Center for Cellular and Molecular Therapeutics at
The Children's Hospital of Philadelphia
(CCMT/CHOP)

AAV8-hFIX19

Environmental Risk Assessment Dossier
(In accordance with Schedule II, SI 500/2003)

Final – January 2013

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AAV-hFIX	Adeno-associated viral vector encoding human coagulation factor IX
AAV2-hFIX9	Single-stranded adeno-associated viral vector, serotype 2, expressing human factor IX under control of the cytomegalovirus promoter (muscle-directed)
AAV2-hFIX16	Single-stranded adeno-associated viral vector, serotype 2, encoding human factor IX under control of the human α1-antitrypsin promoter coupled to the human apolipoprotein E enhancer
AAV8-hFIX19	Single-stranded adeno-associated viral vector, serotype 8, encoding human factor IX under control of the human α1-antitrypsin promoter coupled to the human apolipoprotein E enhancer (construct changes from hFIX16 to hFIX19 include codon-optimization, removal of alternate open reading frames of the factor IX gene, and replacement of *amp* with *kan* resistance in the plasmid used for vector generation)
AAV8-LP1-hFIXco	Self complementary AAV vector, serotype 8, encoding human factor IX
Ad	Adenovirus
APH	Aminoglycoside 3'-phosphotransferase
ApoE	Apolipoprotein E
ARF	Alternate open reading frame
bp	Base pair
BSC	BioSafety Cabinet
BSL	Biosafety Level
CCMT	Center for Cellular and Molecular Therapeutics at The Children's Hospital of Philadelphia
cDNA	Complimentary deoxyribonucleic acid
CHOP	The Children's Hospital of Philadelphia
CRO	Contract research organization
DNA	Deoxyribonucleic acid
DSMB	Data and Safety Monitoring Board
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
FIX	Coagulation factor IX
GCP	Good clinical practice
GMO	Genetically Modified Organism
hAAT	Human alpha 1 antitrypsin
HCC	Hepatocellular carcinomas
HEK	Human embryonic kidney epithelial cell line
hFIX	Human coagulation factor IX
HPV	Human papilloma virus
HSV	Herpes Simplex Virus
IATA	International Air Transport Association
IBC	Institutional Biosafety Committee
IDDS	Investigational Drug Data Sheet

IM	Intramuscular
IMB	Irish Medicines Board
IND	Investigational new drug application
ITR	Inverted terminal repeats
IV	Intravenous
kan	Kanamycin
kb	Kilobases
kDa	kiloDaltons
LCA	Leber's Congenital Amaurosis
LOD	Limit of Detection
LOQ	Limit of Quantitation
LTFU	Long Term Follow-Up
MCB	Master Cell Bank
MSDS	Material Safety Data Sheet
Nab	Neutalising antibodies
NHP	Non-human primate
NPC	Nuclear Pore Complex
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PPE	Personal Protective Equipment
QPCR	Quantitative polymerase chain reaction
rAAV	Recombinant adeno-associated viral vectors
RNA	Ribonucleic acid
SAE	Serious adverse event
ss	Single stranded
SUSAR	Suspected Unexpected Serious Adverse Reaction
vg	Vector genome
WBCT	Whole blood clotting time
WHO	World Health Organisation

1. OBJECTIVE

The objective of this Environmental Risk Assessment (ERA) is to identify and evaluate potential adverse effects of AAV8-hFIX19 on human health and the environment which the deliberate release of the GMO may exert, in accordance with Schedule II of SI 500/2003.

2. GENERAL PRINCIPLE

This ERA has been performed according to the precautionary principle using the methodology set down in Schedule II of SI 500/2003 and Commission Decision 2002/623/EC. These general principles are:

- Identified characteristics of the GMO and its use which have the potential to cause adverse effects should be compared to those presented by the non-modified organism from which it is derived and its use under corresponding situations

- The ERA should be carried out in a scientifically sound and transparent manner based on available scientific and technical data.

- The ERA should be carried out on a case-by-case basis.

3. METHODOLOGY

This Environmental Risk Assessment takes into account the full Technical and Scientific Information on the GMO (AAV8-hFIX19) which are provided in a separate document in accordance with Schedule III of SI 500/2003. The characteristics of the GMO and the nature of the release are summarised below.

3.1. Intended Recipient

AAV8-hFIX19 will be administered by a single peripheral intravenous infusion into eligible, consenting adult males with X-linked severe Haemophilia B.

3.2. Parental organism

The parental virus concerned in this application is a human adeno-associated virus (AAV) with the following viral taxonomy:

Group: Group II (ss DNA)

Family: *Parvoviridae*

Subfamily: Parvovirinae

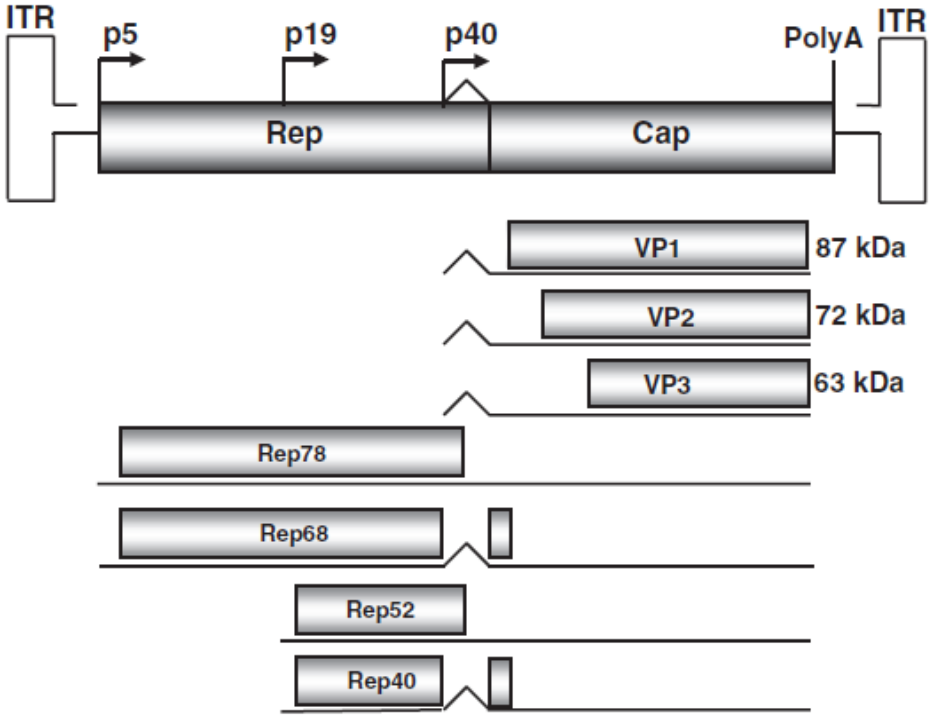
Genus: *Dependovirus*

Adeno-associated virus exists as a non-enveloped icosahedral virion with a diameter of approximately 25nm.

The AAV genome is approximately 4.7 kilobases long and comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames (ORFs): *rep* and *cap* (Figure 1). The former is composed of four overlapping genes encoding Rep proteins required for DNA replication, and the latter contains overlapping nucleotide sequences coding for capsid proteins (VP1, VP2 and VP3) which interact together to form a capsid of an icosahedral symmetry.

The Inverted Terminal Repeat (ITR) sequences comprise 145 bases each and contain all *cis*-acting functions required for DNA replication, packaging, integration into the host genome, and subsequent excision and rescue (Samulski *et al*., 1989).

Figure 1: Genome organisation of wild type adeno-associated viruses



The single-stranded DNA genome of AAV. The inverted terminal repeats (ITRs) flank the two open reading frames *rep* and *cap*. The *rep* gene encodes four nonstructural proteins – Rep78, Rep68, Rep52, and Rep40. The *cap* gene encodes three structural proteins – VP1, VP2, and VP3. The location of the promoters, p5, p19, and p40 are depicted by arrows (Van Vliet *et al*., 2008)

There are several naturally occurring serotypes of human or non-human primate adeno-associated virus (denoted AAV1 to AAV11) and further variants yet to be fully characterised. The serotype of AAV is determined by the capsid of the virion, which is integral to the species / tissue tropism and infection efficiency of AAV.

The human adeno-associated virus (AAV) was discovered in 1965 as a contaminant of adenovirus (Ad) preparations (Atchison *et al*., 1965). It is a globally endemic infection of humans, as demonstrated by the cross-reactivity of antibodies in the population to one or more AAV serotypes. Seroconversion occurs during childhood and is usually concomitant with an adenovirus infection (Reviewed in Tenenbaum *et al*., 2003).

Antibodies against AAV have been variously reported to be present in between 46-96% of individuals studied. Tenenbaum *et al.* (2003) cites studies of adults in Belgium and the USA (85-90% seropositive), France (63%), Brazil (55%), Germany (50%), and Japan (46%).

In a study by Chirmule *et al.* (1999), antibodies to AAV were seen in 96% of the subjects (patients with cystic fibrosis and healthy subjects). Boutin *et al*., (2010) reports the prevalence of IgG cross-reactivity to specific serotypes: AAV1 (67%), AAV2 (72%), AAV5 (40%), AAV6 (46%), AAV8 (38%) and AAV9 (47%).

Although human infections are common, AAV is not known to be a pathogenic virus in humans (reviewed in Tenenbaum *et al*., 2003); AAV has never been implicated as an etiological agent for any disease (Blacklow *et al.*, 1968 a,b, 1971). Wild type AAV is not classified in Risk Groups 2, 3 or 4 in the European Union (EU) according to Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work (Appendix III). It is most appropriately designated a Risk Group 1 biological agent, defined in the EU as 'one that is unlikely to cause human disease'.

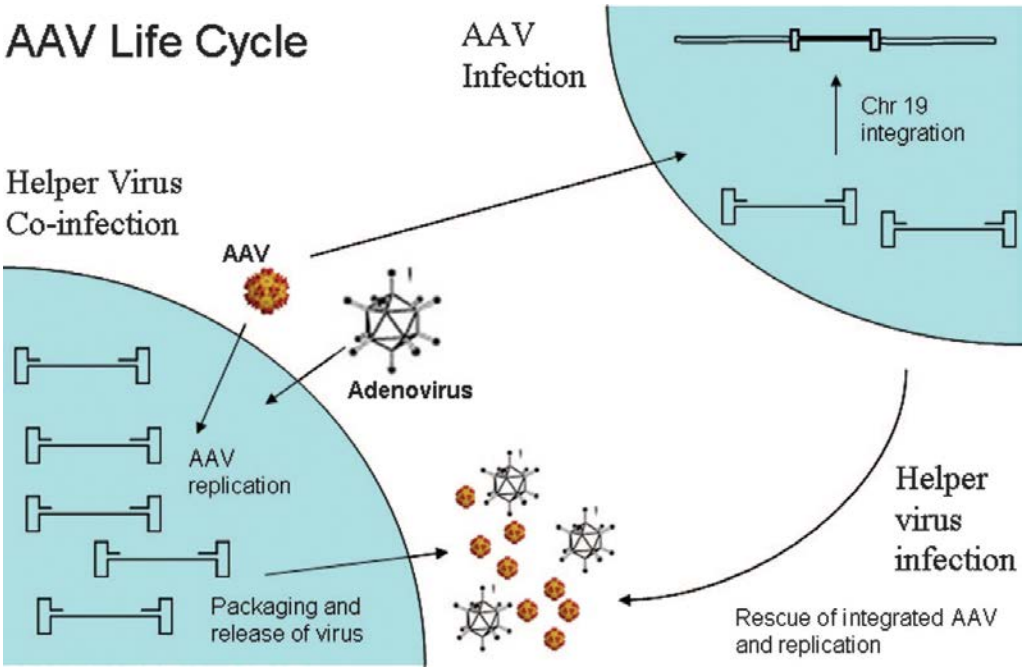
AAV is thought to be spread in nature via inhalation of aerosolized droplets, mucous membrane contact or ingestion.

