexpected effects are being observed, including Suspected Unexpected Serious Adverse Reactions (SUSARS) or Serious Adverse Events (SAEs). Also consider the situation of a test subject having died and an autopsy being required.
All unexpected situations and serious incidences must be reported immediately. See the 'Procedure for unwanted incidences' (www.loketgentherapie.nl)

Since the environmental risk is considered negligible, the patients are not kept in isolation at any point in time. Therefore, no additional measures are required in hypothetical situations where medical care may require interventions or treatments in another physical location.

A5.11. Describe what aftercare will be given if a test subject ends his/her participation in the study prematurely.

Also state to what extent the aftercare deviates from the aftercare for test subjects who have completed the entire study.

The investigator should make all reasonable attempts to retain the test subjects in the trial to allow long term follow-up on patient safety. The test subject will continue to receive the standard of care, which will not be affected by withdrawal from the trial. As AMT-061 is not infectious after being shed and therefore the risk to the environment is considered negligible, no additional measures have been taken for the period after administration.

Monitoring

A5.12. Describe how the monitoring will be set up to identify any spread of the GMO.

In answering this question, pay attention to the method followed, but also to the period during which any positive result can be expected in connection with the applicable scenario. Also state in this context during which period monitoring will take place.

Due to the negligible risk presented by AMT-061 to the environment, as described in above this section, no further monitoring is considered necessary.

A6. Conclusions of the possible environmental effects

Directive 2001/18/EC Annex II under Point D.1 gives a number of aspects that should be used whenever applicable as the basis of the conclusions about the possible environmental effects of the introduction of the GMP into the environment. All these points should be taken into account when drafting the conclusions of the risk analysis.

1. Likelihood of the GMO to become persistent and invasive in natural habitats under the conditions of the proposed release(s).

The GMO could spread from the recipient into natural habitats via blood, urine, faeces, saliva, nasal secretions, and semen.

AAV-based GMO found in body fluids is not infectious (Favre 2001), except for the blood compartment where infectious AAV-based GMO was found briefly after administration. Therefore, persistence through infection is unlikely when the GMO spreads into natural habitats via body fluids, since true shedding will only occur through body fluids such as urine, faeces, and saliva.

Persistence through replication and/or integration is dependent on infection of a (natural) host. As stated above infection of a host following spread via body fluids of the recipient is unlikely. In addition, the GMO has been rendered replication defective, by removal of Rep and Cap sequence from the genome. Integration occurs at a low frequency and at random even when administered IV at a high dose (Paneda 2013).

Taken together, the likelihood of persistence of the GMO into natural habitats is considered extremely unlikely.

2. Any selective advantage or disadvantage conferred to the GMO and the likelihood of this becoming realized under the conditions of the proposed release(s).

No selective advantage has been conferred to the GMO. In contrast, the GMO has been rendered replication-defective through omission of Rep and Cap sequences. Therefore, a selective disadvantage has been conferred to the GMO (vs for example wild type AAV), which is likely to be realized under the conditions of the proposed release (by IV injection). In addition, spread of infectious GMO following release is limited by the fact that the GMO shows poor potential for infection once shed via body fluids as shed material will predominantly contain only DNA fragments of the GMO and is unlikely to contain infectious particle.

3. Potential for gene transfer to other species under conditions of the proposed release of the GMO and any selective advantage or disadvantage conferred to those species.

The likelihood of gene transfer to species other than humans and (some) primates is low, given the host preference of AAV.

As far as (unintentional) gene transfer to humans and primates is concerned, the likelihood is low given the selective disadvantage conferred to the GMO under conditions of proposed release (as explained above).

4. Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO and non-target organisms (if applicable).

No immediate and/or delayed environmental impact is expected, again given the selective disadvantage conferred to the GMO under conditions of proposed release (as explained above).

5. 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).

Under conditions of release, persons working with the GMO could potentially come into contact with the GMO prior to or during administration, when the GMO is handled as distributed, at high concentration and fully functional.
Standard procedures to mitigate spillage and/or aerosol formation will be applied. As explained above, blood samples taken shortly after administration of the GMO should be regarded as containing active (infectious) GMO.
Should persons working with the GMO come into direct contact with the GMO (through inhalation or accidental injection during administration, or via blood samples taken shortly after administration), no immediate and/or delayed effects different from those expected for the recipients (test subjects) are expected: a (dose-dependent) immune response to the GMO could occur that will not affect subjects' general well-being.
It should be noted that humans are natural hosts for AAV, infections are asymptomatic and AAV is not known to cause any noticeable pathology. Similarly, dose-dependent administration of AAV/based GMO´s to humans has been shown to be safe. As noted above, a dose-dependent immune response does occur in a recipient and is without clinical consequence.
Overall, no immediate and/or delayed effects on human health are expected for persons working with the GMO or coming into contact with or in the vicinity of the GMO as it is released.

6. Possible immediate and/or delayed effects on animal health 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.

As explained above, under conditions of release, shedding into the environment can occur via blood, urine, faeces, saliva and semen (i.e. all body fluids). However, concentrations of the GMO in body fluids is low, and shed GMO is not infectious. The exception is blood, up to 3 days after release.
The greatest risk for shedding into the environment lies with urine, faeces, and saliva. As noted previously, GMO present within these body fluids is non-infectious. Therefore, should the GMO be consumed following shedding, a low amount of a non-infectious GMO is consumed.
Therefore, no immediate and or delayed effects on animal health or consequences for the feed or food chain are expected.

7. Possible immediate and/or delayed effects on biogeochemical processes caused by possible direct or indirect interaction between the GMO and the target and non-target organisms in the vicinity of the GMO introduction(s).

Not applicable.

8. Possible change in the current medical practice.

AMT-061 has been designed to deliver the human coagulation factor IX gene to the liver of patients suffering from haemophilia B, enabling the restoration of stable expression of coagulation factor IX thus ameliorating the bleeding phenotype and improving the quality of life of these patients.