Application form

Assessment of clinical research involving gene therapeutics in the Netherlands

-

Bacteria

Clinical study title:

A Phase I/II Open Label Clinical Trial Assessing Safety and Efficacy of Intravesical Instillation of the Recombinant BCG VPM1002BC in Patients with Recurrent Non-Muscle Invasive Bladder Cancer after Standard BCG Therapy

Clinical Study Code:

SAKK 06/14

June 2017

Application form
Assessment of clinical study involving genetically modified bacteria

Part A: Bio-safety aspects

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A3. Production (Public section)
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A7. General (personal) information (Confidential section)- provided separately

Part B: Patient-related aspects (*not included in this form*)

If you have any questions, please get in touch with the Gene Therapy Office
(E-mail: rik.bleijs@rivm.nl, phone: +31-30-2747569).

October 2016

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Part A. Bio-safety aspects

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

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

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

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

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

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

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

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

Specific issues:

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

List of abbreviations

Ag85B	Antigen85B
ATCC	American Type Culture Collection
BCG	Bacille Calmette Guérin
BLAST	Basic local alignment search tool
bp	Base pair
BSL1	BioSafety level 1
CD	Cluster of differentiation
CDS	Coding sequence
CFP-10	10-kDa culture filtrate protein
CFU	Colony Forming Unit
COGEM	Commission on Genetic Modification
CTCAE	Common Terminology Criteria for Adverse Events
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
e.g.	*Exempli gratia* (for example)
EEC	European Economic Community
ESAT-6	6-kDa early secretory antigenic target
EU	European Union
GMO	Genetically modified organism
GMP	Good Manufacturing Practice
hsp60	heat shock protein 60
Hly	Listeriolysin gene
Hyg+	Hygromycin resistance
i.e.	*Id est* (that is)
IenM	Ministry of Infrastructure and Environment
IMP	Investigational medicinal product
LLO	Listeriolysin protein
L. monocytogenes	*Listeria monocytogenes*
LPLT	Last patient last treatment
LDH	Lactate dehydrogenase
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
M. tuberculosis	*Mycobacterium tuberculosis*
min	minute(s)
ML-I	BioSafety level 1
NGS	Next Generation Sequencing
NKI-AVL	Netherlands Cancer Institute Antoni van Leeuwenhoek
NMIBC	Non-muscle invasive bladder cancer
OD600	Optical density measured at wavelength of 600 nm
ORF	open reading frame
PCR	Polymerase Chain Reaction
PEST	P, Proline; E, Glutamic acid; S, Serine; T, Threonine
Ph. Eur.	Pharmacopoea Europaea
PI	Principle Investigator
QoL	Quality of Life
QP	Qualified Person
rBCG	Recombinant Bacille Calmette Guérin
RD	Region of difference
RKI	Robert-Koch Institute
RP2D	Recommended phase 2 dose
RT PCR	Real Time Polymerase Chain Reaction

SAEs	Serious adverse events
SAKK	Swiss Group for Clinical Cancer Research
SI	Sub-investigator
SIIPL	Serum Institute of India, Private Limited
SNP	Single Nucleotide Polymorphism
SOP	Standard operating procedure
SAEs	Serious adverse reactions
SADRs	Serious adverse drug reactions
SUSARs	Suspected unexpected serious adverse reactions
TB	Tuberculosis
TURB	Transurethral resection of the bladder
ureC	Urease C
UV	Ultraviolet
VPM1002BC	IMP: *Mycobacterium bovis* **r**BCGΔ*ureC*::Hly^{+} for immunotherapy
w/o	without
WGS	Whole Genome Sequencing
WHO	World Health Organization

A1. General application details

General information

A1.1. Application title:

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

A Phase I/II Open Label Clinical Trial Assessing Safety and Efficacy of Intravesical Instillation of the Recombinant Bacille Calmette Guérin (rBCG) VPM1002BC in Patients with Recurrent Non-Muscle Invasive Bladder Cancer (NMIBC) after Standard BCG Therapy.

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

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

As part of the phase I/II clinical trial sponsored by the Swiss Group for Clinical Cancer Research (SAKK), sponsor trial code: SAKK 06/14, it is intended to conduct phase II in the Netherlands. The objective of this Phase II clinical trial is to assess the safety and efficacy of intravesical instillation of the rBCG VPM1002BC in patients with NMIBC after standard BCG therapy.