Wild type AAV survives in the environment as a persistent infection in the host vertebrate species or as a latent infection in the nucleus of some infected cells, where it may remain inactive indefinitely, or be reactivated giving rise to secretion of virus (Figure 2).

In its latent state, DNA is maintained either as a stable episome or by integration into the host cell DNA (preferentially into a specific site (*AAVS1*) in human chromosome 19 in ~60% of latently infected cell lines). However, it has been recently demonstrated that only approximately 1 out of 1000 infectious units can integrate (Tenenbaum *et al*., 2003). The mechanism of this site-specific integration requires the AAV Rep proteins.

Productive (lytic) infection develops in cells co-infected with a helper virus such as adenovirus (Atchison *et al*, 1965; Henry, 1973), HPV (Hermonat, 1994; Su and Wu, 1996; Hermonat *et al*, 1997), vaccinia virus (Schlehofer *et al*, 1986) or herpes simplex virus (HSV) (Salo and Mayor, 1979; Buller *et al*, 1981) to help its replication.

Figure 2: Schematic representation of wild type AAV lytic and lysogenic lifecycle



AAV life cycle. AAV undergoes productive infection in the presence of adenovirus coinfection. This is characterized by genome replication, viral gene expression, and virion production. In the absence of adenovirus, AAV can establish latency by integrating into chromosome 19 (AAVS1). The latent AAV genome can be rescued and replicated upon superinfection by adenovirus. Both stages of AAV's life cycle are regulated by complex interactions between the AAV genome and AAV, adenoviral, and host proteins (Daya and Berns, 2008).

There are no known natural predators, preys, parasites, competitors or symbionts associated with AAV (although it does require helper functions of co-infecting viruses for replication in nature as described above). Primate (human) AAV serotypes are not known to actively transfer genetic material to organisms other than primates under natural conditions, although an absence of zoonosis is not documented.

Outside of the host, non-lipid enveloped viruses such as AAV are resistant to low level disinfectants, survive well outside of the laboratory environment and can be easily transmitted via fomites. AAV particles are resistant to a wide pH range (pH 3-9) and can resist heating at 56^{o}C for 1 hour (Berns and Bohenzky, 1987). AAV does not form survival structures but can remain infectious for at least a month at room temperature following simple desiccation or lyophilization.

Wild type AAV is not known to be involved in environmental processes. It does not respire and does not contribute to primary production or decomposition processes. In its virion form, it does not display any metabolic activity.

3.3. Genetic modification and characteristics of the GMO

AAV8-hFIX19 employs the capsid of an adeno-associated virus as a delivery vehicle for the human factor IX gene; the recombinant vector is a non-enveloped icosahedral virion of approximately 25 nm in diameter. Recombinant AAV vectors are derived from the parent virus by removing all of the viral elements except for the inverted terminal repeats (ITR) and inserting the gene or genes of interest and their associated regulatory elements.

AAV8-hFIX19 is pseudotyped with AAV8 capsid proteins, containing a single-stranded DNA genome of 4275 nucleotides consisting of an hFIX expression cassette flanked by the 145 nucleotide inverted terminal repeats derived from AAV type 2. All of the viral coding sequences have been removed and replaced with the hFIX expression cassette.

In order to potentially improve both the safety and potency of the vector, a codon-optimized hFIX gene construct was used; the hFIX cDNA was modified at the nucleotide sequence level, but NOT at the protein sequence level, to potentially achieve higher levels of expression at the same vector dose. The modifications of the hFIX cDNA sequence include amino acid codon usage optimization for expression in *Homo sapiens* and removal of alternate open reading frames (ARF) in the hFIX sequence.

The expression cassette therefore comprises:

- Human α1-antitrypsin (hAAT) liver-specific promoter coupled to the human apolipoprotein E (ApoE) enhancer/hepatocyte control region (Okuyama *et al.*, 1996; Nakai *et al*., 1999; Miao *et al.*, 2000);
- Exon 1 from the human factor IX gene;
- A portion of the human factor IX intron 1;
- Exons 2-8 of the human factor IX gene; and
- Bovine polyadenylation signal sequence.

AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, since it lacks the *rep* and *cap* genes required for rescue/packaging.

Similarly, the mechanism of the site-specific integration into the genome of the host cell involves AAV Rep proteins which are absent in AAV8-hFIX19. Accordingly, recombinant AAV (rAAV) do not integrate site-specifically, and exist in the latent form predominantly as episomal concatamers, though random integration may occur at low level (Miao *et al.*, 1998; Miao *et al.*, 2000).

AAV8-hFIX19 vector is produced by transfecting Human Embryo Kidney (HEK) 293 cells with three plasmid stocks on a batch-by-batch basis:

1) Vector plasmid pAAV2-hFIX19 containing the hFIX expression cassette flanked by AAV2 ITRs;

2) AAV packaging plasmid pAAV8PKv3, containing the AAV2 *rep* and AAV8 *cap* genes coding for non-structural and structural proteins, respectively;

3) Adenovirus helper plasmid pCCVC-AD2HPv2, encoding the adenovirus type 2 genes E2A, E4 and VA RNAs required for AAV replication in HEK293 cells.

The final plasmid stocks used in the manufacture of AAV8-hFIX19 carry the gene for resistance to Kanamycin for selection purposes, and have been amplified and tested to confirm the correct DNA sequence.

Antibiotics are not used in the manufacturing process performed at CCMT for AAV8-hFIX19. The antibiotic kanamycin is used in the manufacturing process for production of the plasmid stocks. The kanamycin resistance genes encoded by the plasmids are not by design part of the final vector, but may be present as residual impurities. Residual plasmid DNA is analysed on a batch-by-batch basis by qPCR.

Note that the required helper functions are provided as a plasmid, NOT a viable adenovirus. Nevertheless, it is possible that replication competent (wild type) AAV particles are produced in the manufacturing process through recombination of AAV *rep*, *cap* and ITR components encoded by different production plasmids. The presence of such contaminants is analysed on a batch-by-batch basis. The recently established replication competent AAV assay (providing improved sensitivity, and replacing an established infectious titre assay used for AAV2 vectors), has an LOD estimated at 10^3 wild type AAV particles in 10^{11} viral genomes.

3.4. Intended release including scale

Following the approval of the Clinical Trial Authorisation for AAV8-hFIX19, it is planned to make the product available to clinical trial site(s) in Ireland for use in accordance with a Clinical Trial Protocol entitled:

'A Phase 1 safety study in subjects with severe Hemophilia B (Factor IX deficiency) using a single-stranded, adeno-associated pseudotype 8 viral vector to deliver the gene for human Factor IX' [Protocol # AAV8-hFIX19-101].

This is a Phase 1/2 safety and dose escalation study of intravenous administration of the AAV8-hFIX19 vector to subjects with severe Haemophilia B. The primary objectives are to evaluate the safety and tolerability of the treatment. A secondary objective is to measure biologic and physiologic activity of the transgene product. It is not known if the doses used in this study will increase the level of FIX in subjects, though based on prior clinical experience it is suspected that all doses tested will result in detectable factor IX levels. The information obtained in this study will guide the design of future studies in which a benefit to subjects may be anticipated.

AAV8-hFIX19 will be administered by a single peripheral intravenous infusion into eligible, consenting adult males with X-linked severe Haemophilia B. Administration will be performed by a medical professional in a medical facility.

The investigational product will only be provided to sites on a subject-by-subject basis, following confirmation of subject eligibility and a review of registration documents/essential documents.

Eligible subjects will be admitted to the hospital on the day of AAV8-hFIX19 infusion. An intravenous catheter will be inserted into a peripheral vein, *e.g.* the median cubital vein, with normal saline fluid infusion. The vector will be thawed and prepared by the investigational pharmacy according to specific Pharmacy Instructions, and kept at room temperature prior to infusion. The vector dose, diluted in normal saline containing human serum albumin at a final concentration of 0.25% and in a volume of 200 mL, will be infused over one hour. The vector will be administered through the catheter

using an appropriate infusion pump. On completion of the infusion the catheter will be flushed with saline.

After vector administration, the catheter will be removed approximately 20 ± 4 hours after the infusion. Haemostasis at the venipuncture site will be secured by applying pressure according to standard protocol for infusing FIX concentrates. Patients will remain in-hospital for approximately 24 hours of observation to monitor for adverse effects related to the procedure.

It is anticipated that the trial will start in Ireland in Q2 2013 and the last patient (worldwide) is expected to be treated by Q3 2015. This estimate is based on the global trial start date of Q3 2012 with an estimated 4 year duration for recruitment and completion of 1 year 'active phase' for the final patient (12 months after treatment).

Doses to be administered for the proposed study in an inter-subject group dose escalation study design are 1×10^{12} vg/kg, 2×10^{12} vg/kg, and 5×10^{12} vg/kg.

The maximum total dose for an 80 kg individual patient is therefore approximately 22 mL as a single treatment (approximately 40×10^{13} vg).

Purified empty capsid material will also be added to the purified full particle vector lot prior to administration at a defined ratio of empty:full capsid particles to allow liver transduction to occur even in the presence of a pre-treatment NAb titre. Empty capsid particles do not contain vector genomes and are therefore genetically and metabolically inert.

The proposed clinical trial aims to recruit 15 evaluable patients across 4 sites in the US, Australia and Ireland. It is therefore reasonable to assume that approximately 4 patients will be treated in Ireland.

3.5. Potential receiving environment

The clinical trial is expected to be performed at a single site in Ireland:

National Centre for Hereditary Coagulation Disorders
St. James's Hospital,
James's Street,
Dublin 8,
Ireland

The investigational product will only be provided to sites on a subject-by-subject basis, following confirmation of subject eligibility and a review of registration documents/essential documents.

A qualified pharmacist with specific training on the protocol will be responsible for gene transfer material receipt from the Sponsor, storage, documentation of traceability of product at the investigational site, preparation (dilution and combination of components) on the day of administration and disposal.

In addition, all study personnel will be trained on the Clinical Trial Protocol and Handling Instructions as part of the study site initiation.

Standard biosafety practices are typically followed by medical facilities when handling injectable medicinal products and medical waste, such as:

- Restricted access
- Secure storage
- Training of personnel
- Availability of Personal Protective Equipment (PPE; laboratory coasts, gowns, gloves and safety glasses)
- Established routine practices for dealing with potentially biohazardous materials such as patient samples/fluids and medical waste (autoclaves, sharps bins, incinerators, disinfectants and appropriate cleanable surfaces).

