Application form

Assessment of clinical research involving gene therapeutics in the Netherlands

7 januari 2009

Application form
Assessment of clinical study involving gene therapeutics

Part A: Bio-safety aspects
Part B: Patient-related aspects

7 januari 2009

CONTENTS

Consent form

The undersigned:

Hereby gives his/her consent to the gene therapy office to send on the application forms and the accompanying file to the relevant assessment authorities.

Gives his/her consent to the assessment authorities to use the fully completed file in making its assessment.

Gives his/her consent to the assessment authorities to inform each other and the gene therapy office about the status of the submitted application.

Signed:

Name Linda Rutgrink, Clinical Project Leader Sanofi-aventis NL B.V.

Date 16/jan/2009

A Bio-safety aspects

This part of the application form provides the information needed for the Ministry of Housing, Spatial Planning and the Environment (VROM) to grant the necessary licences.
All information provided in this form and the accompanying documentation constitutes part of the decision to be made and for this reason is in principle open to inspection by the public; the information will also be available for such public inspection during the procedure.
The applicant may ask for parts of the information provided to be kept confidential. In that case, the applicant must give reasons why the information is of a confidential nature as well as a convincing explanation that the lifting of confidentiality will adversely affect the applicant's competitive position. A publicly available summary of confidential information must be given, containing enough information for a clear general understanding of the application and in order to assess the risk analysis as described in the application and the decision.

An application does not need to be limited to the specific clinical protocol that the applicant wishes to perform. If there are no consequences for the risk analysis, the application can be drawn up with a wider scope, such as for a larger number of patients, an alternative way of administering the GMO, possibly in combination with other non-GMOs. Take note: such additions CANNOT be made later to a decision already given!

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

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

A1 Details of applicant

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

Legal entity

A1.1. Board of Directors, Catharina Hospital

A1.2. Board of Directors

A1.3. Michelangelolaan 2

A1.4. 5623 EJ Eindhoven

A1.5. 040 2399111

Met opmaak: Nederlands (standaard)

Responsible employees (RE)

Should the work described so require, a distinction will be made between an RE (contact person) for work other than the clinical application (RE-1, such as a pharmacist or researcher) and an RE (contact person) for the clinical application (RE-II, such as the treating doctor).

Responsible Employee for work other than the clinical application of the GMO (RE-I)

A1.6. Dr MRHM van Sambeek MD PhD

A1.7. Catharina Hospital

A1.8. Department of Surgery

A1.9. Michelangelolaan 2

A1.10. 5623 EJ Eindhoven

A1.11. Tel: 040 239 7150
Fax: 040 244 3370

A1.12. marc.v.sambeek@cze.nl

Responsible Employee for the clinical application of the GMO (RE-II)

A1.13. Dr MRHM van Sambeek MD PhD

A1.14. Catharina Hospital

A1.15. Department of Surgery

A1.16. Michelangelolaan 2

A1.17. 5623 EJ Eindhoven

A1.18. Tel: 040 239 7150
Fax: 040 244 3370

A1.19. marc.v.sambeek@cze.nl

Environmental Safety Officer (ESO)

A1.20. Title, initial, prefix, surname: dr. K.E. Bakker

A1.21. Institution/company: Erasmus MC

A1.22. Department/section: Dept. Occupational Health & Environmental Protection

A1.23. Correspondence address: P.O. Box 2040
A1.24. Postcode and town/city: 3000 CA Rotterdam

A1.25. Telephone and fax number: tel: 010 – 703 1488; fax; 010-703 3918

A1.26. E-mail address: k.bakker@erasmusmc.nl

Study location

A1.27. At which locations will the intended work take place?
Since the work applied for may only be carried out under the direct control of the licence holder, it is only possible to carry out work at several locations if the licence holder has full control of the way in which the work being applied for is carried out at all locations, in such a way that the licence conditions are complied with. In that case you must state for each location what work will be carried out at which address and in which building. To clarify: you must state for all activities at which location they will be carried out. Apart from the location for the clinical activities with the GMO, you must also state the location or locations of laboratories in which activities with the GMO are carried out under the terms of this licence application, such as procedures with patient samples.

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

Catharinaziekenhuis Eindhoven
A local protocol will be used for all biosafety level 1 activities. This includes receipt, storage, transport, administration and destruction of the product. The intended work will take place in one designated room qualified for biosafety level 1 activities. Decontaminating procedures will be followed according to biosafety level 1 procedures.

A2.1. Application title:
Please give a descriptive title that provides sufficient information on the objectives and aspects relating to the genetic modification. The title should state the type of vector(s) and insertion(s) used and the nature of the application(s).

Phase III gene therapy study using XRP0038 / NV1FGF coding for fibroblast growth factor to provide therapeutic angiogenesis for the treatment of Critical Limb Ischemia.

A2.2. Describe briefly the contents of the application, the objective of the study being aimed for and the intended application of the results
Describe briefly the genetically modified organisms that are to be used, or which may be produced with the application, such as through the recombination of genetic information between organisms or through the integration of genetic material in a genome. Describe the expected action of the gene products of the transgenes and give an explanation of the biological mechanism. Also describe the scientific and public importance of the study, and state how the results of the study may be used in this context.

The information provided will be used as the basis for a brief description of the study in the decision.

Description of the plasmid:

NV1FGF is a recombinant DNA plasmid, constructed by inserting a gene encoding human fibroblast growth factor type 1 (FGF1) into a pCOR plasmid backbone.
The resulting plasmid was named NV1FGF (or pXL3179). .

Expected action of the gene product / biological mechanism:

The purpose of NV1FGF treatment is to provide therapeutic angiogenesis via IM injection of a plasmid vector, allowing subsequent local expression of FGF1 for the treatment of CLI.

The concept of therapeutic angiogenesis is based on the use of angiogenic factors such as Fibroblast Growth Factor (FGF) to promote neovascularisation for the treatment of ischemic tissues.

FGF1 has been shown to activate endothelial cell migration and induce angiogenesis. FGF1, like other FGF family members, has been reported to induce in vitro a complex "pro-angiogenic phenotype" in endothelial cells that recapitulates several aspects of the in vivo angiogenesis process, including the modulation of endothelial cell proliferation, migration, protease production, integrin and cadherin receptor expression, and intercellular gap-junction communication [1,2].

In 1997, a recombinant expression plasmid encoding FGF1 (21-154), was shown to promote angiogenesis in an animal model of experimental hind limb ischemia [11]. This study also showed that efficacy of FGF1(21-154) was significantly higher when fused with a signal peptide to optimize secretion.

Based on these results, same strategy was selected for development of NV1FGF. FGF1(21-154) sequence was fused with the sequence of the heterologous human fibroblast interferon signal peptide in order to use canonical signal peptide induced secretion, known to improve level of secretion. The resulting protein is called sp.FGF1.

The NV1FGF DNA plasmid has no biological activity on its own. After its transfer into the muscle cells following IM injection, NV1FGF is taken up into nuclei of muscle cells near the injection site and results in localized production and release of NV1FGF derived mRNA and sp.FGF1 protein.

Efficacy of IM administration of plasmids encoding sp.FGF1 was evaluated in rabbit and hamster models of hind limb ischemia. In the rabbit model, NV1FGF led to the formation of angiographically visible collateral blood vessels[3]. In the hypercholesterolemic hamster model, NV1FGF significantly increased the angiographic score and the number of small arterioles in the injected ischemic limbs[33].

Scientific and Public Importance of the study:
Treatment is a critical problem in the management of severe Peripheral Artery Disease (PAD). Three therapeutic approaches are usually considered for patients suffering from PAD [12]: 1) risk factor modification such as tobacco, dietary changes, etc.; 2) when possible, percutaneous transluminal angioplasty (PTA) to revascularize the ischemic limb; 3) bypass surgery with the use of prosthetic material or vein graft. Both PTA and especially surgery carry a significant morbidity/mortality in these patients who often suffer concomitantly from an already existing heart or disseminated vascular disease.

In a phase II double blind, randomized, placebo-controlled, multicentre study including 107 evaluable patients with CLI, with non-healing ulcer and no-option for revascularization, 4 IM administrations of NV1FGF at 2- week intervals have shown a similar improvement in ulcer healing as compared to placebo but significantly prolonged the time to first amputation and to first major amputations[30]

The data of this previous study lead us to perform a larger single placebo controlled study involving 490 patients in 30 countries to confirm the benefit of NV1FGF in delaying the time to amputation or to death in CLI patients with skin lesions and unsuitable for standard revascularization.

A2.3. Describe briefly the intended work.

Describe briefly the work to be carried out with the genetically modified organisms. State in chronological order which types of procedures will be carried out, and for which a licence is being applied for (e.g. production, transport, storage and administration of the vector, observation of patients, sampling, transport, storage and processing of samples, waste treatment).

The licence is being applied for transport, storage and administration of the Plasmid DNA, observation of patients, sampling, transport, storage and processing of samples, waste treatment. Work locations: see A1.27
The product is administered via intramuscular injections: 4 into the calf, and 4 into the thigh. The administration (i.e 8 injections) is repeated 3 times at two weeks intervals (i.e. for a total of 4 administrations over a six week period). Syringes and needles need to be discarded in the biohazardous sharp container after use. Used vials must be destroyed at the pharmacy as per Biosafety Level 1 policy.

A2.4. Intended start and end date:

The decision must state a time period within which the procedures will be carried out, and so an end date must be given. The chosen end date will be included in the decision. It is possible to obtain an extension to the decision; please note, however, that any extension procedure must be completed before the decision end date has passed. Instead of the end date, a maximum number of test subjects may also be stated. In that case, the decision end date will correspond to the completion of the study with the last test subject.

Global study-start and end dates: 10 December 2007. End date when 490 patients are reached.
Netherlands start and end dates: May 2008 and 30th December 2011.

Purpose of the introduction into the environment

A2.5. General purpose of the work being applied for:

Please state here the underlying (secondary) purpose of the work, such as the development of a new therapy to treat skin cancer.

The purpose of the work is the development of a new therapy for Critical Limb Ischemia patients.

A2.6. Specific purpose of the work being applied for:

The 'primary purpose' of the project: e.g. phase 1 study, to find out how the GMO is tolerated in the test subjects receiving it.

The primary purpose of the project is a Phase 3 study to demonstrate superiority of NV1FGF over placebo in the prevention of major amputation (of the treated leg) or death, which ever comes first in CLI patients with skin lesions.

A3 Bio-safety details

The preparation administered to the test subject may consist of a cellular organism, live or otherwise (e.g. bacteria, from a viral vector or from naked nucleic acid). Please answer the questions that relate to the preparation to be used.

