

AMT-061  
AAV5-hFIXco-Padua (serotype 5 adeno-associated viral vector containing a codon  
optimised human factor IX Padua gene)

**Belgium**

**Environmental Risk Assessment**

**for Deliberate Release into the Environment of Genetically Modified organisms in  
accordance with Annex II of Directive 2001/18/EC of the European Parliament**

**Version 1.0**

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## 2. LIST OF ABBREVIATIONS

AAV	Adeno-associated viral vector
AAV-n	Adeno-associated viral vector serotype number (1, 2, etc)
AAV5-hFIXco-Padua	Serotype 5 adeno-associated viral vector containing a codon optimised human factor IX gene; the gene is codon-optimised to enhance expression and the product is a hyperactive variant of factor IX carrying an R338L mutation (referred to as "FIX-Padua")
DNA	Deoxyribonucleic acid
FIX	Factor IX
GMO	Genetically modified organisms
hFIXco	Codon optimised human Factor IX
IMP	Investigational Medicinal Product
PCR	Polymerase Chain Reaction
ppm	Parts per million
QPCR	Quantitative Polymerase Chain Reaction
rAAV	Recombinant adeno-associated viral vector
rcAAV	Replication competent adeno-associated viral vector
SV40	Simian virus 40
wt	Wild type

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### 3. ENVIRONMENTAL RISK ASSESSMENT

An environmental risk assessment was performed with respect to the potential risks from AMT 060/AMT 061 and is summarised in this section. Baculovirus (used as a system for AAV vector production) is not in scope of this ERA since the manufacturing process provides assurance that the risk from the presence of baculovirus in the final product is negligible.

#### 3.1 Identification of GMO characteristics which may cause adverse effects

##### Potentially Harmful Effects Related to the GMO (AMT-061)

##### **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 (AAV5-hFIX-Padua / 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

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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  $2 \times 10^{10}$  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., 2008). The triple-infected cell would in this case only produce AAV particles containing the AAV5-hFIX vector genome and particles

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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 et al., 2013; Corallini et al., 2012; Mazzoni et al., 2014). Infection in any case remains subclinical (Garcea et al., 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 et al., 2006). It is not unthinkable that SV40 sequences are present in patients during AAV5-hFIX 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

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the homologous sequences was less than 200 base pairs (Rubnitz et al., 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, levels may range from 50% to 200% of the population mean (Khachidze et al., 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  $2.3 \times 10^{14}$  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 et al., 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 8-9 fold 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.

The toxicity study with AMT-061 confirmed that a single intravenous infusion at an equal dose of  $5 \times 10^{12}$  gc/kg, AMT-060 and AMT-061 returned with a similar circulating FIX protein levels. The study also confirmed that dosing in the dose range of  $5 \times 10^{11}$  to  $9 \times 10^{13}$  gc/kg was well tolerated in non-human primates. No adverse findings were reported, although at a dose of  $9 \times 10^{13}$  gc/kg the overall clotting cascade was affected as shown by prolonged PT and shortened APTT. These effects of AMT-061 on the clotting cascade are likely a consequence of the supra-physiologic FIX activity levels that were reached after infusion of AMT-061 at the

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high dose (reaching up to 500% of normal, at the dose of  $9 \times 10^{13}$  gc/kg which is ~5x the intended clinical dose). Plasma thrombin-antithrombin complex and D-dimer levels were however not affected, suggesting that also at supra-physiological FIX (-Padua) activity levels, the overall clotting cascade was functioning within normal physiological boundaries. Nonetheless, the pharmacodynamic effect on the clotting cascade observed at this dose should be taken into consideration when considering doses higher than the planned clinical dose. The NOAEL for AMT-061 based on the study in non-human primates is set at  $9 \times 10^{13}$  gc/kg.

A potential risk by introducing a Padua-FIX is the onset of an immune response to the neotransgene product. The risk on immunogenicity of Padua-FIX has been investigated in hemophilia B dogs treated by AAV gene therapy (Finn, 2012). These authors report the absence of formation of inhibitory antibodies to the Padua-FIX protein demonstrated on multiple challenges with wild type FIX protein (even > 1 year after stopping immunosuppression). These observations were supported by the lack of IFN- $\gamma$  secretion by T-cells after exposure to wild type FIX protein peptides spanning the 338 residue with either the wild type FIX or Padua FIX amino acid sequence. Finn et al concluded that no detectable immunogenicity to Padua-FIX could be observed (Finn, 2012). These conclusions are aligned with the result of in-silico analysis performed by UniQure. The full-length wild type human FIX sequence as well as the Padua mutation were evaluated for their immunogenic potential by use of an in-silico platform for epitope identification and prediction (EpiMatrix system developed by Epivax, Inc) for both Class I (all nucleated cells) and Class II (antigen presenting cells) HLA. The accuracy of the EpiMatrix system has been thoroughly documented (Koren, 2007). The Padua mutation does not result in a significant change in EpiMatrix hits restricted by Class I or Class II HLA, with minimal observed changes in EpiMatrix score. Altogether it is concluded that the immunogenic difference between the wild type factor IX and the Padua Factor IX is insignificant.