The Investigational Drug Data Sheet (IDDS) provided with the Pharmacy Instructions instruct those involved with dose preparation to use universal precautions and appropriate Personal Protective Equipment. Dose preparation is to be performed in a BioSafety Cabinet (BSC) to reduce the risks posed by the possibility of generation and inhalation of aerosols. Once prepared, the dose (infusion bag) is labelled according to protocol (including a biohazard symbol), double bagged and taken to the administration area in a designated container.

Due to the minimal manipulations involved in dose administration it is not considered necessary to require further precautions at the point of administration, beyond the use of disposable gloves (as specified in the Handling Instructions).

Following administration of AAV8-hFIX19 at a medical facility, used (or partially used) vials of vector/ empty capsids, syringes used in dose preparation, infusion bags and infusion sets are retained and stored in a labeled biohazard bag in the pharmacy at below -60^{o}C for a period of no less than two months, per manufacturer's retention instructions (as specified in the Investigational Drug Data Sheet (IDDS) and Handling Instructions for AAV8-hFIX19). These items will then be returned to the sponsor or destroyed according to sponsor instructions.

Any other disposable instruments or other materials used during the dose preparation procedure will be disposed of in a manner consistent with the standard practice of the institution for potentially biohazardous materials.

In the medical facility, this will involve temporary containment in sharps bins or clearly marked bags (e.g. biohazard, medical waste) prior to autoclaving and/or incineration either on or off site as per local institutional guidelines for handling potentially infectious materials.

All non-disposable equipment and other materials used during the procedure will be cleaned using a chemical disinfectant capable of virucidal activity for the required duration of contact, or sterilized by autoclaving consistent with local institutional guidelines for handling potentially infectious materials.

Typically, standard operating procedures for disposal within medical facilities (where the potential for contamination from other agents is potentially much more hazardous than that presented by AAV8-hFIX19) will be consistent with the guidance given in the WHO Laboratory Biosafety Manual, 3rd Ed (2004) for BSL1/2 as outlined below:

Contaminated (infectious) "sharps":
Hypodermic needles, scalpels, knives and broken glass; should always be collected in puncture-proof containers fitted with covers and treated as infectious.

After use, hypodermic needles should not be recapped, clipped or removed from disposable syringes. The complete assembly should be placed in a sharps disposal container. Disposable syringes, used alone or with needles, should be placed in sharps disposal containers and incinerated, with prior autoclaving if required. Sharps disposal containers must be puncture-proof/-resistant and must not be filled to capacity. When they are three-quarters full they should be placed in "infectious waste" containers and incinerated, with prior autoclaving if laboratory practice requires it. Sharps disposal containers must not be discarded in landfills.

Contaminated (potentially infectious) materials for autoclaving and reuse:
No pre-cleaning should be attempted of any contaminated (potentially infectious) materials to be autoclaved and reused. Any necessary cleaning or repair must be done only after autoclaving or disinfection.

Contaminated (potentially infectious) materials for disposal:
Apart from sharps, which are dealt with above, all contaminated (potentially infectious) materials should be autoclaved in leak-proof containers (e.g. autoclavable, color-coded plastic bags), before disposal. After autoclaving, the material may be placed in transfer containers for transport to the incinerator. If possible, materials deriving from healthcare activities should not be discarded in landfills even after decontamination. If an incinerator is available on the laboratory site, autoclaving may be omitted; the contaminated waste should be placed in designated containers (e.g. color-coded bags) and transported directly to the incinerator. Reusable transfer containers should be leakproof and have tight-fitting covers. They should be disinfected and cleaned before they are returned to the laboratory for further use.

4. IDENTIFICATION OF CHARACTERISTICS WHICH MAY CAUSE ADVERSE EFFECTS

Characteristics which may cause adverse effects are considered based on the pathogenicity of the parent organism (wild-type AAV), the nature of the genetic modifications made to construct AAV8-hFIX19 and the nature of release and receiving environment as summarised in Section 3.

4.1. Potential effects on the environment

Wild type AAV is not known to be involved in environmental processes. It does not respire and does not contribute to primary production or decomposition processes. In its virion form, it does not display any metabolic activity.

There are no known natural predators, preys, parasites, competitors or symbionts associated with wild type AAV (although it does require helper functions of co-infecting viruses for replication in nature as described above. Primate (human) AAV serotypes are not known to actively transfer genetic material to organisms other than primates under natural conditions, although an absence of zoonosis is not documented.

AAV8-hFIX19 is pseudotyped with AAV8 capsid proteins (originally isolated from primates), and contains a single-stranded DNA genome of 4275 nucleotides consisting of an hFIX expression cassette flanked by the 145 nucleotide inverted terminal repeats derived from AAV type 2. All of the viral coding sequences have been removed and replaced with the hFIX expression cassette.

The hFIX cDNA is modified at the nucleotide sequence level, but NOT at the protein sequence level, and therefore does not represent an additional hazard beyond the natural endogenous human protein. Similarly, the regulatory elements in the expression cassette are derived from natural human-specific sequences or in the case of the bovine polyadenylation signal, have been extensively utilised in molecular biology applications with no known risks to the environment.

None of these modifications are anticipated to have any effect on the host range, stability or survival of the GMO when compared to that of the wild-type virus, nor would they be expected to result in the transfer or maintenance of genetic material in the environment (outside humans).

AAV8-hFIX19 itself does not present specific resistance to antibiotics. The virus does not contain any gene that confers resistance to antibiotics of interest in terms of human or animal health.

AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, since it lacks the *rep* and *cap* genes required for rescue/packaging. Therefore, the presence of the expression cassette is expected to confer a severe selective disadvantage to the GMO.

Genetic material may theoretically be transferred to microorganisms, though this would normally require homologous recombination (ie. require replication in the presence of a microorganism with similar ITR sequences). In addition, the expression cassette does not contain microbial promoters, nor would the transgene be anticipated to confer any selective advantage.

A potential contaminant of AAV8-hFIX19 is wild type primate (human) AAV derived from the manufacturing process. Similarly, it is theoretically possible to generate wild type AAV in the recipient which may be shed into the environment. Homologous recombination between AAV8-hFIX19 and a wild type AAV could occur if both were present in the same cell. However, such recombination could only result in the exchange of the hFIX expression cassette with the *rep* and *cap* genes of the wild type virus. It is not possible for the AAV genome to contain both *rep/cap* genes and the transgene, as this is beyond the packaging limit of the virion.

In the rare event that wild type AAV, supplying the requisite replication gene products, were to co-infect a hepatocyte, along with a helper DNA virus such as adenovirus or herpes simplex virus and AAV8-hFIX19 (a triple co-infection), it is possible that vector replication could occur. The resulting virologic outcome would be increased synthesis of AAV8-hFIX19 and wild type AAV.

A further potential contaminant of AAV8-hFIX19 is residual plasmid DNA derived from the manufacturing process. The presence of contaminating plasmids could conceivably lead to limited distribution in the environment of sequences of DNA

contained therein which could persist for a short time in the prevailing conditions prior to degradation. Of most concern would be the genes for kanamycin resistance contained on each plasmid for selection purposes.

4.2. Potential effects on human health

Wild type AAV survives in the environment as a persistent infection in the host vertebrate species or as a latent infection in the nucleus of some infected cells, where it may remain inactive indefinitely, or be reactivated giving rise to secretion of virus.

Outside of the host, non-lipid enveloped viruses such as AAV are resistant to low level disinfectants, survive well outside of the laboratory environment and can be easily transmitted via fomites. AAV particles are resistant to a wide pH range (pH 3-9) and can resist heating at 56^{o}C for 1 hour (Berns and Bohenzky, 1987). AAV does not form survival structures but can remain infectious for at least a month at room temperature following simple desiccation or lyophilization.

AAV is thought to be spread in nature via inhalation of aerosolized droplets, mucous membrane contact or ingestion.

Although AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, since it lacks the *rep* and *cap* genes required for rescue/packaging, it is possible that it could be transmitted to unintended human recipients.

AAV is not known to be a pathogenic virus in humans (reviewed in Tenenbaum *et al*., 2003); AAV has never been implicated as an etiological agent for any disease (Blacklow *et al.*, 1968a,b, 1971).

The transgene encoded by AAV8-hFIX19 is a protein, and is identical to normal human coagulation Factor IX. The gene product is therefore expected to be metabolised naturally.

The human immune response to AAV is reviewed in Daya and Berns (2008). Almost no innate immune response is seen in AAV infection (Zaiss *et al*., 2002). The host defense mechanism at the adaptive level is primarily made up of a humoral response (Xiao *et al*., 1996). Pre-existing antibodies in patients, because of prior infection, account for the humoral response seen toward AAV. In a study by Chirmule *et al.* (1999), antibodies to AAV were seen in 96% of the subjects (patients with cystic fibrosis and healthy subjects), and 32% showed neutralizing ability in an in vitro assay.

The cell-mediated response functions at the cellular level, eliminating the transduced cells using cytotoxic T cells. Cell-mediated responses to AAV vectors have been documented, but this response may be dependent on the route of administration (Brockstedt *et al*., 1999).

Despite the lack of evidence for pathogenicity, correlations have been made between:

i). the occurrence of male infertility and the presence of AAV viral DNA sequences in human semen (Rohde *et al*., 1999)

ii). the occurrence of miscarriage and the presence of infectious AAV in embryonic material as well as in the cervical epithelium (Tobiasch *et al.*, 1998; Walz *et al.*, 1997).

A clear association is hard to establish from these studies, given that co-incident evidence of human papillomavirus infection is present in most subjects (Malhomme *et al.*, 1997), and that AAV DNA can be detected in cervical samples in the majority of women (Burguete *et al.*, 1999) but is very dependent on differences in sample collection between studies (Erles *et al.*, 2001). The possible causal role of AAV in the occurrence of miscarriage is the finding that AAV 2 interferes with mouse embryonic development (Botquin *et al.*, 1994). Furthermore, a significant correlation has been established between the presence of AAV DNA in amnion fluids and premature amniorrhexis and premature labour (Burguete *et al.*, 1999).

An additional, theoretical, risk of AAV infection is the risk of insertional mutagenesis caused by non-site specific integration of the AAV genome into the host-cell genome of infected cells. Such an event carries the risk of malignant transformation leading to cancer. There is no documented causal link between AAV infection and malignancies in humans, but it has been shown that wild type AAV may integrate at sites other than chromosome 19: Schnepp *et al.*, 2005 identified an AAV-cellular DNA junction in a human tissue sample (tonsil) which was mapped to a highly repetitive satellite DNA element on chromosome 1. However, they also demonstrated that the majority of wild-type AAV DNA persisted as circular double-stranded episomes in the tissues following naturally acquired infection.

4.3. Conclusions

In conclusion, based on the nature of the GMO, the parental organism and the receiving environment, the deliberate release of AAV8-hFIX19 is not anticipated to have any direct effects on the environment (other than humans).