State the composition of the genetically modified organism to be administered to the test subject.

Answer:

- O Viral vector (questions A3.1. to A3.14)
- O Bacterial strains (questions A3.15. to A3.27.)
- • Naked nucleic acid (questions 3.28. to A3.30.)
- O Other (Contact the GMO office)

Naked nucleic acid

A3.28. Does the nucleic acid contain sequences of viral origin that may interact with genomes of viruses or other micro-organisms?

Describe the sequences that can interact with genomes of viruses or other micro-organisms, and the chance that this may produce a new GMO. If a GMO might be produced, also give details of potential spreading routes and the risk that spreading may occur.

The NV1FGF construct doesn't contain any sequence of viral origin, however the plasmid construction bears sequences identical to viral sequences (as hCMV promoter). These sequences are *de novo* and *in vitro* synthesized and do not require any viral material.
Firstly, Cytomegalovirus (CMV) promoter sequence in the PCOR plasmid is a human CMV (hCMV) sequence. It is not from viral origin but from recombinant origin. The only way NV1FGF plasmid DNA could recombine with viruses would be inside a myofiber cell that would be the host of a DNA virus. The recombination mode in mammalian cells is mainly based on non homologous sequences; therefore the unlikely potential sequence analogy between hCMV and sequence of viral origin would not increase the potential risk of mutation which is negligible.
Indeed, as mentioned above the probability of NV1FGF plasmid integration in human genome has been evaluated and results indicated that the risk of integration is negligible. In the extremely unlikely event of an homologous recombination, this would lead to the integration of the sequence of viral origin in one of the pCOR plasmid constitutive sequence: hCMV promoter, FGF1-gene, R6K plasmid, etc... Due to pCOR small size, this would prevent expression of the pCOR plasmid. Therefore there will be no impact on health or environment.

Homologous recombination with viruses:
Homologous recombination of the viral sequences of NV1FGF would only be possible with the cytomegalovirus (CMV) or the simian virus 40 (SV40). For homologous recombination to occur a high copy number of the plasmid and the virus have to be present. Both viruses are very unlikely to be present in muscular cells of the subjects, although the possibility that such viruses are present increases with subjects with a deficient immune system. In the extremely unlikely event that recombination with such viruses occurs, it is very unlikely that such (partial) sequences will replicate or express efficiently since they are not advantageous to the virus and most likely decrease the overall replication & expression efficiency of the virus. Therefore the total risk of such a recombination is deemed negligible.

Interaction with bacteria:
In the extremely unlikely event, if an active sequence would become part of the environment, it could not be replicated in a bacteria present in subjects and in the environment. NV1FGF is a pCOR plasmid carrying the sp.FGF1 expression cassette. pCOR are small size plasmids carrying a Conditional Origin of Replication (R6K) which requires a trans-acting replication factor (π) meaning that pCOR can only replicate in a specific E. coli host strain producing π, greatly reducing the potential for propagation in treated patients and in the environment .

Clarification on potential risk to have NV1FGF replication in natural bacteria:

NV1FGF replication is obtained in a specifically laboratory engineered E. Coli strain (E. Coli pir116) that was constructed to produce the R6K replication factor π. The sequence for this factor is not beard by NV1FGF plasmid, making its replication impossible without an external source of π[28].

The question is: what is the probability that NV1FGF plasmid encounters a bacterium susceptible to produce the π factor - probability of presence of such bacteria in the intestine and what would happen?

To evaluate such probability, the following points need to be taken into account:

- This hypothesis implies that NV1FGF would be swallowed. This is highly unlikely as the intended route of administration is via intramuscular injection done by the investigator.

- Secondly, the time during which NV1FGF plasmid would remain complete in the intestine would need to be long enough to allow an efficient transfection of bacteria. However, the actual time is considered as very short taking into account the presence of nucleases in the intestine.

- Furthermore, the breakdown of DNA during food processing and passage through the gastrointestinal tract reduces the likelihood that intact genes capable of encoding foreign proteins will be transferred to gut microflora[31]

- Lastly, the type of bacteria that can bring π factor.
    - O The π factor can only be present in bacteria that bear on their plasmid the natural R6K replication system including the π sequence, i.e. E. Coli bacteria or closely related bacteria (R6K resident bacteria). This indication limits therefore the replication of NV1FGF plasmid in these bacteria.
    - O Importantly, there is no report of a π -like activity in other strains that could allow NV1FGF replication.

In the very unlikely event the three conditions above would be met, what is the Impact of a transfection by NV1FGF of a natural E.Coli strain bearing R6K?

The principle of incompatibility between plasmids would apply [34, 35]. Indeed a bacteria plasmid bearing the R6K system and the NV1FGF bearing the same system (without π sequence) would co-exist. In such a case, the coexistence of the major incompatibility factor present in the oriγ sequence of the R6K system of both systems will lead to the loss of one of the plasmids. Meaning either the bacteria plasmid inducing the lack of π factor needed by NV1FGF, or the NV1FGF plasmid itself: in both cases the replication of NV1FGF plasmid would not be possible or at most very transient.

Taking into account the rational above, it is therefore considered that the probability that NV1FGF replicates in intestinal bacteria would be present in the environment is extremely low if not of zero.

Furthermore, XRP0038/ NV1FGF plasmid does not contain sequences that increase plasmid integration rate, recombinogenic sequences, or sequences closely related to these motifs [13,14,15] The presence of human sequences within NV1FGF does not raise a specific concern regarding integration. Indeed, integration by homologous recombination is several orders of magnitude less frequent than random-integration [16]. In addition, the maximum length of sequence homology between NV1FGF and the human genome is much lower than that required for efficient homologous recombination [17]. Based on these data, NV1FGF shows no particular risk of integration into the host genome.

A3.29. Describe the genetically modified nucleic acid to be used.

In your description please give details in particular of the following aspects:

- *Regulatory sequences, such as promoter, terminator and enhancer sequences;*
- *Structural genes;*
- *Function of the coded proteins in the donor organism (the organism from which the gene was originally isolated or where it naturally occurs is referred to as the donor organism);*
- *Whether the vector or the DNA inserted in the vector contains elements whose origin or function is unknown.*

NV1FGF is a construction of expression plasmid encoding human FGF-1 and using a backbone with a conditional origin of replication (pCOR). pCOR plasmids are very narrow-host range plasmid vectors for non viral gene therapy and DNA vaccination. The pCOR conditional origin of replication does not come from widely-used ColE1- derived plasmids, but from another natural E. coli plasmid, R6K. The pCOR backbone is significantly smaller than standard ColE1-derived plasmids. It contains three key elements: the R6K γconditional origin of replication (ori γ), which requires the R6K πinitiator protein to be functional; a selectable tRNA suppressor gene, and a cer (ColE1 resolution) fragment to resolve
pCOR oligomers. Such plasmids can only replicate in π-producing enterobacteria, considerably limiting their host range. pCOR selection requires expression of a synthetic amber suppressor tRNA gene specific for phenylalanine (sup Phe). This corrects an E. coli argE amber mutation, making it possible for the recombinant host strain to grow on a minimal medium lacking arginine on which bacteria without the plasmid cannot grow. We engineered a specific E. coli host, (XAC- 1 pir-116), supporting pCOR replication and selection. Its genome contains: i) a copy-up mutant of pir (gene encoding π), pir-116 and ii) the argEam gene for plasmid selection. The construction strategy used to create a plasmid containing sp.FGF-1 expression cassette led to the following features. sp.FGF-1 is a fusion gene between sequences encoding the secretion signal peptide (sp) from human fibroblast interferon γ and the naturally occurring truncated form of human FGF-1 from amino acids 21 to 154. Expression of sp.FGF-1 is driven by the human cytomegalovirus (CMV) immediate early enhancer / promoter module. The late polyadenylation signal from Simian Virus 40 is inserted downstream of sp.FGF-1 to ensure proper and efficient transcription termination and subsequent polyadenylation of sp.FGF-1 transcript.
The sp.FGF-1 expression cassette was inserted into a pCOR backbone creating a closed circular plasmid. This plasmid is propagated in the E. coli host XAC-1 pir-116.

In summary, in addition to an optimized expression cassette, pCOR plasmids, exhibit the following main features:

- pCOR are small size plasmids carrying a Conditional Origin of Replication (R6K) which requires a trans-acting replication factor (π) meaning that pCOR can only replicate in a specific E. coli host strain producing π, greatly reducing the potential for propagation intreated patients and in the environment.

Clarification on potential risk to have NV1FGF replication in natural bacteria:

NV1FGF replication is obtained in a specifically laboratory engineered E. Coli strain (E. Coli pir116) that was constructed to produce the R6K replication factor π. The sequence for this factor is not beard by NV1FGF plasmid, making its replication impossible without an external source of π[28].

The question is: what is the probability that NV1FGF plasmid encounters a bacterium susceptible to produce the π factor - probability of presence of such bacteria in the intestine and what would happen?

To evaluate such probability, the following points need to be taken into account:

- This hypothesis implies that NV1FGF would be swallowed. This is highly unlikely as the intended route of administration is via intramuscular injection done by the investigator.

- Secondly, the time during which NV1FGF plasmid would remain complete in the intestine would need to be long enough to allow an efficient transfection of bacteria. However, the actual time is considered as very short taking into account the presence of nucleases in the intestine.

- Furthermore, the breakdown of DNA during food processing and passage through the gastrointestinal tract reduces the likelihood that intact genes capable of encoding foreign proteins will be transferred to gut microflora[31].

- Lastly, the type of bacteria that can bring π factor.
  - O The π factor can only be present in bacteria that bear on their plasmid the natural R6K replication system including the π sequence, i.e. E. Coli bacteria or closely related bacteria (R6K resident bacteria). This indication limits therefore the replication of NV1FGF plasmid in these bacteria.
  - O Importantly, there is no report of a π -like activity in other strains that could allow NV1FGF replication.

In the very unlikely event the three conditions above would be met, what is the Impact of a transfection by NV1FGF of a natural E.Coli strain bearing R6K?

The principle of incompatibility between plasmids would apply[34]. Indeed a bacteria plasmid bearing the R6K system and the NV1FGF bearing the same system (without π sequence) would co-exist. In such a case, the coexistence of the major incompatibility factor present in the oriγ sequence of the R6K system of both systems will lead to the loss of one of the plasmids. Meaning either the bacteria plasmid inducing the lack of π factor needed by NV1FGF, or the NV1FGF plasmid itself: in both cases the replication of NV1FGF plasmid would not be possible or at most very transient.

