Response to Questions

Question 1:

The acceptance criteria for the batch as listed in chapter A3.3 of the submission should also be listed in the public part of the submission. These are now only listed in the confidential part.

Answer to question 1:

Acceptance criteria referenced in A3.3 of the original application have been added to table 4 in the response to A3.2. In addition, the text of A3.3 has been adapted. Changes to the application form are included below and marked red.

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?

Test	Method	Acceptance criteria [1]	Method Sensitivity
Replication competent-AAV (rcAAV)	Bio-assay	< 10 rcAAV per 2×10^{10} gc [2]	10 rcAAV per 2 $\times10^{10}$ gc[2] (LOD [3])
Residual infectious baculovirus	Bio-assay	< 6.8 iu [4]/mL	6.8 iu/mL (LOD)
Residual baculovirus DNA	Q-PCR	≤ 8×10^{-9} geq / 1.0 x 10^{13} gc	5.0x10^{-10} geq/mL (LOQ [5])
Rep full-length sequences	Q-PCR	≤ 9×10^{8} copies/1.0 x 10^{13} gc	2.2$\times10^{7}$ copies/mL (LOQ)

[1]Acceptance criteria are in place on the active substance. [2](AAV5-hFIX-) genome copies. [3]Limit of Detection. [4]Infectious units. [5]Limit of Quantitation.

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 A3.2.

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 as outlined in A3.2. ~~Related acceptance criteria are provided as confidential information (see quality tests assessing environmental risk related parameters).~~

Question 2:

In your environmental risk assessment, you need to discuss the possible immunological or harmfull effects of the mutated factor IX protein, since this protein can possibly be regarded as foreign.

Answer to question 2:

Section A5.2. of the application form has been adapted to include a section on potential harmful effects including thrombosis and potential immunological effects. The revised text (red marked) is included below. In addition, these adverse effects are included in Table 8 presented in A5.5.

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

1..1 Risks related to the FIX transgene and the Padua modification

Increased FIX activity: AMT-060 related findings

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.

Increased FIX activity: AMT-061 related findings

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 toxicity study with AMT-061 confirmed that a single intravenous infusion at an equal dose of 5×10^{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 5×10^{11} to 9×10^{13} gc/kg was well tolerated in non-human primates. No adverse findings were reported, although at a dose of 9×10^{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 high dose (reaching up to 500% of normal, at the dose of 9 x 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 x10^{13} gc/kg.

Immunological responses

A potential risk by introducing a Padua-FIX is the onset of an immune response to the neo-transgene product. The risk on immunogenicity of FIX-Padua has been investigated in hemophilia B dogs treated by AAV gene therapy (Finn 2012). These authors report the absence of formation of inhibitory antibodies or T-cell responses against FIX following AAV-mediated expression of FIX-Padua, even after multiple challenges with wild type FIX protein (even > 1 year after stopping immunosuppression). These observations were supported by the lack of IFN-ɣ secretion by T-cells after exposure to peptides spanning the 338 residue with either the wild type FIX or FIX-Padua 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 FIX-Padua sequence 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 FIX and the Padua variant of FIX is insignificant.

In conclusion, the only risk associated with the Padua-FIX modification would be unintended achievement of supra-physiological levels of circulating FIX activity, either as the result of intended or unintended exposure. It has been reported that only in patients with these supra-physiological 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 of vector preparation or more. In addition, the probability that such unintended exposure would result in overexpression of FIX expression levels is extremely low, as explained above. Finally, the probability that overexpression of FIX or FIX-Padua 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 FIX in third parties due to unintentional exposure will result in observable effects is therefore negligible.

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.

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
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.

Question 3:

You should include the preclinical study report for of AMT-061 performed in Cynomolgus macaques in your application. The report can be submitted as confidential.

Answer to question 3:

The preclinical report on AMT-061 presenting the efficacy and safety study in Cynomolgus Monkeys is requested in the confidential part of the dossier. The study confirmed that a single intravenous infusion of AMT-061 is well tolerated by cynomolgus monkeys. No adverse findings were reported. The text in the public part of A5.2. is adapted to reflect the main conclusions.

Question 4:

You should add to the confidential section in A3.2 a short description of the Q-PCR test for the presence of Rep full-length sequences in the product.

Answer to question 4:

The confidential section in A3.2 is adapted to include a short description on the Q-PCR test for the presence of Rep full length sequences in the product. The response package includes both a track changed and clean version of the submitted confidential information.

Question 5:

For the 4 quality tests for the environmental risk related parameters in table 4 of section A3.2 validation reports should be submitted. These can be submitted as confidential.

Answer to question 5:

In response to the question the following is added to section A3.2 of the application form.
In the confidential section an overview is provided on the validation of the relevant assays including Replication competent-AAV, Residual Infectious Baculovirus, Residual Baculovirus DNA to Genome Copies and Rep full-length sequences to Genome Copy. These assays have also been used in the previous clinical trial that was approved by the GMO office for the phase I/II clinical trials on AMT-060 which is a similar AAV based vector as explained in the application. The test methods, were validated for a similar viral vector in full adherence to ICH Q2 guidelines. For each test method, matrix verification was performed for AMT-060 in order to ensure that the method was also valid for the analysis of AAV5-hFIX. No changes have been made to the method procedure. The method is performing equivalently between AMT-060 and AMT-061 and since all the assay controls are performing as expected, the assays are considered suitable for its intended use.

Question 6:

In section A4.3 of the application you should specify how the treatment room is cleaned and disinfected after treatment of a patient

Answer to question 6:

The following text (in red) is added to A4.3:

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

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. After administration the treatment room will be decontaminated with a disinfectant. A 250 ppm chlorine solution will be used for regular disinfection on used surfaces. In case of a spill, the surface will be treated with 1000 ppm chlorine solution.

Question 7:

In sections A4.7 and A4.8 it is written that biosamples are taken from treated patients. It is unclear what samples and when they are taken. This should be clarified. Are these only blood and semen, as mentioned in A4.8 or also other types of patient samples?

Answer to question 7:

Sampling of blood and semen to determine vector DNA levels will be performed at baseline and at specific time points post-baseline, by means of quantitative (real-time) polymerase chain reaction (QPCR). Sampling should continue for the individual subject and for a specific matrix until three consecutive negative samples have been detected for the subject for that particular type of matrix. Vector genome detection will be performed at Charles River Preclinical Services (United Kingdom). Based on the wish of the subject semen samples can be collected at home prior to attending the visit (at the visit day or at the day before the visit day). Also, the frequency of semen sampling may be reduced (to be agreed between investigator and subject) as long as the subject uses a condom during sexual intercourse until three consecutive negative samples have been detected. In case a subject is not able to provide semen samples due to a medical condition, this should be recorded by the investigator in the subjects' medical record.

Section A5.1. presents an overview on the clinical shedding assessment for AMT-060. These data demonstrate that shedding of vector DNA was the highest observed in the whole blood where it peaked the day after administration and subsequently rapidly declined. 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. These results justify the selection of blood and semen sampling to monitor any potential shedding.

Details on what samples are taken for study purposes and when these samples are taken is provided in sections A4.7. and A4.8.

Question 8:

You should submit the article "Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B" from Miesbach et al 2017.

Answer to question 8:

The requested article is provided electronically in the current response package. In addition a paper of Koren et al (2007) is included which is referenced in the response to Question 2.