The potential direct effects in humans are limited to the transmission of AAV8-hFIX19 to an unintended human recipient. These potential adverse effects are expected to be the same as those which may be anticipated in patients receiving the treatment (immune response, potential for insertional mutagenesis and potential for germline transmission).

Indirect effects of the release are limited to the consequences of the release of wild type AAV (through contamination of the medicinal product during manufacture or following recombination in the recipient's cells followed by shedding into the environment) and the possible fate of contaminating DNA sequences derived from the manufacturing process.

5. EVALUATION OF POTENTIAL CONSEQUENCES / MAGNITUDE OF EFFECT

The potential consequences of the possible adverse effects identified in Section 4 of the ERA are considered in this section.

5.1. Direct effects

The potential direct effects in humans are limited to the transmission of AAV8-hFIX19 to an unintended human recipient.

5.1.1. Magnitude of effect

Cases of the transmission of AAV8-hFIX19 to an unintended human recipient are likely to be isolated. The medicinal product will be administered to (and handled by) a limited number of individuals. It is estimated that 4 patients will receive the treatment at the medical facility in Ireland. Any inadvertent exposure will be self-limiting, since AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, as it lacks the *rep* and *cap* genes required for rescue/packaging.

The most likely individuals who may be affected would be:

- Healthcare workers involved in the preparation and administration of AAV8-hFIX19, and obtaining clinical samples as per the Clinical Trial Protocol (blood, saliva)
- Laboratory workers involved in sample preparation and analysis of blood, saliva, urine, semen as per the Clinical Trial Protocol
- Close contacts of the treated individual (partners and family members) who may potentially be exposed to any shed vector via saliva, semen, urine or faeces.

The capacity for widespread dissemination of AAV8-hFIX19 is expected to be severely limited due to:

- Attenuation of the GMO rendering it even less replication competent than the parental virus (AAV2/8), by deletion of the replication genes.
- Intravenous administration to eligible patients by medical professionals in a medical facility.
- Limited host and tissue tropism (human/primate) of the parental virus (AAV2/8)
- Low and transient incidence of shedding of infective virus from treated individuals (Nathwani *et al.*, 2011a,b)
- High levels of existing adaptive immunity in the human population

5.1.2. Potential consequences

The potential direct effects in humans are limited to the transmission of AAV8-hFIX19 to an unintended human recipient. The consequences of the transmission of AAV8-hFIX19 to an unintended human recipient are expected to be no greater than those which may be anticipated in patients receiving the treatment (immune response, potential for insertional mutagenesis and potential for germline transmission).

Neither wild type AAV nor the experimental vector AAV8-hFIX19 is known to be pathogenic to humans. AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, since it lacks the *rep* and *cap* genes required for rescue/packaging.

The transgene encoded by AAV8-hFIX19 is a protein, and is identical to normal human coagulation Factor IX. The gene product is therefore expected to be metabolised naturally. It is clear, based on data from infusion of FIX concentrates (ie. the Factor IX protein) into patients with haemophilia B, that circulating FIX levels as high as 100% of normal are not associated with ill-effects since the protein circulates as a zymogen (inactive precursor). In patients receiving the full, high dose of AAV8-hFIX19, it is postulated that hFIX expression will lead to circulating levels of FIX in the region of 2-10% of normal. Thus, the effect of expression from an inadvertently acquired 'dose' of AAV8-hFIX19 may be expected to have a negligible effect on circulating hFIX in a non-haemophiliac.

The possible effects of direct administration to patients are summarised below. In the case of transfer of vector to an unintended human recipient, the risks are expected to be considerably reduced, since the vector is not able to replicate and the 'dose' which may conceivably be transferred (from e.g. aerosol, splashing or fomites) will be orders of magnitude lower than that received by patients.

Potential risks of direct administration of AAV8-hFIX19 to human haemophiliacs

The long-term safety of recombinant AAV vectors in humans is unknown; however, AAV vectors have been delivered to several hundred human subjects to date, in trials for cystic fibrosis, rheumatoid arthritis, Leber's congenital amaurosis, α_1-antitrypsin deficiency, congestive heart failure, lipoprotein lipase deficiency, as well as haemophilia, and have been remarkably free of vector-related adverse events (Mingozzi and High, 2011).

In earlier trials of AAV mediated hFIX delivery in the treatment of haemophilia B, eight men were injected via the hepatic artery with AAV2-hFIX16 at doses ranging from 8×10^{10} vg/kg to 2×10^{12} vg/kg, during a period 2001-2004. Long-term follow-up has revealed no vector-related late complications (Manno *et al*., 2006; Wellman *et al*., 2012). In a subsequent trial conducted in 2009-2012, ten men were injected intravenously with doses of a self-complementary AAV8-hFIX (AAV8-LP1-hFIXco) ranging from 2×10^{11} vg/kg to 2×10^{12} vg/kg, again with no late complications noted to date (Nathwani *et al*., 2011a; Davidoff *et al*., 2012).

Potential immune response

Administration of AAV8-hFIX19 is likely to lead to the production of a humoral immune response to AAV and might lead to an antibody response to factor IX protein. In clinical trial results with similar constructs to date, anti-AAV neutralizing antibodies have been detected in all subjects after vector infusion; however, none have shown anti-FIX neutralizing antibodies (inhibitors). An antibody directed against the vector capsid may affect the response to a subsequent administration of vector and subjects in this trial may be precluded from subsequent treatment with AAV vectors. There is a possibility that subjects infused with AAV8-hFIX19 will develop neutralising antibodies to FIX, although this has not occurred in any of the eight subjects previously infused with an AAV2-hFIX16 vector via the hepatic artery (Manno *et al*., 2006), in any of the ten subjects receiving a self-complementary AAV8-hFIX vector (AAV8-LP1-hFIXco) by peripheral vein infusion (Nathwani *et al*., 2011a; Davidoff *et al*., 2012), or in the eight subjects injected with an AAV2-FIX9 vector in skeletal muscle (Manno *et al*., 2003).

A cell-mediated response to AAV8-hFIX19 may give rise to elevated transaminases. Two subjects infused with either $4x10^{11}$ vg/kg or $2x10^{12}$ vg/kg of AAV2-hFIX16 developed asymptomatic elevation of transaminases beginning four weeks after vector infusion and resolving to baseline by twelve weeks post vector infusion without specific treatment (Manno *et al.*, 2006). Two of six reported subjects infused with AAV8-LP1-hFIXco, those at the high dose of $2x10^{12}$ vg/kg, developed elevation of hepatic transaminases (one above the upper limit of normal). Both of these subjects were treated with a short, tapering course of prednisolone and resolved the elevated transaminases; one suffered a modest reduction in FIX levels, but remained with a level greater than his baseline, while the other maintained his FIX level throughout the reported period of observation (Nathwani *et al.*, 2011a). The level of transaminase elevations noted in previous trials are not high; this may be considered an issue that affects efficacy, rather than one that affects safety, since the transaminase elevation to date has been self-limited and asymptomatic.

As previously stated, the potential for the induction of a significant immune response to AAV8-hFIX19 is expected to be diminished in unintended recipients, since the scale of exposure would likely be orders of magnitude less than that received by patients.

Potential for insertional mutagenesis

AAV vectors have the potential to integrate into the genome of transduced cells. The major potential risk resulting from integration into the host cell DNA is an enhanced risk of malignant transformation leading to cancer.

It is assumed that the greatest potential for integration would be into cells within the liver, but given the results of tissue distribution studies of NHPs with similar (AAV8 pseudotyped) vectors (AAV8-LP1-hFIXco; Nathwani *et al.*, 2011b; see Section II.C.2.(i) 3), the potential for integration into cells of other tissues also exists.

At high multiplicity of infection, wild type AAV integrates into human chromosome 19 in ~60% of latently infected cell lines. However, it has been recently demonstrated that only approximately 1 out of 1000 infectious units can integrate (Tenenbaum *et al.*, 2003). The mechanism of this site-specific integration involves AAV Rep proteins which are absent in AAV8-hFIX19. Accordingly, recombinant AAV (rAAV) do not integrate site-specifically. Random integration of vector sequences has been demonstrated in established cell lines but only in some cases and at low frequency in primary cultures and *in vivo*. In contrast, Duan *et al.*, 1998 demonstrated prolonged persistence of head-to-tail circular intermediates in muscle tissue at 80 days postinfection, suggesting that a large percentage of rAAV genomes may remain episomal. It has also been shown that, following liver transduction, (Miao *et al.*, 1998; Miao *et al.*, 2000) rAAV is stabilized predominantly in a non-integrated form; however, integration does occur at some low level.

The site(s) of integration have been analyzed using 454 pyrosequencing and bioinformatics analysis to characterize > 1000 integration sites from mouse liver injected at high doses with an AAV2-hFIX16 vector. These data (Li *et al.*, 2011) confirmed earlier reports (Nakai *et al.*, 2005) of preferential integration into actively transcribed genes, CPG islands, and GC-rich regions. Note that these data are relevant, since both AAV2 and AAV8 vectors utilize the AAV2 ITRs, the vector element

involved in integration. It is important to note that the integration mechanism of AAV differs from that of other viruses, such as retroviruses. Retroviral vectors contain the proteins needed to cause double-stranded DNA breaks for integration. AAV vectors do not contain such proteins and must rely on the cellular machinery. In fact, it is currently thought that AAV vectors may preferentially integrate into genomic DNA regions that are already broken (Miller *et al.*, 2004).

A mouse model of AAV infusion into liver during the neonatal period led to an increased number of hemangiosarcomas and hepatocellular carcinomas (HCC) after prolonged periods, and for at least some of the HCC tumors, an integration event in mouse chromosome 12 was associated with the tumor tissue but not the normal adjacent tissue (Donsante *et al.*, 2007). Other studies have not confirmed this association, although the studies may have been hampered by inadequate numbers of mice (Li *et al.*, 2011). Still, the risk of such an event is suspected to be low based on absence of malignant transformation in large haemophilic animals (haemophilia B dogs) followed for over ten years after AAV vector infusion (Niemeyer *et al.*, 2009; Tim Nichols, personal communications). In addition, liver ultrasounds carried out in 4/7 human subjects injected with AAV2-hFIX16 from 2001-2004 have shown no evidence of liver tumors. Of the remaining 3 subjects: 1 has died of causes unrelated to gene transfer, and autopsy showed no evidence of tumor in the liver; 1 was lost to follow-up; and 1 has declined to undergo a liver ultrasound but remains in good health (Wellman *et al.*, 2012).

Again, the potential for insertional mutagenesis mediated by AAV8-hFIX19 is expected to be diminished further in unintended recipients, since the scale of exposure would likely be orders of magnitude less than that received by patients.