Taking into account the rational above, it is therefore considered that the probability that NV1FGF replicates in intestinal bacteria would be present in the environment is extremely low if not of zero.

- pCOR do not contain an antibiotic resistance gene and selection of pCOR is based on suppression of an auxotrophy of the bacterial host;
- pCOR are at least comparable in level of transgene expression to standard (ColE1) plasmids in vitro and in vivo;
- pCOR backbone does not contain known immunostimulatory sequences (ISS) and should have less, if any, immunostimulatory properties compared to standard ColE1-derivedplasmids.

The plasmid map of NV1FGF is shown in Figure 1 (§ A3.30).

FGF1 is a mitogen and chemotactic agent acting via binding to FGF receptors. The mitogenic activity of FGF1 is potentiated by heparin/heparan sulfate (HS) which extends its biological

half-life and is required for binding FGF1 to its high affinity receptor by formation of a ternary complex among ligand, receptor and heparin/HS [4]. The sites of receptor binding and interaction with heparin/HS described for FGF1 [4 -7] are located within the FGF121-154 sequence. This suggests that the 3 FGF1 isoforms provide a similar FGF1-FGFR-heparin/HS ternary complex, the structural basis for the FGF1 biological activity. The functional significance of the amino-terminal extension of FGF1(21-154) and (FGF1(15-154) versus FGF1(1-154) has not been established [8]. The biological activities for heparin affinity and mitogenic activities for endothelial cells of FGF1(1-154) and FGF1(21-154) have been shown to be similar [9]. The competitive binding property for binding to endothelial cell-derived receptor and mitogenic activities for endothelial cells of FGF1(1-154) and FGF1(15-154) have also been shown to be similar [10].

For further information see A2.2.

A3.30. Provide a diagram (map) and a description of the structure of the genetically modified nucleic acid.

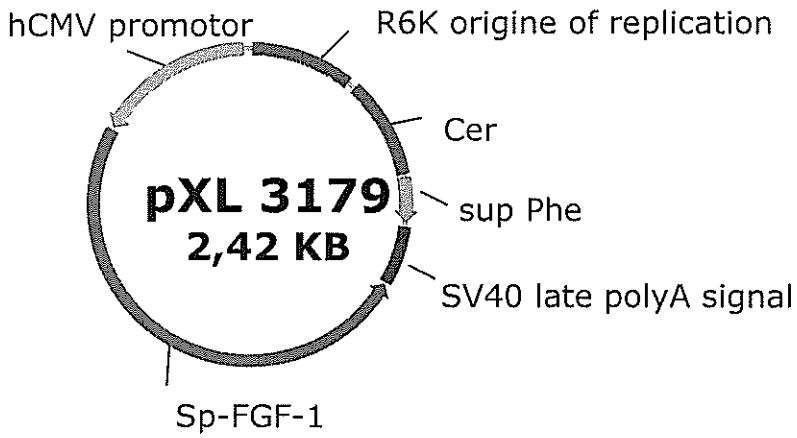

Figure 1 – NV1FGF plasmid map, also referred to as pXL 3179 (see section A3.29).

A4 Other information

Environment-related information originating from earlier experiments

A4.1. Describe the results originating from earlier (pre-)clinical studies with the GMO, and which are important for the environmental risk assessment.
When answering this question, please describe the results achieved with an identical or comparable GMO, to the extent that these are relevant for the environmental risk analysis of the application in question, e.g. details of shedding, duration of latent presence of the vector/the GMO, spread of the vector/the GMO and potential interaction with other micro-organisms (including viruses) are important.

Expression of FGF-1 protein or/and FGF-1 mRNAs in the injected muscle was demonstrated after NV1FGF I.M. administration in various animal species (mice, rats and dogs) and in CLI patients. Expression of NV1FGF-derived FGF-1 protein was detected by immunohistochemistry in the myofibers of the injected muscle only. Expression of NV1FGF-derived mRNA was shown to be local and restricted to a few cm around the injection site as shown in animals (20 cm^3) and CLI patients (mRNA expression centered on the theoretical needle path in muscle biopsy samples measuring 5X3X2 cm^3).

Biodistribution studies reported in the literature document a limited dissemination of naked plasmid DNA after I.M. administration and a lack of persistence of plasmid sequences at distant sites, including in the gonads [18,19,20,21].

Studies conducted in rats with NV1FGF show that IM NV1FGF leads to transient dissemination of NV1FGF sequences into blood and distal organs. The NV1FGF sequences were mostly products of plasmid degradation (non-coding sequences) with no capability to express FGF1 protein. There was no evidence of NV1FGF-derived mRNA expression in the assessed organs suggesting that the transient distribution of NV1FGF sequences did not led to expression in organs other than the injected muscle. Metabolic studies indicated that the NV1FGF plasmid is rapidly degraded when present in blood: the half-life of NV1FGF sequences in blood was shown to be 6.00 min in CLI patients. In conclusion, these data indicated that the NV1FGF sequences transiently disseminated are mostly product of plasmid degradation with no capability to drive FGF1 expression. The transient presence of NV1FGF sequences is not associated with NV1FGF-derived mRNA expression in organs other than the injected muscle.

NV1FGF IM toxicity studies in rats did not show any treatment-related effects other than at the injection site. No adverse effects were observed in organs distal from the injected muscle including eyes, gonads and kidney. In addition, direct injection of very high levels of NV1FGF did not result in any adverse effects.

The rat is considered as a relevant species to assess the potential toxicity of NV1FGF based on the high sequence homology (amino acid sequence alignment of both human and rat FGF1 show 97% of identity from position 21 to 154, the isoform of the NV1FGF-related FGF1), on exploratory toxicity studies conducted with recombinant human FGF1 in the rat and studies to assess the potential for anti-hFGF1 antibodies raised following repeated IM administration of NV1FGF to the rat.

Methods used:

Various assay methods were developed to investigate the pharmacokinetics of NV1FGF-derived DNA, NV1FGF-derived mRNA, and FGF1 protein, as shown in the table below.

Analyte/sample	Method	Range	Lower limit of quantification
NV1FGF-derived DNA sequences (69-bp) / tissue, blood	PCR (TaqMan kit)	$10\sim10^5$ copies or $10\sim10^6$ copies[a] / µg genomic DNA	10 copies / µg genomic DNA
NV1FGF-derived DNA sequences (825-bp) / tissue, plasma	PCR (TaqMan kit)	$10^2\sim10^5$ copies / µg genomic DNA (muscle), $10^2\sim10^8$ copies / 100 ng genomic DNA or 10µL plasma	10^2 copies / µg genomic DNA (muscle), 10^2 copies / 100 ng genomic DNA (other tissues) or 10 µL plasma
NV1FGF-derived mRNA (69-bp) / tissue	RT-PCR (TaqMan kit)	$10^3\sim10^7$ in vitro RNA transcript copies / 8.7 µg total RNA. $10\sim10^5$ cDNA copies / 600 ng total RNA	10^3 in vitro RNA transcript copies / 8.7 µg total RNA. 10 cDNA copies / 600 ng total RNA
FGF1 protein / serum, plasma, tissue	ELISA (Quantikine FGF-1 immunoassay kit)	31.25~2000 pg/mL serum or plasma	45 pg/mL serum or plasma
	Immuno- histochemical assay	Qualitative (tissue)	-

[a] 1 µg of NV1FGF corresponds to $3.7x10^{11}$ copies

NV1FGF-derived DNA sequences: NV1FGF sequences were assessed in total DNA extracted from selected tissues, using Southern blot analysis and real-time PCR assays. Southern blot analysis allows visualizing various sizes of DNA sequences (ie, intact NV1FGF plasmid and various products of NV1FGF degradation). However, its insufficient sensitivity (10^7 copies of NV1FGF sequence) makes it irrelevant for assessing tissue distribution of NV1FGF following IM administration (levels below 10^7 copies of NV1FGF sequence).

Therefore, 2 real-time PCR assays were developed:

One assay has been designed to specifically amplify a 69 base pair (bp) DNA sequence that encompasses the junction between the CMV promotor and the beginning of the sp.FGF-1 coding sequence in NV1FGF. The limit of quantitation of this assay is 10 copies /10 µl of plasma or /µg DNA. This assay can detect intact NV1FGF and NV1FGF degradation fragments containing the 69 bp target sequence. It cannot discriminate between NV1FGF molecular species with ability to drive FGF-1 expression and those with no potential to express FGF-1.

NV1FGF sequences with capability to express FGF-1 are monitored using a second quantitative Real-Time PCR assay designed to specifically amplify an 825 bp DNA sequence spanning from the CMV promotor to the SV40 polyadenylation signal region in NV1FGF. This amplicon represents the minimal NV1FGF sequence required for FGF-1 expression. The limit of quantitation of this assay is 100 copies /10 µl of plasma or /µg DNA. This assay can detect intact NV1FGF and NV1FGF degradation fragments with capability to express FGF-1.

NV1FGF derived mRNA was quantified using real-time RT-PCR assays qualified for the muscle tissue and for a series of other tissues (distal organs).

MetFGF121-154 protein was assessed using:
- Human FGF1 immuno-histochemical assay on histological muscle sections
- Human FGF1 ELISA or plasma samples

In human:

To date, 2 Phase 1 studies (PM101 and PM105) and 3 Phase 2 studies (PM201, PM202, and PM211) have been performed. All studies were conducted according to Good Clinical Practice and other applicable regional and local regulations.
No studies have been performed in healthy subjects. Studies PM101 (randomized patients N =51), PM105 (randomized patients N = 6), PM201 (randomized patients N =125), and PM202 (randomized patients N =71) randomized CLI patients and study PM211 enrolled Intermitent Claudication patients (randomized patients N =36). Summaries of these studies are provided in the Table below.