In conclusion, 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. It has been reported that only in patients with these supraphysiological levels of Padua-FIX (>700% of normal) thrombosis may be observed (Simioni, 2009).

In case of intended exposure, i.e. in patients, the scenario of reaching extreme levels of circulating FIX activity is highly unlikely. Exposure to a dose 5x higher than the intended clinical dose needs to occur to reach supra-physiological levels, as shown in the non-human primates. It is therefore concluded that the risk of thrombosis following intended exposure to AMT-061 is negligible.

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.

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### Environmental exposure to AMT-061

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, analysis of patient samples are not within the scope of the current application as these activities will be performed within laboratories which are covered through different licenses.

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 et al., 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 AAV5-hFIX 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 AAV5-hFIX 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.

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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 about 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  $9 \times 10^{13}$  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 infectious vector particles. 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 et al., 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,

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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.

**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.

**3.2 Evaluation of the magnitude of the potential consequences and likelihood of occurrence of adverse effects: Estimation of the risk posed by each identified characteristic of the GMO**

**Likelihood estimation**

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.

**Risk estimation**

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 1.

**Table 1: Estimation of the risk posed by each identified characteristics of the GMO**

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
Immunogenicity to Padua FIX	self-inoculation	negligible	negligible	negligible
	exposure	negligible	negligible	negligible
Tumorigenicity to humans	self-inoculation	negligible	low	negligible
	exposure	negligible	negligible	negligible
Thrombosis following supraphysiological Padua FIX activity	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.

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

Therefore, no specific risk management measures are deemed necessary. Nevertheless, standard biosafety measures (outlined below) are implemented which relate to general hospital procedures which by no means relate to any risk management to reduce the already negligible risk related to the gene therapy with AMT-061:

**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 additional protective measures like safety glasses, gloves and mouth-nose mask.
- 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 are all disposables and are disposed of as biohazard waste in accordance with the local hospital procedures and GMO requirements. Non-disposable materials (tools, devices) are cleaned with a disinfectant with viricidal activity, e.g. a chlorine releasing disinfectant lyke hypochlorite containing 0.1% available chlorine (1000 ppm) after usage and then autoclaved, if possible. Contact surfaces are disinfected with a similar disinfectant.

These standard precautions will be described in the Investigational Medicinal Product handling manual which will be provided to all clinical staff involved in the preparation and administration of AMT-061.

**Accidental exposure to AAV5-hFIX**

Accidental exposure of health care professionals to AAV5-hFIX should be treated according to the measures listed in Table 2 below. These are standard measures for which it should be indicated that they are not needed to reduce the already negligible risk.

**Table 2: Measures for occupational exposure with AAV5-hFIX**

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. A spill kit will be provided to the clinical sites and pharmacy. 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

The spill procedure will be described in the Investigational Medicinal Product (IMP) handling manual.

### **3.3 Application of management strategies for risks from the deliberate release of GMO**

#### Risk Management

#### **Inclusion and exclusion criteria needed for risk mitigation**

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.

#### Measures to prevent the spread of the GMO to third parties

#### **Administration in the clinic**

Following normal clinical practices standard precautions are applied to mitigate spillage and/or aerosol formation of hazardous substances, similar to for example the application of

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cytotoxic agents. As it is concluded that AAV5-hFIX as proposed in this application poses only a negligible risk to the environment, no additional measures have been taken.

Accidental exposure and/or spilling of AAV5-hFIX are mitigated by the following standard procedures:

- Personal administering AAV5-hFIX will wear protective clothing and additional protective measures like a safety glass, gloves and mask.
- The hospital pharmacy will provide the infusion bag connected to a pre-filled tubing with sterile diluent. When spillage and/or aerosol formation will occur, this will be only diluent.
- The pre-filled tubing of the infusion bag will be connected to the main infusion tubing, which has 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 any other ancillary items used are all disposables and are disposed of as biohazard waste according local hospital procedures and GMO requirements. 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.

#### **After Administration Patient Contact**

As AMT-061 is not infectious after being shed and therefore the risk to the environment is considered negligible, standard hospital hygiene measures are sufficient and no additional measures have been taken for the period after IMP administration.

#### **Procedure in Case of Unexpected Situations and Serious Incidences**

##### Procedures when medical reasons require changes in the risk management

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.

##### **Aftercare when test subject prematurely ends participation in the 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 to identify spread of the GMO**

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

#### **3.4 Determination of the overall risk of the GMO**

The overall risk of AMT-061 to people and the environment can be considered negligible (please refer to Section 3.3. Table 1).

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### **3.5 Conclusion on the potential environmental impact from the release of the GMO**

#### Summary and Conclusions

#### **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 et al., 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 et al., 2013).

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

#### **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.

#### **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).

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**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).

**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.

**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.

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**Possible change in the current medical practice**

AAV5-hFIX 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.

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