Potential for germline transmission

In the AAV2-FIX16 liver gene transfer trial (Manno *et al.*, 2006), vector genome sequences were detected in semen; semen samples from all eight subjects cleared within sixteen weeks. A series of non-clinical studies was performed to demonstrate: 1) the transient nature of this finding in both humans and animals; and 2) that vector sequences were not detected in spermatocytes (Arruda *et al.*, 2001; Couto and Pierce, 2003; Couto *et al.*, 2004; Manno *et al.*, 2006). The sponsor has performed a series of studies using rabbits, a valuable model for assessing germline transmission risk for humans. It was determined that following intravenous injection of AAV2, the duration of detection of vector genomes in the semen is dose-dependent and time-dependent, with genomes diminishing over time until they are completely undetectable; AAV infectious particles were only present up to day 4 post-injection and undetectable thereafter (Schuettrumpf *et al.*, 2006). The sensitivity of the nested PCR used in these studies was greater than 1 copy per 6000 cells. Rabbit studies were also performed with AAV8 vectors (Favaro *et al.*, 2009). In these studies, AAV2 and AAV8 vectors were compared, showing that the kinetics of vector clearance from semen was dose- and time-dependent but serotype-independent. Furthermore, AAV2 or AAV8 sequences were detected in the semen of vasectomized animals that lack germ cells, leading to the conclusion that the genitourinary tract, as well as the testis, contributes significantly to vector shedding in the semen. These studies are supported by findings in humans (Nathwani *et al.*, 2011a; see Section II.C.2.(i) 3), in which vector genomes were detected in semen only briefly after AAV8-LP1-hFIXco vector systemic administration; clearance was reported by 15

days post-administration. These results suggest that the risk of inadvertent germline transmission in males by AAV8 vectors is low, similar to that of AAV2.

Given the dose-dependent kinetics of clearance from semen, the risk of germline transmission mediated by AAV8-hFIX19 is expected to be diminished further in unintended recipients, since the scale of exposure would likely be orders of magnitude less than that received by patients.

5.2. Indirect effects

Indirect effects of the release are limited to the consequences of the release of wild type AAV (through contamination of the medicinal product during manufacture or following recombination in the recipient's cells followed by shedding into the environment) and the possible fate of contaminating DNA sequences derived from the manufacturing process.

5.2.1. Magnitude of effect

Cases of the transmission of a replication competent AAV contaminant of AAV8-hFIX19 to an unintended human recipient are likely to be isolated, even if such a contaminant existed. The medicinal product will be administered to (and handled by) a limited number of individuals. It is estimated that 4-8 patients will receive the treatment at the medical facility in Ireland. Any inadvertent exposure will be limited in its potential for spread, since wild type AAV requires the presence of a co-infecting helper virus for replication.

The generation of a wild type AAV from the experimental product requires homologous recombination of AAV8-hFIX19 with a co-infecting wild type AAV and a co-infecting helper virus (triple infection). It is not possible to generate a replication competent AAV containing the transgene. The number of individuals in whom exposure could occur is again limited to healthcare and laboratory workers involved in the Clinical Trial Protocol, and close contacts of the treated individuals (see Section 5.1.1).

The capacity for widespread dissemination of wild type AAV derived from the treatment is expected to be limited due to:

- Requirement for a co-infecting helper virus for replication
- Intravenous administration to eligible patients by medical professionals in a medical facility
- Limited host and tissue tropism (human/primate) of the parental virus (AAV2/8)
- Low and transient incidence of shedding of infective virus from treated individuals (Nathwani *et al.*, 2011a,b)
- High levels of existing adaptive immunity in the human population

In relation to possible contaminating DNA derived from the plasmids used in the manufacture of AAV8-hFIX19, the magnitude of effect is likely to be minimal. These DNA sequences are not capable of replication independently. Following administration

to a patient, residual DNA sequences will be degraded via normal routes. It is possible they will persist in the patient cells if the sequences are encapsidated, but again they will be unable to replicate independently. The maximum exposure to the environment which could be envisaged would be as a consequence of an untreated spill or breakage prior to administration. Given the low quantities of residual contaminating plasmid DNA in the product (as quantified for each batch of AAV8-hFIX19), the disposal procedures in place at medical facilities, and the small scale of the release, the amount of residual plasmid DNA disseminated into the environment is expected to be negligible.

5.2.2. Potential consequences

The presence of wild-type AAV, either as a contaminant of AAV8-hFIX19 derived during manufacture, or as an indirect consequence of homologous recombination with an existing wild type AAV in an intended human recipient, is not considered to constitute an increased risk to the environment, since it is already globally endemic and is not associated with pathogenesis.

The consequences of dissemination of DNA derived from the plasmids used to manufacture AAV8-hFIX19 are theoretically uptake and integration into microbial genomes, although infection of microbes by AAV is not described to our knowledge. Such an event could result in transient expression of a plasmid gene, but the genes present are unlikely to confer any selective advantage and consequently be quickly lost. The possible exception is the transfer of the gene for Kanamycin resistance, which would confer a selective advantage in certain environments, though likely not in sewerage and water systems.

5.3. Conclusions

The potential magnitude of any identified direct or indirect effect of AAV8-hFIX19 on the environment is low.

For those unintended individuals that may be exposed to AAV8-hFIX19, the potential adverse effects are expected to be of a lower severity than those expected in patients receiving considerably higher doses, and of no greater severity than wild type AAV, which is not known to be pathogenic. The consequences of indirect effects of release of residual plasmid DNA or wild type AAV are low.

In conclusion, the potential consequences in the case of exposure of AAV8-hFIX19 (or a contaminating or derived wild type AAV) to an unintended individual, or the dissemination of contaminating residual plasmid DNA to the environment, are expected to be LOW LEVEL and ISOLATED.

6. EVALUATION OF LIKELIHOOD OF OCCURRENCE OF IDENTIFIED ADVERSE EFFECT

The potential direct adverse effects and the magnitude and consequence of those effects described in the preceding sections of the ERA are dependent on the likelihood of exposure of unintended recipients or the environment to AAV8-hFIX19, wild type AAV (as a contaminant of, or derived from the experimental vector) or residual plasmid DNA.

This in turn is influenced by the manner, scale and environment of release (see Sections 3.4 and 3.5), the potential mechanisms of exposure and the specific risk management measures in place to minimise exposure (see Section 8).

6.1. Likelihood of transmission of AAV8-hFIX19 to an unintended human recipient

Wild type AAV survives in the environment as a persistent infection in the host vertebrate species or as a latent infection in the nucleus of some infected cells, where it may remain inactive indefinitely, or be reactivated giving rise to secretion of virus (Figure 2).

AAV is thought to be spread in nature via inhalation of aerosolized droplets, mucous membrane contact or ingestion.

Outside of the host, non-lipid enveloped viruses such as AAV are resistant to low level disinfectants, survive well outside of the laboratory environment and can be easily transmitted via fomites. AAV particles are resistant to a wide pH range (pH 3-9) and can resist heating at 56^{o}C for 1 hour (Berns and Bohenzky, 1987). AAV does not form survival structures but can remain infectious for at least a month at room temperature following simple desiccation or lyophilization.

The survival and route of transmission of AAV8-hFIX19 is not expected to be different from that of wild type AAV. However, AAV8-hFIX19 is unable to replicate, even in the presence of a helper virus due to the deletions of the *rep* and *cap* genes.

The route of exposure will therefore most likely be through direct contact with the investigational product, as opposed from potential secondary exposure resulting from shedding of the vector from treated patients.

The potential for direct exposure to AAV8-hFIX19 will be limited to those healthcare professionals trained in the Clinical Trial Protocol (ie. those involved in dose preparation and administration). The likelihood of exposure during dose administration (attaching or removing the pre-prepared infusion bag/kit to the patient via a pre-existing intravenous catheter) is considered extremely low.

During dose preparation by a trained pharmacist, several manipulations are required to prepare the correct dose, potentially involving multiple vials of AAV8-hFIX19. In this case, the risk of exposure is limited by the use of a Biosafety Cabinet during dose preparation (as well as Personal Protective Equipment). The precautions to be taken (including use of Personal Protective Equipment (PPE)) during dose preparation and administration are described in the Investigational Drug Data Sheet (IDDS) and Handling Instructions for AAV8-hFIX19 (see Section 8 for details). The availability of appropriate PPE is universal in a hospital setting.

The most-likely route of exposure will be via accidental needle-stick injury.

Therefore, given the scale of the release in Ireland (treatment of approximately 4-8 patients on a single occasion), it is considered that the likelihood of exposure of a healthcare professional to AAV8-hFIX19 is very low.

Such exposure would be limited to the healthcare professional involved, since the virus is unable to replicate even in the presence of a helper virus.

The likelihood of secondary exposure to the virus from clinical samples is also considered very low, given the scale of release, low level transient shedding of the vector (Nathwani *et al.*, 2011a,b) and the universal precautions routinely applied when obtaining or analysing clinical samples (which may potentially contain considerably more hazardous viruses than AAV8-hFIX19).

Instructions for the disposal of waste and for the handling of accidental spills and breakages are provided in the Investigational Drug Data Sheet (IDDS) and Handling Instructions for AAV8-hFIX19 (see Section 8 for details). Appropriate waste disposal procedures for potentially infective materials are universally applied in a healthcare setting.

In addition, routine cleaning procedures and practices are universally applied in a healthcare setting, where the potential for contamination from other agents is potentially much more hazardous than that presented by AAV8-hFIX19.

Finally, the likelihood of secondary exposure to close contacts of patients who receive the treatment is also considered very low. AAV8-hFIX19 cannot replicate in the human body, and shedding through saliva, urine, faeces and semen is expected to be low level and transient. In the Clinical Trial Protocol, patients are required to use an effective barrier contraceptive until at least two consecutive semen samples after vector administration are negative for vector sequences (see Section 8). Note that detection of a vector sequence does not imply infective virus.

6.2. Likelihood of transmission of wild type AAV to an unintended human recipient

Exposure to wild type AAV may theoretically be mediated by the investigational product by:

1. Contamination of AAV8-hFIX19 with replication competent (wild type) AAV during manufacture

2. Homologous recombination of an existing wild type AAV with AAV8-hFIX19 in a patient's cells (co-infected with a helper virus)

In each of these scenarios, the likelihood of transmission is considered even lower than that of transmission of AAV8-hFIX19. The potential routes of exposure will be the same as described for the AAV8-hFIX19 vector itself (Section 6.1).

Each batch of the experimental product is tested for the absence of replication competent AAV to assure low levels of contamination. Thus the quantity of wild type AAV which may be present in the product is low. The likelihood of a homologous recombination event within the patient's cells is also very low in itself, requiring a triple infection and further since the vector target cells (liver) are not the natural target cells of helper viruses.

6.3. Likelihood of exposure of the environment to residual plasmid sequences

Each batch of the experimental product is tested prior to lot release for the amount of residual plasmid DNA in the product. These DNA sequences are not capable of replication independently. Following administration to a patient, residual DNA sequences will be degraded via normal routes. It is possible they will persist in the patient cells if the sequences are encapsidated, but again they will be unable to replicate independently. Therefore the only likely route of contamination of the environment would be through exposure of the product itself to microorganisms.

Instructions for the disposal of waste and for the handling of accidental spills and breakages are provided in the Investigational Drug Data Sheet (IDDS) and Handling Instructions for AAV8-hFIX19 (see Section 8 for details). Appropriate waste disposal procedures for potentially infective materials are universally applied in a healthcare setting.