Study Number + Title (Country)	Main objectives	Design	NV1FGF Total Dose (number of patient with at least 1 injection)	Overall follow-up
PM101-A Phase 1, multi-center, open-label, safety and tolerability study of single and repeated administrations of escalating dose(s) of NV1FGF administered by intramuscular injection in patients with severe peripheral artery occlusive disease	Primary: Clinical and safety/tolerability Secondary: Pharmacodynamic based on $TcPO_2$, ABI and clinical response on ulcer healing and amputation. Biological activity of NV1FGF on collateral artery development.	Open-label, escalating doses	Single administration: 0.5 mg (n=3), 1 mg (n=3), 2 mg (n=3), 4 mg (n=6), 8 mg (n=6), 16 mg (n=6) Multiple administrations: 1 mg (n=3), 2 mg (n=3), 4 mg (n=6), 8 mg (n=6) and 16 mg (n=6)	6 months
PM105- Phase 1, double blind, parallel-group, multi-center, gene expression (synthesis of FGF-1 mRNA) and tolerability study of increasing single dose of NV1FGF administered by intra-muscular injection in patients with severe peripheral artery occlusive disease planned to undergo amputation above the ankle	Primary: NV1FGF expression in injected tissues following single intramuscular administration of NV1FGF Secondary: NV1FGF biodistribution	Double blind, randomized, parallel groups	Single administration: 0.5 mg (n=2), 2 mg (n=2) and 4 mg (n=2).	6 months
PM201. Phase 2, randomized, double-blind, placebo-controlled, parallel group, efficacy and safety study of NV1FGF in patients with severe peripheral artery occlusive disease	Primary: Efficacy based on ulcer healing endpoint and safety. Secondary: Efficacy on pain, amputation, death and biomarkers ($TcPO_2$, ABI)	Double blind, randomized, placebo controlled, parallel groups	Placebo (n=61) NV1FGF 16 mg (n=57)	12 months
PM202. Phase 2 randomized, double-blind, placebo-controlled, parallel group, efficacy and safety study of different doses and schedules of administration of NV1FGF in patients with severe peripheral artery occlusive disease	Primary: Efficacy based on $TcPO_2$ endpoint and safety. Secondary: NV1FGF biodistribution	Double blind, randomized, placebo controlled, parallel groups	Placebo (n=16) NV1FGF 2 mg (n=11) NV1FGF 8 mg (n=10) NV1FGF 16 mg 4x4x4x4 (n=11) NV1FGF 16 mg 8x0x8x0 (n=11)	12 months

Study Number + Title (Country)	Main objectives	Design	NV1FGF Total Dose (number of patient with at least 1 injection)	Overall follow-up
			NV1FGF 16 mg 8x8x0x0 (n=11)	
PM211 (Part I). Phase 2, double-blind, randomized, placebo-controlled, parallel group and dose-finding, safety and efficacy study with intramuscular injections of NV1FGF in patients with intermittent claudication	Primary: Safety of NV1FGF. Secondary: NV1FGF biodistribution	Double blind, randomized, placebo controlled, parallel groups	Placebo (n=10) NV1FGF 16 mg (n=15) NV1FGF 32 mg (n=9)	6 months

PHARMACOKINETICS AND METABOLISM IN HUMANS

Intramuscular administration of NV1FGF leads to the local production of NV1FGF mRNA and finally FGF1 protein. Therefore, in order to evaluate NV1FGF pharmacology in clinical studies, NV1FGF DNA, NV1FGF mRNA, and FGF1 protein were measured, when appropriate, in injected muscles and in blood and urine. The assays used for those measures and their lower limit of quantification (LLOQ) are presented in the Table below.

Analyte	Assay	Purpose	Blood	Injected muscle	Urine	Assay LLOQ [a]
NV1FGF DNA	Real-Time PCR with short amplicon (69 bp)	Quantify NV1FGF DNA (entire NV1FGF and NV1FGF fragments containing the 69 bp sequence)	X	X	X	Phase 1 studies: 10 copies/µg genomic DNA; 10 copies / 10 µl plasma or urine DNA extract Phase 2 studies: 20 copies/10 µl plasma DNA extract; 50 copies/10 µl urine DNA extract
	Real-Time PCR with long amplicon (825 bp) specific for FGF1 expression cassette	Quantify NV1FGF sequences with capability to express FGF1	X	X	X	Phase 1 studies: 100 copies/µg genomic DNA; 100 copies/10 µl plasma or urine DNA extract Phase 2 studies: 20 copies/10 µl plasma DNA extract; 50 copies/10 µl urine DNA extract
NV1FGF mRNA	Real-Time RT-PCR	Quantify NV1FGF expression (mRNA)		X		6000 NV1FGF mRNA copies/ 6 µg total RNA
FGF1 protein	FGF1 immunohistochemistry	Quantify FGF1-expressing cells		X		NA [b]
	FGF1 ELISA	Quantify circulating FGF1	X			Phase 1 studies: 45 pg $FGF1_{15-154}$ / ml serum [c] Phase 2 studies: 600 pg $FGF1_{21-154}$ / ml plasma [c]
FGF receptors (R1, R2, R3, R4)	Immunohistochemistry	Detect the presence of receptors to FGF1		X		NA [2]

Analyte	Assay	Purpose	Blood	Injected muscle	Urine	Assay LLOQ [a]
Anti-FGF1 antibody	ELISA	Quantify human antibodies to FGF1	X			Anti-FGF-1 antibody levels in serum were measured using ELISA. The LOQ of the anti-FGF-1 antibodies ELISA is 5000 ng reference antibody/mL

[a] Lower Limit Of Quantification

[b] Not Applicable

[c] NV1FGF encodes sp.FGF1, ie, human FGF1 amino acids 21-154 (FGF121-154) fused to the human fibroblast interferon β signal peptide. The "Quantikine FGF-acidic Immunoassay" ELISA (R&D Systems) is used to quantify circulating levels of human FGF1 in clinical studies. This assay was developed and validated for Phase 1 studies using recombinant human FGF-acidic (FGF1 amino acids 15-154 (FGF115-154)). The sensitivity of the assay (Minimal Detectable Dose: MDD) is less than 15 pg FGF115-154/ml human serum or plasma. The Limit of Quantification of the assay (LOQ) was set at 3 times the MDD, ie, 45 pg FGF115-154/ml human serum or plasma. The assay was validated for Phase 2 studies using recombinant human FGF1 amino acids 21-154 (FGF121-154). The assay LOQ for FGF121-154 is 600 pg/ml in human plasma and human serum.

The LLOQ was lower in the Phase 1 program compared to the Phase 2 program. This is explained by the fact that the initial assays used in the Phase 1 program were further developed and validated according to ICH guidelines for the Phase 2 program. Despite this change in LLOQ, conclusions of the Phase 2 pharmacokinetic studies are in line with the Phase 1 studies.

Pharmacokinetics in patients
NV1FGF pharmacokinetics was determined in PM101, PM105, PM202 and PM211 clinical studies following different doses/regimens and at different time points (Table below).

Summary of clinical pharmacology studies as per protocols - pharmacokinetics

Study	Group (n randomized)	Pharmacokinetic sampling
PM101 (US)	(n=3): 0.5 mg n=3): 1 mg n=3): 2 mg (n=6): 4 mg (n=6): 8 mg (n=6): 16 mg (n=3): 2 x 0.5 mg (n=3): 2 x 1 mg (n=6): 2 x 2 mg (n=6): 2 x 4 mg (n=6): 2 x 8 mg	FGF1 protein in serum at baseline and 1, 2, 3, 7, 11, and 23 weeks after first NV1FGF administration. NV1FGF DNA in plasma and urine at baseline and 1, 2, 3, 7, and 11 weeks after first NV1FGF administration. NV1FGF DNA in muscle tissue of the treated limb when possible following amputation.
PM105 (US/EU)	(n=2): 0.5 mg (n=2): 2 mg (n=2): 4 mg	NV1FGF DNA in plasma at baseline and 15 minutes, 2 and 4 hours, 3 to 8 days, 4, and 8 weeks after NV1FGF administration. NV1FGF DNA, NV1FGF mRNA, FGF1 protein, and FGF1 receptors in muscle tissue from the amputated part of the limb (3 to 8 days after NV1FGF administration).
PM201 (EU)	(n=66): placebo (n=59): 4 x 4 mg	NV1FGF DNA and mRNA in muscle tissue of the amputated part of the limb in case of amputation (in Germany). NV1FGF DNA in gonads, brain, lung, heart, kidney, liver, spleen, muscle from injected limb, muscle other than from injected limb, and blood when possible in case of patient death.
PM202 (US)	(n=16): placebo (n=11): 2 x 8 mg (n=11): 2 x 8 mg (n=11): 4 x 4 mg (n=11): 4 x 2 mg (n=11): 4 x 0.5 mg	FGF1 protein in plasma at baseline and 2, 4, 6, 8, 12[a], 24[a], and 51[a] weeks after first NV1FGF administration. NV1FGF DNA in plasma at baseline and 2, 4, 6, 8, 12[a], 24[a], and 51 weeks after first NV1FGF administration. NV1FGF DNA in urine at baseline and 2, 4, 6, 8, 12[a], 24[a], and 51[a] weeks after first NV1FGF administration. When possible, NV1FGF DNA in sperm at baseline and 12 and 24 weeks after first NV1FGF administration. NV1FGF DNA in gonads, brain, lung, heart, kidney, liver, spleen, muscle from injected limb, muscle other than from injected limb, and blood when possible in case of patient death
PM211 (US/EU)	(n=12): placebo (n=15): 4 x 4 mg (n=9): 4 x 8 mg	Patients enrolled in Part I, measurement of : FGF1 protein in plasma at baseline and 2, 8, and 26[a] weeks after first NV1FGF administration. NV1FGF DNA in plasma at baseline and 15, 30, 45, and 60 minutes, 2, 8, and 26 weeks after first NV1FGF administration.

[a] Will be measured only if previous time point is positive.

Each NV1FGF administration consisted of 4 intramuscular injections in the calf and 4 intramuscular injections in the thigh, except in 27 of 51 patients in study PM101, where NV1FGF administration consisted in 2 intramuscular injections in the calf and 2 intramuscular injections in the thigh. A 2.5 mL dose of NV1FGF solution was delivered per injection site. The 2.5 mL volume was chosen to

provide an optimal volume for gene transfer (large volume) and an optimal volume for local tolerance (small volume).

PM101
PM101 was a multi-center Phase 1, open-label, single and repeated administrations, (2 administrations at 2 weeks interval) dose-escalating study from 0.5 mg to 16 mg conducted in 51 patients with severe PAD. Circulating NV1FGF sequences with the capability to express sp. FGF1 21-154 could not be quantified, including at the first assessment 1 week post-NV1FGF administration. No increase of circulating levels of FGF1 compared to baseline values was observed. NV1FGF did not provoke a specific humoral immune reaction to FGF1. A 69 bp NV1FGF-specific sequence was quantified in the urine of 4 of 51 treated patients. This was observed at only 1 time point per patient and not at any particular time point throughout the 11 weeks post-NV1FGF administration. The maximal level of 69 bp sequence was 126 copies/10 µl urine. A 69 bp NV1FGF-specific sequence could be quantified for up to 11 weeks post NV1FGF administration in muscle biopsies collected at injection sites from the amputated part of the limb. [32]

PM105
PM105 was a multi-center Phase 1 study aimed to assess NV1FGF biodistribution and NV1FGF expression in injected tissues following single intramuscular administration of 0.5, 2 and 4 mg NV1FGF. Six eligible patients with severe PAD who planned to undergo major amputation were randomized. Patients underwent amputation 3 to 5 days after NV1FGF administration. Muscle samples were collected from injection sites and distant sites (ie, locations the most distant possible from any injection site) of the amputated limb. This study showed that NV1FGF was degraded in blood following intramuscular administration, that myofibers of patients with severe PAD were able to be transfected with NV1FGF and to express FGF1 transgene NV1FGF mRNA and FGF1 protein, that expression of the FGF1 transgene in muscle was localized to the injection site and that FGF1 receptors were present in ischemic muscles of all tested patients (5). These data rationalize (local expression and presence of FGF receptors) the potential for efficacy of NV1FGF in humans.