Therefore, given the small scale of the release and the handling and disposal procedures in place, it is considered that the likelihood of exposure of the environment to residual plasmid DNA sequences is very low.

6.4. Conclusions

Consideration of the manner, scale and environment of release, the mode of transmission and survivability of the parent organism (wild-type AAV), and the risk management measures in place, it is considered that the likelihood of exposure of an unintended recipient to AAV8-hFIX19 or wild type AAV mediated by AAV8-hFIX19 administration is low or very low.

The likelihood of exposure of the environment to residual plasmid DNA sequences is also very low.

In conclusion, the likelihood of exposure of AAV8-hFIX19 (or a contaminating or derived wild type AAV) to an unintended individual, or the dissemination of contaminating residual plasmid DNA to the environment, is LOW.

7. ESTIMATION OF RISK POSED BY EACH IDENTIFIED CHARACTERISTIC

The risk posed by AAV8-hFIX19 on human health (specifically an unintended recipient) or the environment is considered by combining the estimated consequences of the effect with the estimated likelihood of effect (in accordance with 2001/18/EC and 2002/623/EC). This estimation is made with reference to the risk attributed to the parental organism (wild type AAV) for context.

7.1. Risk associated with the parental organism (wild type AAV)

For context, it is useful to consider the risk associated with wild type AAV itself.

The human adeno-associated virus (AAV) was discovered in 1965 as a contaminant of adenovirus (Ad) preparations (Atchison *et al.*, 1965). It is a globally endemic infection of humans, as demonstrated by the cross-reactivity of antibodies in the population to

one or more AAV serotypes. Seroconversion occurs during childhood and is usually concomitant with an adenovirus infection (Reviewed in Tenenbaum *et al*., 2003).

Antibodies against AAV have been variously reported to be present in between 46-96% of individuals studied. Tenenbaum *et al.* (2003) cites studies of adults in Belgium and the USA (85-90% seropositive), France (63%), Brazil (55%), Germany (50%), and Japan (46%).

In a study by Chirmule *et al.* (1999), antibodies to AAV were seen in 96% of the subjects (patients with cystic fibrosis and healthy subjects). Boutin *et al*., (2010) reports the prevalence of IgG cross-reactivity to specific serotypes: AAV-1 (67%), AAV2 (72%), AAV5 (40%), AAV6 (46%), AAV8 (38%) and AAV9 (47%).

Although human infections are common, AAV is not known to be a pathogenic virus in humans (reviewed in Tenenbaum *et al*., 2003); AAV has never been implicated as an etiological agent for any disease (Blacklow *et al*., 1968a,b, 1971).

Wild type AAV and its clinical effects are therefore widespread and well understood.

Wild type AAV is not classified in Risk Groups 2, 3 or 4 in the European Union (EU) according to Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work (Appendix III). It is most appropriately designated a Risk Group 1 biological agent, defined in the EU as ‘one that is unlikely to cause human disease’.

Similar classifications of hazard have been assigned to AAV according to the definitions of the World Health Organisation (WHO), and in the US, Canada and Australia as summarized in Table 1.

It should be noted that these classifications are based on the effects on healthy workers, and does not consider effects on individuals with altered susceptibility which may be as a result of pre-existing disease, medication, compromised immunity, pregnancy or breast feeding.

Conversely, the classification does not consider genetically modified micro-organisms which are attenuated or have lost known virulence genes, or carry a transgene.

Table 1: Biosafety classifications for wild type AAV outside the EU

Territory	Category	Definition	Reference
WHO	**Risk Group 1** (no or low individual and community risk)	A microorganism that is unlikely to cause human or animal disease.	WHO Laboratory Biosafety Manual, 3rd Ed (2004).
US	**Risk Group 1**	Agents that are not associated with disease in healthy adult humans.	NIH Recombinant DNA Guidelines (USA, 2011). Appendix B-I. Not listed under 42CFR73.3 – Select Agents and Toxins.
Canada	**Risk Group 1** (low individual and community risk)	Any biological agent that is unlikely to cause disease in healthy workers or animals.	Canadian Laboratory Safety Guidelines (2004). Human Pathogens and Toxins Act. S.C. 2009, c. 24. Not listed in Schedules II, II, IV.
Australia/NZ	**Risk Group 1** (low individual and community risk)	A microorganism that is unlikely to cause human or animal disease.	Standard AS/NZS 2243.3:2010. Safety in laboratories Part 3: Microbiological safety and containment. Standards. Association of Australia/New Zealand. Not listed in Tables 3.4, 3.7 or 3.8.

7.2. Risk associated with the transmission of AAV8-hFIX19 to an unintended recipient

The consequences of transmission of AAV8-hFIX19 to an unintended human recipient are considered 'low' (See Section 5.1), and no greater than those which may be anticipated in patients receiving the treatment, or by infection with wild type AAV (see above).

Neither wild type AAV nor the experimental vector AAV8-hFIX19 is known to be pathogenic to humans. AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, since it lacks the *rep* and *cap* genes required for rescue/packaging.

The transgene encoded by AAV8-hFIX19 is a protein, and is identical to normal human coagulation Factor IX. The gene product is therefore expected to be metabolised naturally. It is clear, based on data from infusion of FIX concentrates (ie. the Factor IX protein) into patients with haemophilia B, that circulating FIX levels as high as 100% of normal are not associated with ill-effects since the protein circulates as a zymogen (inactive precursor). In patients receiving the full, high dose of AAV8-hFIX19, it is postulated that hFIX expression will lead to circulating levels of FIX in the region of 2-10% of normal. Thus, the effect of expression from an inadvertently acquired 'dose' of AAV8-hFIX19 may be expected to have a negligible effect on circulating hFIX in a non-haemophiliac.

The possible effects of direct administration to patients are the development of an immune response, potential for insertional mutagenesis and potential for germline transmission. In the case of transfer of vector to an unintended human recipient, these risks are expected to be considerably reduced, since the vector is not able to replicate and the 'dose' which may conceivably be transferred (from e.g. aerosol, splashing or fomites) will be orders of magnitude lower than that received by patients.

Further the magnitude of any adverse effect is expected to be low, and confined to isolated cases, given that AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus.

The likelihood of transmission of AAV8-hFIX19 is considered 'low' (see Section 6.1).

Its mode of transmission and survivability are unchanged by the genetic modifications made to the wild type virus. However, the scale of the release (approximately 4 patients in Ireland), limited number of unintended recipients who may be at risk of exposure, and precautions in place for dose preparation and administration, handling of clinical samples and disposal/ decontamination (see Section 8) combine to significantly reduce the likelihood of exposure. The likelihood of secondary exposure through vector shedding is also low, due to the inability of the vector to replicate (even in the presence of helper virus) and the low and transient levels of vector shedding anticipated. The use of barrier contraception until 2 consecutive semen samples are negative for vector DNA reduces the likelihood of secondary transmission further (see Section 8).

Thus, through a combination of the low level consequences of transmission and the low likelihood of this occurring, the overall risk posed by AAV8-hFIX19 to the unintended recipient is considered **LOW** (with reference to Table 2: Guideline on Environmental

Risk Assessments for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs)).

7.3. Risk associated with transmission of wild type AAV to an unintended human recipient

The consequences of transmission of wild type AAV (mediated by the experimental vector) to an unintended recipient is considered 'low' (See Section 5.2).

Exposure to wild type AAV may theoretically be mediated by the investigational product by:

1. Contamination of AAV8-hFIX19 with replication competent (wild type) AAV during manufacture

2. Homologous recombination of an existing wild type AAV with AAV8-hFIX19 in a patient's cells (co-infected with a helper virus)

The presence of wild type AAV, either as a contaminant of AAV8-hFIX19 derived during manufacture, or as an indirect consequence of homologous recombination with an existing wild type AAV in an intended human recipient, is not considered to constitute an increased risk to human beings or the environment, since it is already globally endemic and is not associated with pathogenesis.

The likelihood of transmission of wild type AAV is considered even lower than that of transmission of AAV8-hFIX19. The potential routes of exposure will be the same as described for the AAV8-hFIX19 vector itself (Section 6.1). However, each batch of the experimental product is tested for the absence of replication competent AAV to assure low levels of contamination. Thus the quantity of wild type AAV which may be present in the product is low. The likelihood of a homologous recombination event in the patient's cells is also very low in itself.

Thus, through a combination of the low level consequences of transmission and the low likelihood of this occurring, the overall risk posed by wild type AAV (mediated by the experimental vector) to the unintended recipient is considered **LOW** (with reference to Table 2: Guideline on Environmental Risk Assessments for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs)).

7.4. Risk to the environment associated with residual plasmid DNA sequences

The magnitude and consequences of exposure of the environment to residual plasmid sequences in AAV8-hFIX19 are considered low.

The consequences of dissemination of DNA derived from the plasmids used to manufacture AAV8-hFIX19 are theoretically uptake and integration into microbial genomes, although infection of microbes by AAV is not described to our knowledge. Such an event could result in transient expression of a plasmid gene, but the genes present are unlikely to confer any selective advantage and consequently be quickly lost. The possible exception is the transfer of the gene for Kanamycin resistance, which would confer a selective advantage in certain environments, though likely not in sewerage and water systems.

Each batch of the experimental product is tested for the amount of residual plasmid DNA in the product. These DNA sequences are not capable of replication independently. Following administration to a patient, residual DNA sequences will be degraded via normal routes. It is possible they will persist in the patient cells if the sequences are encapsidated, but again they will be unable to replicate independently. Therefore the only likely route of contamination of the environment would be through exposure of the product itself to microorganisms.

Instructions for the disposal of waste and for the handling of accidental spills and breakages are provided in the Investigational Drug Data Sheet (IDDS) and Handling Instructions for AAV8-hFIX19 (see Section 8 for details). Appropriate waste disposal procedures for potentially infective materials are universally applied in a healthcare setting.

Therefore, given the small scale of the release and the handling and disposal procedures in place, it is considered that the likelihood of exposure of the environment to residual plasmid DNA sequences is very low.

Thus, through a combination of the low level consequences of exposure and the low likelihood of this occurring, the overall risk posed by residual plasmid DNA sequences to the environment is considered **LOW** (with reference to Table 2: Guideline on Environmental Risk Assessments for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs).

8. APPLICATION OF MANAGEMENT STRATEGIES FOR RISKS

8.1. Design of viral construct

AAV8-hFIX19 is pseudotyped with AAV8 capsid proteins, containing a single-stranded DNA genome of 4275 nucleotides consisting of an hFIX expression cassette flanked by the 145 nucleotide inverted terminal repeats derived from AAV type 2. All of the viral genes have been removed and replaced with the hFIX expression cassette.

Homologous recombination between AAV8-hFIX19 and a wild type AAV could occur if both were present in the same cell. However, such recombination could only result in the exchange of the hFIX expression cassette with the *rep* and *cap* genes of the wild type virus. It is not possible for the AAV genome to contain both *rep/cap* genes and the transgene.

8.2. Control of release

Following the approval of the Clinical Trial Authorisation for AAV8-hFIX19, it is planned to make the product available to clinical trial site(s) in Ireland for use in accordance with a Clinical Trial Protocol entitled:

‘A Phase 1 safety study in subjects with severe Hemophilia B (Factor IX deficiency) using a single-stranded, adeno-associated pseudotype 8 viral vector to deliver the gene for human Factor IX’ [Protocol # AAV8-hFIX19-101].

The manufacture, supply and traceability of AAV8-hFIX19 is therefore tightly controlled and monitored in accordance with medicines regulation.

The investigational product will only be provided to sites on a subject-by-subject basis, following confirmation of subject eligibility and a review of registration documents/essential documents.

A qualified pharmacist with specific training on the protocol will be responsible for gene transfer material receipt from the Sponsor, storage, documentation of traceability of product at the investigational site, preparation (dilution and combination of components) on the day of administration and disposal.

Adequate records of study drug receipt and disposition will be maintained by the study site Investigational Pharmacy, and records of receipts, investigational drug orders, dispensing records, and disposition forms will be examined during the course of the study. The purpose of these records is to ensure that the investigational new drug will not be distributed to any person outside the terms and conditions set forth in the Clinical Trial Protocol. The study medication is to be prescribed by the Investigator or designee and may not be used for any purpose other than that described in the Clinical Trial Protocol.

8.3. Dose preparation and administration precautions

AAV8-hFIX19 will be administered by a single peripheral intravenous infusion into eligible, consenting adult males with X-linked severe Haemophilia B. Administration will be performed by a medical professional in a medical facility.

AAV8-hFIX19 vector is derived from a virus and should be considered and handled as an infectious agent. Given the non-replicative nature of the modified organism, the nature of the transgene, and the fact that the parent organism is not known to be pathogenic, BSL-1 procedures are appropriate in the clinical setting. As a precaution, the Sponsor designates the AAV8-hFIX19 vector as BSL-2.

Key handling practices and containment requirements for BSL-1 and BSL-2 organisms obtained from globally available biosafety guidelines are presented in Table 2 and Table 3.

As a minimum, institutional risk assessments are required to determine appropriate precautions for the work tasks and processes.

Standard biosafety practices are similar amongst the guidelines and are typically followed by medical facilities when handling injectable medicinal products and medical waste, such as:

- Restricted access
- Secure storage
- Training of personnel
- Availability of Personal Protective Equipment (PPE; laboratory coats, gowns, gloves and safety glasses)

- Established routine practices for dealing with potentially biohazardous materials such as patient samples/fluids and medical waste (autoclaves, sharps bins, incinerators, disinfectants and appropriate cleanable surfaces).

The Investigational Drug Data Sheet (IDDS) provided with the Pharmacy Instructions instruct those involved with dose preparation to use universal precautions and appropriate Personal Protective Equipment. Dose preparation is to be performed in a BioSafety Cabinet (BSC) to reduce the risks posed by the possibility of generation and inhalation of aerosols. Once prepared, the dose (infusion bag) is labelled according to protocol (including a biohazard symbol), double bagged and taken to the administration area in a designated container.

Due to the minimal manipulations involved in dose administration it is not considered necessary to require further precautions at the point of administration, beyond the use of disposable gloves (as specified in the Handling Instructions).

Instructions for dealing with spills/breakages and accidental exposure are provided in the Handling Instructions.

The worker protection measures proposed for the preparation and administration of AAV8-hFIX19 are therefore in line with those recommended globally for the handling of BSL-1/2 organisms in a research setting. An additional precaution of dose preparation in a BioSafety Cabinet is also specified.

Table 2: Summary of Recommended Containment Precautions for BSL-1 Infectious Agents

Region	Practices	Personal Protective Equipment	Primary barriers	Secondary Barriers (Facilities)
WHO Laboratory Biosafety Manual (3rd Ed., 2004). Pages 2-3 and 9-15.	Good microbiological technique	Laboratory coats and gloves; eye, face protection, as needed	No primary barriers required	Laboratory bench and sink required
EU Directive 90/219/EEC as amended. Annex IV; Table IA	Good microbiological laboratory practices	Suitable protective clothing	No primary barriers required	Bench - Surfaces resistant to water, acids, alkalis, solvents, disinfectants, decontamination agents and easy to clean Autoclave on site
US Biosafety in Microbiological and Biomedical Laboratories (5th Ed., 2009). Page 59.	Standard microbiological practices	Laboratory coats and gloves; eye, face protection, as needed	No primary barriers required	Laboratory bench and sink required
Canada Canadian Biosafety Guidelines 3rd Ed., 2004. Page 19-22	Good microbiological laboratory practices	Laboratory coats and gloves; eye, face protection, as needed	No primary barriers required	Laboratory bench and sink required Autoclave available
Australia/ New Zealand AS/NZS 2243.3:2010 Safety in laboratories - Microbiological safety and containment Pages 38-40	Limited access Biohazard warning signs Staff shall be trained in the clean-up of microbiological spills	Laboratory coats and gloves; eye, face protection, as needed	No primary barriers required	Laboratory bench and sink required

Table 3: Summary of Recommended Containment Precautions for BSL-2 Infectious Agents

Region	Practices	Personal Protective Equipment	Primary barriers	Secondary Barriers (Facilities)
WHO Laboratory Biosafety Manual (3rd Ed., 2004). Pages 2-3 and 9-19.	BSL-1 plus: Limited access Biohazard warning signs	As BSL-1	BSCs for procedures with a high potential for producing aerosols	BSL-1 plus: Autoclave or other means of decontamination should be available in appropriate proximity
EU Directive 90/219/EEC as amended. Annex IV; Table IA	BSL-1 plus: Limited access Biohazard warning signs	As BSL-1 plus: Gloves (optional)	Minimise aerosol dissemination	BSL-1 plus: Autoclave in the building
US Biosafety in Microbiological and Biomedical Laboratories (5th Ed., December 2009). Page 59	BSL-1 plus: Limited access Biohazard warning signs "Sharps" precautions Biosafety manual defining any needed waste decontamination or medical surveillance policies	As BSL-1	BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials	BSL-1 plus: Autoclave available
Canada Canadian Biosafety Guidelines 3rd Ed., 2004. Page 22-23	BSL-1 plus: Limited access Biohazard warning signs Training and/or supervision of staff commensurate with their anticipated activities in the containment area Written procedures for emergencies (eg. spills/ clean up) must be available	As BSL-1	BSCs must be used for procedures that may produce infectious aerosols and that involve high concentrations or large volumes of biohazardous material.	As BSL-1
Australia/ New Zealand AS/NZS 2243.3:2010 Safety in laboratories - Microbiological safety and containment. Pages 40-44	BSL-1 plus: Instruction and training in handling infectious microorganisms shall be provided to laboratory personnel with regular updates	As BSL-1	If the work produces a significant risk from the production of infectious aerosols, a biological safety cabinet shall be used.	BSL-1 plus: Autoclave available

8.4. Waste treatment

Waste generated from the preparation and infusion of AAV8-hFIX19 will be limited to:

- Used vials of the Investigational Medicinal Product
- Used vials of empty capsid (containing no DNA)
- Used preparation equipment in the pharmacy; syringes, needles, vials
- Used Infusion bags and infusion kits
- Bags used to transport potentially contaminated equipment to and from the pharmacy
- Used swabs and items used to clean injected area
- Personal Protective Equipment used during dose preparation and administration

Following administration of AAV8-hFIX19 at a medical facility, used (or partially used) vials of vector/ empty capsids, syringes used in dose preparation, infusion bags and infusion sets are retained and stored in a labeled biohazard bag in the pharmacy at below -60^{o}C for a period of no less than two months, per manufacturer's retention instructions (as specified in the Investigational Drug Data Sheet (IDDS) and Handling Instructions for AAV8-hFIX19). These items will then be returned to the sponsor or destroyed according to sponsor instructions.

Any other disposable instruments or other materials used during the dose preparation procedure will be disposed of in a manner consistent with the standard practice of the institution for potentially biohazardous materials.

Typically, standing procedures for disposal within hospitals (where the potential for contamination from other agents is potentially much more hazardous than that presented by AAV8-hFIX19) will be consistent with the guidance given in the WHO Laboratory Biosafety Manual, 3rd Ed (2004) for materials potentially contaminated with BSL1/2 infective agents (see Section 3.5).

The genetic modifications made during the construction of AAV8-hFIX19 from wild type AAV do not affect its sensitivity to physical and chemical inactivation.

Effective disinfectants require a minimum of 20 minutes contact time. Adeno-Associated virus is susceptible to sodium hypochlorite (1-10% dilution of fresh bleach), alkaline solutions at pH >9 and 5% phenol. Alcohol is not an effective disinfectant against AAV. AAV is inactivated by autoclaving for 30-45 minutes at 121^{o}C (CCMT/CHOP MSDS; adeno-associated viral vectors).

8.5. Product Labelling

The product labelling and information contains essential information to minimise the risk of exposure to an unintended individual or the environment. Copies of the documents referenced below are provided as Appendices to a separate document in accordance with Schedule III of SI 500/2003.

- The Investigational Medicinal Product is supplied as a frozen liquid at a volume of 1 mL in a 1.5 mL polypropylene sterile cryogenic screw cap vial and labelled according to Pharmaceutical Law (Annex 13 of EU GMP guidelines), containing at least:

  Investigational product name (AAV8-hFIX19); manufacturer (CCMT, Children's Hospital of Philadelphia PA, USA); specific lot number; date of manufacture; vial number; storage instructions (< -60°C); Clinical Trial Protocol number (Protocol # AAV8-hFIX19-101) and investigational product warning (For Clinical Trial Use Only).

- The Investigational Drug Data Sheet (IDDS) / Pharmacy Instructions for AAV8-hFIX19 contain instructions on product receipt from the Sponsor, storage and documentation of traceability at the investigational site.

- The Investigational Drug Data Sheet (IDDS) / Pharmacy Instructions contain instructions on dose preparation, including use of a BioSafety Cabinet, Personal Protective Equipment and storage (bagged with biohazard label) / disposal of potentially contaminated items.

- The Investigational Drug Data Sheet (IDDS) / Pharmacy Instructions contain instructions for labelling and packaging the prepared dose (in infusion bag) for transport to the administration site. This label includes a Biohazard symbol.

- The Clinical Trial Protocol requires patients to be hospitalised for the first 24 hours following infusion.

- The Clinical Trial Protocol and Patient Information (as Part of the Informed Consent document) describe the frequency and procedure for obtaining clinical samples.

- The Clinical Trial Protocol and Patient Information (as Part of the Informed Consent document) describe the requirement for the use of barrier contraception until 2 consecutive monthly semen samples are shown to be negative for vector sequences.

- The Handling Instructions (to be supplied to all personnel involved in dose preparation and administration) contain information on the administration procedure (including personal protective equipment), disposal procedures and procedures to be followed in the case of spills or occupational exposure.

8.6. Monitoring activities

8.6.1. Monitoring during treatment of patients

Patients will be monitored throughout treatment by the Principal Investigator and delegates. An independent Data Safety Monitoring Board (DSMB) will oversee data and safety monitoring, with interval meetings based on DSMB guidelines. Independent

Contract Research Organisations (CROs) will be used for study monitoring and data management activities. Any serious adverse event will be reported in the appropriate time-frame to the Sponsor, and as required to each of the national regulatory agencies according to pharmaceutical legislation.