PM202
PM202 was a Phase 2, randomized, double-blind, placebo-controlled, 6 parallel group, efficacy and safety study of different doses and schedules of administration of NV1FGF (4 x 0.5 mg, 4 x 2 mg, 4 x 4 mg, 2 x 8 mg at 2 weeks interval and 2 x 8 mg at 4 weeks interval versus placebo) in patients with severe PAD. NV1FGF administration did not lead to quantifiable levels of FGF1 in circulation at any dose or regimen. During the treatment period, sporadic quantifiable levels of NV1FGF sequences were detected in the plasma with no relation to study treatment. During the follow-up period, quantifiable levels of NV1FGF sequences were not detected.
NV1FGF sequences in urine have been measured sporadically with no relation to study treatment or visit. Clinical data indicate no dissemination of quantifiable levels of NV1FGF coding sequences in the urine samples collected at various time points post administration. In Patient 620-0014, breast cancer specimens were collected. There was no NV1FGF detected in either the surrounding tissue or within the tumor. Presence at a very low level (below LLOQ) of non-functional fragment was detected in surrounding tissue (Real-Time PCR for 69 bp amplicon).
Furthermore, there was no evidence of humoral immune response to FGF1 at any dose regimen or at any dose level.

PM211
PM211 was a Phase 2, randomized, placebo-controlled study conducted in patients with intermittent claudication. In PM211, pharmacokinetic analysis was intended to determine pharmacokinetic parameters for NV1FGF sequences in plasma and to confirm the lack of FGF1 systemic exposure after 4 administrations every 2 weeks of 4 mg (total dose 16 mg) or 8 mg (total dose 32 mg) of NV1FGF. NV1FGF half-life in plasma was very short, between 5 and 6 minutes. Exposure for later time points were consistent with prior observations. Furthermore, there was no evidence of circulating FGF1 and no evidence of humoral immune response to FGF1 at any dose level.

Pharmacokinetic/pharmacodynamic relationship

Pharmacokinetic analysis at early time points (0 to 1 hour) indicate that, following intramuscular administration, NV1FGF half-life in plasma is very short (5 to 6 minutes). There was no evidence of NV1FGF accumulation in the circulation from repeated administrations. Furthermore, there was no evidence of circulating FGF1 and no evidence of humoral immune response to FGF1 at any dose level. These data provide evidence that NV1FGF is a local treatment, with no particular risk of exposure to functional sequences at distance of injection site.

Based on clinical studies, the treatment of patients with Critical Limb Ischemia (studies PM101, PM105, PM201, PM202) and with Intermittent Claudication (PM211), a total of 192 patients, single and repeated IM injections of NV1FGF, were well tolerated. Additionally, clinical data (PM101 and PM202) indicated that trial subject does not excrete coding sequence of NV1FGF.

Summary:
The risk that any active sequence becomes part of the environment is extremely low. NV1FGF is rapidly degraded after IM administration when present in blood. The half-life of NV1FGF sequences (825 bp) in circulating blood is 3 min. following IV administration in rats and 5-6 min. following IM administration in human. Additionally, clinical data indicate no dissemination of quantifiable levels of NV1FGF sequences in the urine samples collected at various time points post administration.

Furthermore, XRP0038/ NV1FGF plasmid does not contain sequences that increase plasmid integration rate, recombinogenic sequences, or sequences closely related to these motifs [13,14,15]. The presence of human sequences within NV1FGF does not raise a specific concern regarding integration. Indeed, integration by homologous recombination is several orders of magnitude less frequent than random-integration [16]. In addition, the maximum length of sequence homology between NV1FGF and the human genome is much lower than that required for efficient homologous recombination [17]. Based on these data, NV1FGF shows no particular risk of integration into the host genome.
A nonclinical study to document the risk of integration has been conducted. Following the injection of NV1FGF at a clinically relevant dose into rat muscles, levels of NV1FGF were found to be ≤27 copies associated with 1 µg of High Molecular Weight (HMW) genomic DNA after one round of purification. These data are similar to those obtained with plasmid DNA vaccines [23, 24, 25, 26, 27]. If these copies were integrated, the frequency would be 3 to 2 orders of magnitude below the spontaneous mutation rate [22], indicating that the risk of mutation is negligible.

These data as well as previous studies indicating a transient distribution of NV1FGF
sequences to the gonads (mostly product of degradation with no evidence of transgene expression in gonads at any assessed time point, nor evidence of persistence of NV1FGF sequences in gonads) and no treatment-related adverse effects in the gonads provide no evidence of risk of inadvertent germ line integration.

Homologous recombination with viruses:
Homologous recombination of the viral sequences of NV1FGF would only be possible with the cytomegalovirus (CMV) or the simian virus 40 (SV40). For homologous recombination to occur a high copy number of the plasmid and the virus have to be present. Both viruses are very unlikely to be present in muscular cells of the subjects, although the possibility that such viruses are present increases with subjects with a deficient immune system. In the extremely unlikely event that recombination with such viruses occurs, it is very unlikely that such (partial) sequences will replicate or express efficiently since they are not advantageous to the virus and most likely decrease the overall replication & expression efficiency of the virus. Therefore the total risk of such a recombination is deemed negligible.

Interaction with bacteria:
In the extremely unlikely event an active sequence would become part of the environment, it could not be replicated in a bacteria present in subjects and in the environment. NV1FGF is a pCOR plasmid carrying the sp.FGF1 expression cassette. pCOR are small size plasmids carrying a Conditional Origin of Replication (R6K) which requires a trans-acting replication factor (π) meaning that pCOR can only

replicate in a specific E. coli host strain producing π, greatly reducing the potential for propagation in treated patients and in the environment.

Clarification on potential risk to have NV1FGF replication in natural bacteria:

NV1FGF replication is obtained in a specifically laboratory engineered E. Coli strain (E. Coli pir116) that was constructed to produce the R6K replication factor π. The sequence for this factor is not beard by NV1FGF plasmid, making its replication impossible without an external source of π[28].

The question is: what is the probability that NV1FGF plasmid encounters a bacterium susceptible to produce the π factor - probability of presence of such bacteria in the intestine and what would happen?

To evaluate such probability, the following points need to be taken into account:

- This hypothesis implies that NV1FGF would be swallowed. This is highly unlikely as the intended route of administration is via intramuscular injection done by the investigator.

- Secondly, the time during which NV1FGF plasmid would remain complete in the intestine would need to be long enough to allow an efficient transfection of bacteria. However, the actual time is considered as very short taking into account the presence of nucleases in the intestine.

- Furthermore, the breakdown of DNA during food processing and passage through the gastrointestinal tract reduces the likelihood that intact genes capable of encoding foreign proteins will be transferred to gut microflora [31].

- Lastly, the type of bacteria that can bring π factor.
O The π factor can only be present in bacteria that bear on their plasmid the natural R6K replication system including the π sequence, i.e. E. Coli bacteria or closely related bacteria (R6K resident bacteria). This indication limits therefore the replication of NV1FGF plasmid in these bacteria.
O Importantly, there is no report of a π -like activity in other strains that could allow NV1FGF replication.

In the very unlikely event the three conditions above would be met, what is the Impact of a transfection by NV1FGF of a natural E.Coli strain bearing R6K?

The principle of incompatibility between plasmids would apply [34, 35]. Indeed a bacteria plasmid bearing the R6K system and the NV1FGF bearing the same system (without π sequence) would co-exist. In such a case, the coexistence of the major incompatibility factor present in the oriγ sequence of the R6K system of both systems will lead to the loss of one of the plasmids. Meaning either the bacteria plasmid inducing the lack of π factor needed by NV1FGF, or the NV1FGF plasmid itself: in both cases the replication of NV1FGF plasmid would not be possible or at most very transient.

Taking into account the rational above, it is therefore considered that the probability that NV1FGF replicates in intestinal bacteria would be present in the environment is extremely low if not of zero.

With regards recombination with virus:
Firstly, Cytomegalovirus (CMV) promoter sequence in the PCOR plasmid is a human CMV (hCMV) sequence. It is not from viral origin but from recombinant origin. The only way NV1FGF plasmid DNA could recombine with viruses would be inside a myofiber cell that would be the host of a DNA virus. The recombination mode in mammalian cells is mainly based on non homologous sequences; therefore the unlikely potential sequence analogy between hCMV and sequence of viral origin would not increase the potential risk of mutation which is negligible. Indeed, as mentioned above the probability of NV1FGF plasmid integration in human genome has been evaluated and results indicated that the risk of integration is negligible. In the extremely unlikely event of an homologous recombination, this would lead to the integration of the sequence of viral origin in one of the pCOR

plasmid constitutive sequence: hCMV promoter, FGF1-gene, R6K plasmid, etc... Due to pCOR small size, this would prevent expression of the pCOR plasmid.

Additionally, with regards the chance of plasmid uptake by other cells in the body (most importantly the reproductive cells) absorb the plasmid: Nonclinical and clinical data indicate that IM administration of NV1FGF result in expression of NV1FGF-derived FGF1 in myofibers located around the injection site only. Biodistribution data collected in the rat indicate a transient systemic distribution of DNA sequences that are mostly product of plasmid degradation without the intact expression cassette and therefore without the capability to express FGF1 transgene. An additional nonclinical rat study, using a sensitive quantitative RT-PCR assay, indicated that IM administration of NV1FGF did not led to expression of NV1FGF-derived mRNA in organs other than the injected muscle. In particular there was no evidence of NV1FGF-derived mRNA expression in the gonads from day 1 to 3 months post-administration. These data suggest no chance that cells other than the myofibers at the injected site can express NV1FGF.

Conclusion:

Based on these data there is no evidence that support an environmental risk. There a no significant possibility that NV1FGF will spread from the patient to other persons or to the environment. It is a product classified as biosafety level I. Institutional biosafety policies and procedures will be followed.

Production of the GMO or nucleic acid preparation

A4.2. State under whose responsibility the production of the GMO or nucleic acid is carried out.

Answer:

O Production will be by and under the responsibility of the applicant and forms part of this licence application:
O Production will be by and under the responsibility of the applicant but does not form part of this licence application:
O A separate application for production will be submitted for contained use
O Reference is made for the production to an existing licence for contained use: (State here the number of the relevant GMO licence)
• Production will be under the responsibility of third parties. If production is in the Netherlands, please state the number of the relevant GMO licence. Please state if production is outside the Netherlands.

Production is outside the Netherlands by sanofi-aventis Deutschland GmbH in Frankfurt, Germany. More information can be found in the "Copy of the Manufacturer's Authorisation", appendix I.

A4.3. During which steps of the production process does quality control take place, which test methods are used and how are the tests carried out?
Give an overview of the production process of the GMO or nucleic acid preparation and describe the points in the production process at which quality control takes place. State which controls are carried out and which methods are used for the controls.

NV1FGF is produced by classical E. coli fermentation and, after chemical lysis of the bacteria, the product is submitted to subsequent purification by chromatography in order to obtain the drug substance (DS). At the broth harvest level, cell growth (dry cell weight), microorganism contamination and plasmid stability are tested. The clarified lysate following lysis process is tested for the ratio impurities/plasmid DNA. The eluate from last

chromatography is tested for percentage of plasmid open circular forms and plasmid concentration is determined following diafiltration.
The drug product is a solution for injection of plasmid DNA at a concentration of 0.2 mg/mL in aqueous, trometamol-buffered 0.9% NaCl. It is supplied for parenteral administration as a sterile, non-pyrogen, injectable, clear solution. The pH of the solution is about 7.4.

It is manufactured by dissolving and mixing the mentioned ingredients, followed by sterilizing filtration and filling in vials. Quality Control tests during manufacturing are measurement of pH and determination of maximum acceptable bioburden (the latter according EMEA guideline CPMP/QWP/486/95).

The purity of the product in term of DNA species, DNA purity and other contaminant is as follows:

	TEST	Specifications
DNA Species	% of supercoiling (IE HPLC)	> 80%
	Plasmid size (agarose gel)	2178 – 2662 bp
	Restriction map(agarose gel)	Sizes as expected
	Dimers (CE)	≤ 3%
	Trimers (CE)	≤ 2%
	Tetramers (CE)	≤ 2%
DNA Purity	E. Coli DNA (PCR)	≤ 100 ppm
	E. Coli RNA (RT-PCR)	≤ 100 ppm
	E. Coli proteins (ELISA)	≤ 1.0 ppm
	Oligonucleotide (IE HPLC)	< 20 ppm
	Ammonium sulfate (IE HPLC)	< 100 ppm
Other contaminants	Bacterial endotoxin test	< 5 EU/ml
	Sterility	pass

A4.4. Which criteria are imposed on a batch of the GMO before it is released for the application in question?
State which criteria are used to reject a batch.

The drug substance is tested for appearance, identity, purity, potency quantity and general safety tests. Each release test has defined acceptance criteria. All release tests should be compliant to allow DS batch release.
For identity DNA plasmid size is measured using agarose gel electrophoresis before and after digestion with site specific endonucleases.
For identity, product related impurities as well as process related impurities are measured. The percentage of multimeric forms of plasmid is measured using capillary electrophoresis,

the percentage of supercoiled and depurinated forms using ion exchange HPLC. Remaining contaminants from the host are tested: host cell proteins using a specific ELISA assay, host cell DNA using Q-PCR and host cell RNA using Q-RT-PCR. The impurities from the process are tested using ion exchange HPLC: the oligonucleotide (leachable from the resin of chromatographic column) and ammonium sulfate (last buffer used before final diafiltration).
The dose is measured using ion exchange HPLC.
For potency a specific in vitro bioassay is used to compare each batch to a reference standard.
As this drug substance is dedicated to manufature an injectable, bioburden, endotoxine level and pH are also measured.

The drug product is tested for appearance, purity, potency, identity, quantity and general safety tests. Each release test has defined acceptance criteria. All release tests should be compliant to allow DP batch release.
For purity the percentage of supercoiled and depurinated forms are measured using ion exchange HPLC. The potency and the identity are controled using a specific bioassay and the dose using HPLC.
As this drug product is an injectable product, the following standard tests are also performed: appearance, sterility assessment and measurement of endotoxin level, pH, and extractable volume.

Aspects forming part of the study

A4.5. How many test subjects will take part in the study?

The study is planned to enrol 490 patients internationally. Patients will be randomized either to placebo or to NV1FGF group.

A4.6. Which doses will be administered and at what times during the study will they be administered?

XRP0038/NV1FGF or placebo will be administrated at 2-week intervals for a total of 4 administrations (4 mg each, total administered dose: 16mg) scheduled on day 1, day 15 +/-2, day 29 +/-2 and day 43 +/-2. Each administration implies 8 intramuscular injections of placebo or NV1FGF (0.5 mg each), 4 in the calf and 4 in the thigh. This dose has been administered to subjects in previous clinical studies.

The placebo to be used has a standard saline placebo formulation similarly to the placebo used in previous phase I-II clinical trials. The administration of an empty plasmid was not considered for ethical reasons.
As detailed in A4.1, the dose and regimen to be used do not influence the environmental risk analysis. As based on (pre)clinical studies with this plasmid DNA, the maximum dose that is to be administered (16 mg) does not have an adverse effect on people or the environment.

A4.7. How will the GMO preparation be administered to the test subject?

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

At each administration of XRP0038/NV1FGF or XRP0038/NV1FGF placebo, intra muscular injections will be performed in the leg to be treated. Investigators will be advised to administer in areas of good striated muscle and over an area of possible collateral blood flow, at distance from an artery or a nerve.
If a superficial spill occurs on the skin, skin should be disinfected with soap and water.

Although there are no significant environmental risks, standard requirements for all biosafety level 1 products will be followed:
Gloves should be worn for the product administration by the person administrating the product. After use, the needles, syringes and gloves must be discarded in a biohazard container. Empty vials should be put first in a sealed plastic bag before destruction by the pharmacy, as per biosafety level 1 policy.

A4.8. Are samples taken from the test subjects that do or may contain GMOs, and which tests are carried out with these samples?
Give an overview of the samples and state for which tests they are used. Describe how the sampling will take place. State whether GMO material is expected to be present in these samples.
Blood samples will only be taken for haematology and Biochemistry centralized analysis [gloves are worn when handling blood samples at the central lab level]. Those samples are unlikely to contain GMOs as NV1FGF plasmid is rapidly degraded after IM administration (half-life ~5 to 6 min).

Assuming nevertheless that the samples might contain GMO, this risk is low as per COGEM Table 1 classification: NV1FGF is a non transferable plasmid which presents an 'origin of replication' (R6K) with a CMV promoter and a polyadenylation signal.

In the extremely unlikely event an active sequence (full expression cassette) would become part of the environment, it could not be replicated in a bacteria present in subjects and in the environment. NV1FGF is a pCOR plasmid carrying the sp.FGF1 expression cassette. pCOR are small size plasmids carrying a Conditional Origin of Replication (R6K) which requires a trans-acting replication factor (π) meaning that pCOR can only replicate in a specific E. coli host strain producing π, greatly reducing the potential for propagation in treated patients and in the environment.

A4.9. Will the test subject be admitted to hospital for the study and which criteria will be used for his/her discharge?
When answering this question, also state whether hospital admission is prescribed apart from medical reasons for the purpose of protection against potentially negative effects on human beings and the environment.

There is no need for the study patient to be admitted to hospital for the study purpose: the study procedures, including NV1FGF or PLACEBO administration will require no more than 2 hours. However, study patients can be hospitalized because of their severe condition, but no criteria is to be used for their discharge.

Risk analysis

A4.10 State according to which scenario the genetically modified organism and/or a derivate from the nucleic acid preparation can disperse from the test subject into the environment.

State in your answer the scenarios in which the GMO may disperse in the environment. Explain the level of risk that spread will actually occur. Also describe in your answer whether the number of test subjects and/or the dosage to be administered affects the risks to be identified. Please give further details in questions A4.11 to A4.13.

IM administration was shown in animals and CLI subjects to induce FGF1 expression in a restricted area of the injected muscle only, not in distant organs.
Preclinical data indicate that IM administration of NV1FGF leads to a transient tissue distribution of NV1FGF sequences, mostly product of plasmid degradation with no capability to express FGF1 transgene. Indeed, NV1FGF –derived mRNA was restricted to the injected muscle only. NV1FGF is rapidly degraded when present in blood: the half-life of NV1FGF sequences in blood was shown to be 6 min in CLI patients. Clinical data indicate no dissemination of quantifiable levels of NV1FGF sequences in the urine samples collected at various time points post administration.

Furthermore, XRP0038/ NV1FGF plasmid does not contain sequences that increase plasmid integration rate, recombinogenic sequences, or sequences closely related to these motifs [13,14,15]. The presence of human sequences within NV1FGF does not raise a specific concern regarding integration. Indeed, integration by homologous recombination is several orders of magnitude less frequent than random-integration [16]. In addition, the maximum length of sequence homology between NV1FGF and the human genome is much lower than that required for efficient homologous recombination [17]. Based on these data, NV1FGF shows no particular risk of integration into the host genome.
A nonclinical study to document the risk of integration has been conducted. Following the injection of NV1FGF at a clinically relevant dose into rat muscles, levels of NV1FGF were found to be ≤27 copies associated with 1 µg of High Molecular Weight (HMW) genomic DNA after one round of purification. These data are similar to those obtained with plasmid DNA vaccines [23, 24, 25, 26, 27]). If these copies were integrated, the frequency would be 3 to 2 orders of magnitude below the spontaneous mutation rate [22], indicating that the risk of mutation is negligible.

These data as well as previous studies indicating a transient distribution of NV1FGF sequences to the gonads (mostly product of degradation with no evidence of transgene expression in gonads at any assessed time point, nor evidence of persistence of NV1FGF sequences in gonads) and no treatment-related adverse effects in the gonads provide no evidence of risk of inadvertent germ line integration.

Assuming nevertheless that the patient's body secretions might contain GMO, this risk is low as per COGEM Table 1 classification: NV1FGF is a non transferable plasmid which presents an 'origin of replication' (R6K) with a CMV promoter and a polyadenylation signal.