After vector administration, vital signs will be monitored hourly for 6 hours and then every 2 hours for 6 hours and then at 4-hour intervals. Patients will remain in hospital for approximately 24 hours of observation to monitor for adverse effects relating to the procedure.

A comprehensive battery of laboratory evaluations, comprising primarily of blood tests, will be conducted at regular intervals to assess safety throughout the first year following administration. Safety tests will include PCR testing for vector shedding in saliva, blood and urine at each weekly visit until week 12 (or until 2 consecutive samples are negative). PCR testing of semen will be performed at each monthly visit until 2 consecutive samples are negative.

Factor IX activity/antigen will be monitored weekly until 12 weeks, and monthly thereafter until Month 12.

8.6.2. Follow-up of patients after treatment

Patients will be subject to long term follow-up as described below:

In Years 2 to 5 following vector administration, an annual physical exam will be performed with complete history and a subset of laboratory evaluations for safety and Factor IX activity (as a surrogate test to indicate vector persistence).

The subject's body fluids will be monitored only during the first year of the study for persistent vector sequences. It is not anticipated, based on non-clinical studies and prior clinical experience, that testing for vector sequences will be necessary in the long-term follow-up period.

Subjects will be encouraged to monitor themselves and to assist in reporting adverse events; they will be provided with laminated wallet-sized cards with investigator contact information. Additionally, health care professionals from the subject's home haemophilia centre, who are not otherwise associated with the clinical trial, will be notified to provide prompt reports of adverse events to the investigators.

Investigators will maintain in the case history records of exposures to mutagenic agents and other medicinal products along with subjects' adverse event profiles.

Clinical information will focus on information pertaining to new malignancies, new incidence or exacerbation of a pre-existing neurological disorder, new incidence or exacerbation of a prior rheumatologic or other autoimmune disorder, and new incidence of a hematologic disorder.

For the subsequent ten years, subjects will be contacted at a minimum of once per year. A clinical questionnaire, administered by telephone call or at the subject's home haemophilia treatment centre, will focus on information pertaining to new malignancies, new incidence or exacerbation of a pre-existing neurological disorder, new incidence or exacerbation of a prior rheumatologic or other autoimmune disorder, and new incidence of a hematologic disorder. It will ask subjects to describe any adverse events, including any unexpected illness and/or hospitalization, and provide a description of exposures to mutagenic agents and other medicinal products. Additionally, ultrasound of the target organ, the liver, will be performed every three years for the fifteen year long-term follow-up period.

8.6.3. Monitoring of unintended recipients

No monitoring of unintended recipients is planned or considered necessary.

8.7. Conclusions

Appropriate risk management strategies are in place to minimise the risks of exposure to unintended individuals or the environment. Appropriate monitoring strategies are proposed to gather further information on safety, persistence and shedding prior to further (wider-scale) development.

9. DETERMINATION OF OVERALL RISK OF THE GMO

Following the approval of the Clinical Trial Authorisation for AAV8-hFIX19, it is planned to make the product available to clinical trial site(s) in Ireland for use in accordance with a Clinical Trial Protocol entitled:

'A Phase 1 safety study in subjects with severe Hemophilia B (Factor IX deficiency) using a single-stranded, adeno-associated pseudotype 8 viral vector to deliver the gene for human Factor IX' [Protocol # AAV8-hFIX19-101].

Doses to be administered for the proposed study in an inter-subject group dose escalation study design are 1×10^{12} vg/kg, 2×10^{12} vg/kg, and 5×10^{12} vg/kg.

The maximum total dose for an 80 kg individual patient is therefore approximately 22 mL as a single treatment (approximately 40×10^{13} vg).

The proposed clinical trial aims to recruit 15 evaluable patients across 4 sites in the US, Australia and Ireland. It is therefore reasonable to assume that approximately 4-8 patients will be treated in Ireland.

AAV8-hFIX19 will be administered by a single peripheral intravenous infusion into eligible, consenting adult males with X-linked severe Haemophilia B. Administration will be performed by a medical professional in a medical facility.

AAV8-hFIX19 employs an adeno-associated virus as a delivery vehicle for the human factor IX gene; the recombinant vector is a non-enveloped icosahedral virion of approximately 25 nm in diameter.

AAV8-hFIX19 is pseudotyped with AAV8 capsid proteins, containing a single-stranded DNA genome of 4275 nucleotides consisting of an hFIX expression cassette flanked by the 145 nucleotide inverted terminal repeats derived from AAV type 2. All of the viral genes have been removed and replaced with the hFIX expression cassette.

AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, since it lacks the *rep* and *cap* genes required for rescue/packaging.

Although human infections are common, wild type AAV is not known to be a pathogenic virus in humans. Wild type AAV is not classified in Risk Groups 2, 3 or 4 in the European Union (EU) according to Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work (Appendix III). It is most appropriately designated a Risk Group 1 biological agent, defined in the EU as 'one that is unlikely to cause human disease'.

There are no known natural predators, preys, parasites, competitors or symbionts associated with AAV (although it does require helper functions of co-infecting viruses for replication in nature as described above). Primate (human) AAV serotypes are not known to actively transfer genetic material to organisms other than primates under natural conditions, although an absence of zoonosis is not documented.

Wild type AAV is not known to be involved in environmental processes. It does not respire and does not contribute to primary production or decomposition processes. In its virion form, it does not display any metabolic activity.

None of the genetic modifications made to wild type AAV during construction of AAV8-hFIX19 would be expected to alter its effect on environmental processes. As such, there is no expected impact to the environment as a whole following the release of AAV8-hFIX19.

Based on the nature of the GMO, the parental organism and the receiving environment, the deliberate release of AAV8-hFIX19 is not anticipated to have any direct effects on the environment (other than humans).

The potential direct effects in humans are limited to the transmission of AAV8-hFIX19 to an unintended human recipient. These potential adverse effects are expected to be the same as those which may be anticipated in patients receiving the treatment (immune response, potential for insertional mutagenesis and potential for germline transmission).

Indirect effects of the release are limited to the consequences of the release of wild type AAV (through contamination of the medicinal product during manufacture or following recombination in the recipient's cells followed by shedding into the environment) **and the possible fate of contaminating DNA sequences derived from the manufacturing process**.

The potential magnitude of unintended spread within the human population is considered low. Cases of the transmission of AAV8-hFIX19 to an unintended human recipient are

likely to be isolated, and transmission of AAV vectors to any unintended human recipient has not been reported. The medicinal product will be administered to (and handled by) a limited number of individuals. It is estimated that 4-8 patients will receive the treatment at the medical facility in Ireland. Any inadvertent exposure will be self-limiting, since AAV8-hFIX19 is unable to replicate independently, even in the presence of a helper virus, as it lacks the *rep* and *cap* genes required for rescue/packaging.

For those unintended individuals that may be exposed to AAV8-hFIX19, the potential adverse effects are expected to be of a lower severity than those expected in patients receiving considerably higher doses, and of no greater severity than wild type AAV, which is not known to be pathogenic.

The presence of wild-type AAV, either as a contaminant of AAV8-hFIX19 derived during manufacture, or as an indirect consequence of homologous recombination with an existing wild type AAV in an intended human recipient, is not considered to constitute an increased risk to the environment, since it is already globally endemic and is not associated with pathogenesis.

The consequences of dissemination of DNA derived from the plasmids used to manufacture AAV8-hFIX19 are theoretically uptake and integration into microbial genomes, although infection of microbes by AAV is not described to our knowledge. Such an event could result in transient expression of a plasmid gene, but the genes present are unlikely to confer any selective advantage and consequently be quickly lost. The possible exception is the transfer of the gene for Kanamycin resistance, which would confer a selective advantage in certain environments, though likely not in sewerage and water systems.

Therefore, the potential consequences in the case of exposure of AAV8-hFIX19 (or a contaminating or derived wild type AAV) to an unintended individual, or the dissemination of contaminating residual plasmid DNA to the environment, are expected to be LOW LEVEL and ISOLATED.

The survival and route of transmission of AAV8-hFIX19 is not expected to be different from that of wild type AAV.

The route of exposure will therefore most likely be through direct contact with the investigational product, as opposed from potential secondary exposure resulting from shedding of the vector from treated patients.

The potential for direct exposure to AAV8-hFIX19 will be limited to those healthcare professionals trained in the Clinical Trial Protocol (ie. those involved in dose preparation and administration). Procedures are in place to minimise this risk, including information provided to healthcare personnel relating to dose preparation, administration and disposal of potentially contaminated items. Dose preparation is performed in a BioSafety cabinet.

The likelihood of secondary exposure to close contacts of patients who receive the treatment is also considered very low. AAV8-hFIX19 cannot replicate in the human body, and shedding through saliva, urine, faeces and semen is expected to be low level and

transient. The Clinical Trial Protocol specifies that patients are required to use an effective barrier contraceptive until at least two consecutive semen samples after vector administration are negative for vector sequences (see Section 8).

The likelihood of transmission of wild type AAV is considered even lower than that of transmission of AAV8-hFIX19. The potential routes of exposure will be the same as described for the AAV8-hFIX19 vector itself. However, each batch of the experimental product is tested for the absence of replication competent AAV to assure low levels of contamination. Thus the quantity of wild type AAV which may be present in the product is extremely low. The likelihood of a homologous recombination event in the patient's cells is also very low in itself.

Given the small scale of the release and the handling and disposal procedures in place, it is considered that the likelihood of exposure of the environment to residual plasmid DNA sequences is very low.

In conclusion, the likelihood of exposure of AAV8-hFIX19 (or a contaminating or derived wild type AAV) to an unintended individual, or the dissemination of contaminating residual plasmid DNA to the environment, is LOW.

The risk posed by AAV8-hFIX19 on human health (specifically an unintended recipient) and the environment is considered by combining the estimated consequences of the effect with the estimated likelihood of effect (in accordance with 2001/18/EC and 2002/623/EC). This estimation is made with reference to the risk attributed to the parental organism (wild type AAV) for context and 'Table 2: Guideline on Environmental Risk Assessments for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs)'.

Thus, through a combination of the low level consequences of transmission and the low likelihood of this occurring, **the overall risk posed by AAV8-hFIX19 to the unintended recipient is considered LOW.**

Similarly, through a combination of the low level consequences of transmission and the low likelihood of this occurring, **the overall risk posed wild type AAV (mediated by the experimental vector) to the unintended recipient is considered LOW.**

Finally, through a combination of the low level consequences of exposure and the low likelihood of this occurring, **the overall risk posed by residual plasmid DNA sequences to the environment is considered LOW.**

Appropriate risk management strategies are in place to minimise the risks of exposure to unintended individuals or the environment. Appropriate monitoring strategies are proposed to gather further information on safety, persistence and shedding prior to further (wider-scale) development.

In conclusion, overall the environmental risks associated with the deliberate release of AAV8-hFIX19 under the conditions of release proposed, and with the precautions and monitoring activities proposed, is considered acceptable.

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