Table 1 - Criteria risk groups naked DNA – COGEM advice CGM/041223-02 December 23rd, 2004

Group	Criteria sequenties
I high risk	- naked DNA which is replication competent in mammalian cells. - naked DNA which could become part of viruses, i.e. due to the presence of viral packing signals (such as Ψ); - naked DNA which could be efficiently absorbed by bacteria due to the presence of DNA uptake sequences (DUS), such as the the specific *Neisseria* DUS 5'- GCCGTCTGAA-3' and the *H. influenzae* DUS 5'-AAGTGCGGT-3'.
II medium risk	- non-transferable plasmids with viral sequences with the exception of the CMV promotor and the polyadenylation signal. - non-transferable plasmids with sequences which have the intention to promote or cause recombination of naked DNA (of parts hereof) with, or integration of the naked DNA (of parts hereof) in, the genome. Such sequences are, among others, the recombination signal of the *Hepatitis B virus*, retroviral LTR, bacteriophage integrases, VDJ-recombination signalsequences, Alu elements en hypervariabel minisatellite sequences; - non-transferable plasmids with protein coding sequences that have transforming or oncogenic properties.
III low risk	- non transferable plasmids, based on the 'origin of replication' (ori) which are present in plasmids such as pBR322, pUC (ColE1 ori) and p15A (pACYC-series plasmids) which contain a kanamycin-resistence gene which is active in prokaryotes - non-transferable plasmids with a CMV promoter active in eukaryotic cells, a polyadenylation signal and the eukaryotic VEGF-2 gene

In case there is a spill of body secretions on a human skin, the plasmid can not enter the cells in these conditions. In order to lead to gene transfer and expression in a given tissue, plasmids need to be present in the extracellular space in close contact to the cell membrane of living cells so the DNA plasmid can be taken up within the cytoplasm and subsequently within the nucleus. It is estimated that at least 105 plasmid copies per cell are required in the extracellular compartment to ensure that a few DNA molecules are taken up into the nucleus of non-mitotic cells such as a skeletal myofiber[29]. Indeed, in addition to the extracellular (plasmid retention and plasmid degradation due to endonucleases) and cellular barriers, intracellular obstacles restrict the translocation of plasmid vectors into the nucleus (restricted cytoplasm mobility, metabolic instability, endo-lysosmal entrapment, nuclear envelope barrier) Therefore a high number of copies is directly injected within the muscle mass (living cells) to result in gene transfer and expression. Gene transfer is not expected to succeed when the plasmid ends up on the skin.

In the extremely unlikely event an active sequence would become part of the environment, it could not be replicated in a bacteria present in subjects and in the environment. NV1FGF is a pCOR plasmid carrying the sp.FGF1 expression cassette. pCOR are small size plasmids carrying a Conditional Origin of Replication (R6K) which requires a trans-acting replication factor (π) meaning that pCOR can only replicate in a specific E. coli host strain producing π, greatly reducing the potential for propagation in treated patients and in the environment.

Clarification on potential risk to have NV1FGF replication in natural bacteria:

NV1FGF replication is obtained in a specifically laboratory engineered E. Coli strain (E. Coli pir116) that was constructed to produce the R6K replication factor π. The sequence for this factor is not beard by NV1FGF plasmid, making its replication impossible without an external source of π[28].

The question is: what is the probability that NV1FGF plasmid encounters a bacterium susceptible to produce the π factor - probability of presence of such bacteria in the intestine and what would happen?

To evaluate such probability, the following points need to be taken into account:

- This hypothesis implies that NV1FGF would be swallowed. This is highly unlikely as the intended route of administration is via intramuscular injection done by the investigator.

- Secondly, the time during which NV1FGF plasmid would remain complete in the intestine would need to be long enough to allow an efficient transfection of bacteria. However, the actual time is considered as very short taking into account the presence of nucleases in the intestine.

- Furthermore, the breakdown of DNA during food processing and passage through the gastrointestinal tract reduces the likelihood that intact genes capable of encoding foreign proteins will be transferred to gut microflora [31].

- Lastly, the type of bacteria that can bring π factor.
    - O The π factor can only be present in bacteria that bear on their plasmid the natural R6K replication system including the π sequence, i.e. E. Coli bacteria or closely related bacteria (R6K resident bacteria). This indication limits therefore the replication of NV1FGF plasmid in these bacteria.
    - O Importantly, there is no report of a π -like activity in other strains that could allow NV1FGF replication.

In the very unlikely event the three conditions above would be met, what is the Impact of a transfection by NV1FGF of a natural E.Coli strain bearing R6K?

The principle of incompatibility between plasmids would apply [34, 35]. Indeed a bacteria plasmid bearing the R6K system and the NV1FGF bearing the same system (without π sequence) would co-exist. In such a case, the coexistence of the major incompatibility factor present in the oriγ sequence of the R6K system of both systems will lead to the loss of one of the plasmids. Meaning either the bacteria plasmid inducing the lack of π factor needed by NV1FGF, or the NV1FGF plasmid itself: in both cases the replication of NV1FGF plasmid would not be possible or at most very transient.

Taking into account the rational above, it is therefore considered that the probability that NV1FGF replicates in intestinal bacteria would be present in the environment is extremely low if not of zero.

Gut flora is not a possible host for the plasmid since original E.Coli strain in gut flora does not display the gene for pi protein which is mandatory for plasmid replication.

There is no significant possibility that NV1FGF will spread from the patient to other persons or to the environment. It is a product classified as biosafety level I. Institutional biosafety policies and procedures will be followed.

A4.11 State which potentially harmful effects may be linked to exposure of human beings or the environment to the GMO.

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

Assuming despite the unlikelihood, that NV1FGF could spread from the patient to other persons or to the environment via body secretions containing the GMO (with the full expression cassette), and assuming that the plasmid would be transferred into another person cells (without being inactivated by the air), the quantity of product able to do so will be very limited.

In case this small quantity is not degraded in the circulation and reaches functional cells potentially leading to local FGF1 expression, angiogenesis (development of new capillaries and arterioles) could theoretically occur locally, provided that the level of FGF1 expression is high enough to stimulate the FGF receptors and that receptor single activation is deemed enough to induce this phenomenon.

No risk to human beings and the environment are anticipated

A4.12 Give an estimate of the chance that the adverse effects described in A4.11 could actually occur.

Give a reasoned estimate of the chance of the aspects described in A4.10 and A4.11, also taking account of the number of test subjects and the dosage.

The risk that the adverse effects described above (angiogenesis in the site where GMO is transferred) occur depends on the following risks:

- The risk that GMO (with the full expression cassette) is contained in the body secretions (NV1FGF has a local distribution, and was not detected in organs distant from the injection site).
- The risk that the GMO is transferred from the body secretions of a treated patient to another person and enter the latter cells (an active transfer would be required : A spill of solution/secretion on the skin, on the respiratory mucosa, can not transfer the GMO).
- The ability for a very low quantity of the GMO (and therefore a very low quantity of FGF1 expressed) to induce a clinically significant angiogenesis (i.e. leading to an adverse effect).

Given the previous, assuming that this risk exists, it is very low.

A4.13 Describe the risks that could occur as a consequence of the application of the GMO.

Describe the risks in such a way that makes clear how the risks can be reduced through risk management.

NV1FGF IM toxicity studies in rats did not show any treatment-related effects other than at the injection site. No adverse effects were observed in organs distal from the injected muscle including eyes, gonads and kidney. In addition, direct injection of very high levels of NV1FGF did not result in any adverse effects.

The safety of NV1FGF administration has been assessed in 5 clinical trials with a total of 192 patients who received at least 1 injection of NV1FGF. Current data indicate that single and repeated IM injections of NV1FGF were well tolerated at the site of injection. No data indicate an increased incidence of adverse events at distance of injection site.

See A4.1 for further environmental risks

Risk management

A4.14 Which inclusion and exclusion criteria are adopted in the selection of test subjects and what is the effect of these criteria on environmental safety?
Give an overview of the inclusion and exclusion criteria that are necessary for the protection of the environment or which criteria might possibly have an effect on safety for human beings and the environment.

No specific exclusion/inclusion criteria are needed with respect to environmental safety

A4.15 What limitation to the scope of the study, in relation to the number of test subjects and the dosages to be used, is used as part of risk management measures?
If you have already answered that the number of test subjects, the dosage of the GMO or nucleic acid preparation affect the risks to human beings and the environment, please state whether and, if so, which measures will be taken to manage the risks relating to these aspects.

No risk is associated with the number of subjects. The dosage and mode of administration of NV1FGF have been assessed in previous clinical trials under Biosafety level 1 regulations, with no data indicating the need for specific risk management measures.

A4.16 Describe which measures are provided for in respect of the hospitalisation of the test subject.
When answering this question, please emphasise those aspects that are important in preventing spread in the environment of the test subject. Also state which discharge criteria are to be adopted.

As mentioned above, there is no significant risk of spread in the environment of NV1FGF. No specific measures are needed in case of hospitalization.

A4.17 Describe which measures will be taken to prevent the spread of the GMO to third parties (including medical personnel).
For example, give an overview of relevant (hospital hygiene) measures that will be taken.

Not applicable, other than standard biosafety level 1 guidelines should be adhered to as defined in hospital protocols.

A4.18 Describe what aftercare will be given if a test subject ends his/her participation in the study prematurely.
Also state to what extent the aftercare deviates from the aftercare for test subjects who have completed the entire study.

If the study patient ends his participation during the treatment phase, he will be asked to complete the remaining study visits as per protocol. If the patient ends his participation during the follow-up period, he will be asked to have a final assessment at 12 month. This evaluation could include any of the assessments requested by the protocol or any other procedures that the investigator feels are medically necessary.

A4.19 Describe the procedures to be followed if changes in the risk management are required for medical reasons.

Consider situations in this respect where a test subject needs to be taken out of isolation, e.g. because treatment in intensive care is required, or where unforeseen effects are observed.

Not applicable

A4.20 Describe which samples can be expected to contain GMOs, and for these samples state how sampling will take place and how the samples will be processed further.
When answering the question, also state how the spread of the GMO during sampling and testing will be prevented. State which physical containment will be used with the further processing. If the activities concerned do not form part of the present licence application, you are referred to a licence for work under Contained Use.

Blood samples will be taken for haematology and Biochemistry centralized analysis at all study visits. Most samples will be taken before the GMO administration except one CPK sampling at Day 15) that is requested from 2 to 48 hours after the investigational product administration. Gloves are worn for the blood drawing at the hospitals. Blood samples will be put in a transparent bag, and then in a protective gel bag, and then in special shipping box with all identifications required. At the central lab level, gloves are also worn when handling the blood samples.

As detailed above (question A 4.8), those blood samples are unlikely to contain GMOs with full expression cassette and assuming nevertheless that the samples might contain GMO, this risk is low. NV1FGF is a non transferable plasmid which presents an 'origin of replication' (R6K) with a CMV promoter and a polyadenylation signal.

Monitoring and waste processing

A4.21 How is the GMO preparation detected after being administered?
State, if applicable, when GMO components are detected during or after administration and why detection at that particular moment of the test is regarded as important. Describe the nature of the samples that are tested, the method used and the detection limit that can be achieved.

Not applicable, based on previous results, the administration of NV1FGF via the IM route was confirmed to be appropriate for local treatment of CLI. Hence, no samples are taken to detect NV1FGF preparation in the Phase 3 trial.

A4.22 Describe how the monitoring will be set up to identify any spread of the GMO.
In answering this question, pay attention to the method followed, but also to the period during which any positive result can be expected in connection with the applicable scenario. Also state in this context during which period monitoring will take place.

Not applicable as detailed above (A4.21).

A4.23 Give an overview of the nature and quantity of the waste produced and describe how the waste will be disposed of.
State which waste flows can be distinguished. State which waste flows may potentially contain the GMO and how the GMO is prevented from being released into the environment via the waste flows.

XRP0038 / NV1FGF product is glass vials at hospital/site level and will be administered to the patients via Intramuscular injection. The waste on site would consist of partially used/ unused clinical supply (including syringes/needles). Syringes and needles need to be discarded in the biohazardous sharp container after use. These are disposed as required by hospital protocols as mentioned before at A1.27. A detailed treatment log of the returned to sponsor/destroyed on site IP will be established with the investigator (or the pharmacist) and counter signed by the investigator and the monitoring team. The investigator or a designated person will not destroy the unused IP unless the sponsor provides written authorization.

A4.24 Describe the hospital hygiene measures used to prevent the spread of the genetically modified organism.
If existing guidelines are followed, please state which ones. Additional or deviating measures should also be described.

Biosafety level I guidelines and Microbiological Good Practice should be adhered to. Hospital protocols for are applicable as mentioned before at A1.27. In case of accidental inoculation of the GMO to the hospital staff for example, the event should be reported immediately to the sponsor Pharmacovigilance.

Signature:

On behalf of the legal entity date: January 9, 2009
Name: Dr P Batenburg
Chairman Board of Directors Catharina Hospital

REI (non-clinical application) date: January 9, 2009
Name: Dr MRHM van Sambeek
Surgeon, Department of Surgery, Catharina Hospital

REII (clinical application) date: January 9, 2009
Name: Dr MRHM van Sambeek
Surgeon, Department of Surgery, Catharina Hospital

ESO date January 16, 2009
Name: Dr. K.E. Bakker

Appendix

1. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev 2005; 16:159-78.
2. Javerzat S, Auguste P, Bikfalvi A. The role of fibroblast growth factors in vascular development. Trends Mol Med 2002; 8: 483-9.
3. Witzenbichler B, Mahfoudi A, Soubrier F, Le Roux A, Branellec D, Schultheiss HP, Isner JM. Intramuscular gene transfer of fibroblast growth factor-1 using improved pCOR plasmid design stimulates collateral formation in a rabbit ischemic hindlimb model. J Mol Med 2006;84(6):491-502
4. Pellegrini L, Burke DF, Von Delft F, Mulloy B, Blundell TL. Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. Nature 2000 407: 1029-1034. (PDB id:1e0o)
5 DiGabriele AD, Lax I, Chen DI, Svahn CM, Jaye M, Schlessinger J, Hendrickson WA. Structure of a heparin-linked biologically active dimer of fibroblast growth factor. Nature 1998; 25;393(6687):812-817. (PDB id:2axm)
6. Plotnikov AN, Hubbard SR, Schlessinger J, Mohammadi M. Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specificity. Cell 2000; 101(4):413-424. (PDB id:1evt)
7. Ogura K, Nagata K, Hatanaka H, Habuchi H, Kimata K, Tate S, Ravera MW, et al. Solution structure of human acidic fibroblast growth factor and interaction with heparin-derived hexasaccharide. J Biomol NMR 1999;13(1):11-24.
8. Burgess WH, Mehlman T, Marshak DR, Fraser BA, Maciag T. Structural evidence that endothelial cell growth factor beta is the precursor of both endothelial cell growth factor alpha and acidic fibroblast growth factor. Proc Natl Acad Sci U S A. 1986;83:7216-20.
9. Burgess WH, Mehlman T, Friesel R, Johnson WV, Maciag T. Multiple forms of endothelial cell growth factor . Rapid isolation and biological and chemical characterization. J Biol Chem. 1985;260:11389-92.
10. Schreiber AB, Kenney J, Kowalski J, Thomas KA, Gimenez-Gallego G, Rios-Candelore M, et al. A unique family of endothelial cell polypeptide mitogens: the antigenic and receptor cross-reactivity of bovine endothelial cell growth factor, brain-derived acidic fibroblast growth factor, and eye-derived growth factor-II. J Cell Biol. 1985;101:1623-6.
11. Tabata H, Silver M, Isner JM. Arterial gene transfer of acidic fibroblast growth factor for therapeutic angiogenesis in vivo: critical role of secretion signal in use of naked DNA. Cardiovascular Research 1997; 35: 470-9.
12. Isner JM, Rosenfield K. Redefining the treatment of peripheral artery disease. Role of percutaneous revascularization. Circulation. 1993 Oct;88(4 Pt1):1534-57.
13 Aoki H, Kajino K, Arakawa Y, Hino O, Molecular cloning of a rat chromosome putative recombinogenic sequence homologous to the hepatitis B virus encapsidation signal. Proc. Natl. Acad. Sci. USA, 93(14): 7300-04, 1996.
14 Kang Y-K, Park JS, Lee C-S, Yeom YI, Chung A-S, Lee K-K. Efficient integration of short interspersed element-flanked foreign DNA via Homologous recombination., J. Biol. Chem., 274(51): 36585-91, 1999.
15 Rudiger NS, Gregersen N, Kielland-Brandt MC. One short well conserved region of lusequences is involved in human gene rearrangements and has homology with prokaryotic chi. Nucleic Acids Res., 23(2): 256-60, 1995.
16 Yanez RJ, Porter ACG. Therapeutic gene targeting. Gene Ther., 5(2): 149-59, 1998.
17 Hasty P, Rivera-Perez J, Bradley A. The length of homology required for gene targeting in embryonic stem cells. Mol. Cell. Biol., 11(11): 5586-91, 1991.
18 Winegar et al., Determination of Tissue Distribution of an Intramuscular Plasmid Vaccine Using PCR and *In Situ* DNA Hybridzation. Hum. Gene Ther. 1996, 7, 2185-2194;
19 Parker et al., Plasmid DNA Malaria Vaccine: Tissue Distribution and Safety Studies in Mice and Rabbits. Hum. Gene Ther. 1999, 10, 741-748;
20 Parker et al., Safety of a GM-CSF adjuvant-plasmid DNA malaria vaccine. Gene Ther. 2001, 8, 1011-1023;

21 Kim et al., Potential DNA Vaccine Integration Into Host Cell Genome. Arch. Pharm. Res. 2003, 26, 493-498
22 Cole J, Skopek TR. International Commission for protection against environmental mutagens and carcinogens. Working papers no. 3. Somatic mutant frequency, mutation rates and mutation spectra in the human population in vivo. Mutat. Res., 304: 33-105, 1994.
23 Nichols WW, Ledwith BJ, Manam SV, et al. Potential DNA vaccine integration into host cell genome. Annals New York Academy of Sciences, 772: 30-39, 1995.
24 Martin T, Parker SE, Hedstrom R, Le T, Hoffman SL, Norman J, Hobart P, Lew D. Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. Hum Gene Ther.10: 759-768, 1999.
25 Ledwith BJ, Manam S, Troilo PJ, et al. Plasmid DNA vaccines: Investigation of integration into host cellular DNA following intramuscular injection in mice. Intervirology, 43: 258-272, 2000.
26 Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, Griffiths II TG, Niu Z, Denisova L, Follmer TT, Pacchione SJ, Wang Z, Beare CM, Bagdon WJ, Nichols WW. Plasmid DNA vaccines: Tissue distribution and effects of DNA sequences, adjuvants and delivery method on integration into host DNA. Intervirology, 43: 273-281, 2000.
27 Wang Z, Troilo PJ, Wang W, Griffiths II TG, Pacchione SJ Barnum AB, Harper LB, Pauley CJ, Niu Z, Denisova L, Follmer TT, Rizzuto G, Ciliberto G, Fattori E, Monica NL, Manam S, Ledwith BJ. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. Gene Therapy, 11: 711-721, 2004.
28 Soubrier F, Cameron B, Manse B, Somarriba S, Dubertret C, Jaslin G, Jung, G, Le Caer C, Dang D, Mouvault JM, Scherman D, Mayaux JF, Crouzet J. pCOR: a new design of plasmid vectors for nonviral gene therapy. Gene Therapy, 6: 1482-1488, 1999.
29 Lechardeur et al., Intracellular routing of plasmid DNA during non-viral gene transfer. Advanced Drug Delivery Reviews 57 (2005) 755– 767.
30 Nikol S, VanBelle E, Diehm C, Visona A, Capogrossi M, Ferreira-Maldent N, et al. Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. Program and abstracts of the American College of Cardiology 55th Annual Scientific Session; March 11-14, 2006; Atlanta, Georgia. Smaller Trial Late Breaking Clinical Trials I. Abstract 405-10.
31 D.A Jonas et al., Safety Considerations of DNA in Food, Ann Nutr Metab 2001;45:235–254.
32 Comerota AJ, Throm RC, Miller KA, Henry T, Chronos N, Laird J, et al. Naked plasmid DNA encoding fibroblast growth factor type 1 for the treatment of end-stage unreconstructible lower extremity ischemia: preliminary results of a phase 1 trial. J Vasc Surg. 2002 May;35(5):930-62.
33 Caron A, Michelet S, Caron A, Sordello S, Ivanov MA, Delaere P, Branellec D, Schwartz B, Emmanuel F. Human FGF-1 gene transfer promotes the formation of collateral vessels and arterioles in ischemic muscles of hypercholesterolemic hamsters. J Gene Med. 2004;6:1033-45
34 Novick R, Hoppensteadt F. On plasmid incompatibility. Plasmid 1978; 1: 421–434.
35 Filutowicz M et al. DNA and protein interactions in the regulation of plasmid replication. J Cell Sci Suppl 1987; 7: 15–31.).