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

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

VPM1002BC is an investigational medicinal product (IMP) whose active agent is a genetically modified organism (GMO). It is an immunotherapeutic agent based on the live attenuated *Mycobacterium bovis* BCG strain (Bacille Calmette Guérin). VPM1002BC was generated by incorporation of the gene for listeriolysin into the urease C (*ureC*) gene of *M. bovis* BCG. Listeriolysin is a pore-forming protein whose activity is strongly restricted to an acidic milieu, such as the pH 5.5-6 environment in phagosomes (1). Urease C is a *M. bovis* BCG enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia, thus contributing to alkalization, and therefore, inhibiting the maturation of mycobacterium-containing phagosomes of infected cells (2). Incorporation of the gene for listeriolysin into the *ureC* gene of *M. bovis* BCG resulted in disruption of the urease C activity and introduction of listeriolysin activity. Inactivation of the urease C gene ensures an acidic phagosomal pH necessary for listeriolysin activity. Listeriolysin in the acidic milieu of urine is of no concern, since it is constantly diluted in urine and excreted from the bladder. Furthermore, under normal conditions no cells are found in urine.

The exact name of the GMO is rBCGΔ*ureC*::Hly^{+}: (VPM1002BC).

As part of the Phase I/II SAKK06/14 clinical trial, VPM1002BC will be applied by instillation into the bladder. This application concerns SAKK 06/14 phase II clinical trial in the Netherlands. *M. bovis* BCG has been used as immunotherapy against NMIBC since first being reported in 1976 (3) as well as a vaccine for the prevention of tuberculosis (TB) all over the world since 1921. Worldwide, more than 200,000 patients suffering from NMIBC are treated with *M. bovis* BCG per year, with a probability of

disease to recur in 30-50% of cases (4). Also, intravesical therapy with *M. bovis* BCG is connected to frequent side effects, which are however in most cases of moderate severity.

Although *M. bovis* BCG has been used as a therapy for NMIBC since many years, exact mechanisms of its antitumor effects are still under investigation. It is known that multiple cell types are important for *M. bovis* BCG-mediated antitumor activity and the mechanisms are generally grouped in three stages: i) infection of urothelial cells or bladder cancer cells by *M. bovis* BCG ii) induction of immune responses and iii) induction of antitumor effects (5). Of particular importance for antitumor activity of *M. bovis* BCG is the T cell response (6). However, the potential of the standard *M. bovis* BCG currently used in clinical application to induce cluster of differentiation (CD)8^+ T cell activation seems to be suboptimal (7).

Therefore *M. bovis* BCG was modified genetically, with the aim of inducing a better immune response and improving its safety/tolerability by designing VPM1002BC. VPM1002BC was generated by incorporation of the gene for listeriolysin into the *ureC* gene of *M. bovis* BCG, which resulted in disruption of the urease C. Absence of urease C activity allows for development of an ideal pH environment for activity of listeriolysin in phagosomes of VPM1002BC-infected cells. Activated listeriolysin perforates the phagosomal membrane, resulting in leakage of phagosomal content (i.e. VPM1002BC antigens and phagosomal enzymes) into the cytosol, allowing cross-presentation of VPM1002BC antigens and induction of apoptosis of infected macrophages, promoting activation of bystander antigen presenting cells, and finally resulting in enhanced T cell-mediated immune responses (8). At the same time, listeriolysin's activity is tightly controlled through its narrow pH optimum and other molecular features (see below), limiting its activity to the phagosome. It is not active in the cytosol of the cells and does not lyse the outer cell membrane. Likewise, listeriolysin is not active in the blood (blood pH 7.4) so that hemolysis is not an issue. Listeriolysin in the acidic milieu of urine is of no concern, since it is constantly diluted in urine and excreted from the bladder. Furthermore, under normal conditions no cells are found in urine.

It is expected that modifications introduced into VPM1002BC will improve its antitumor activity in patients with NMIBC when compared to classical treatment with BCG.

A1.4. Describe briefly the intended work.

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

The SAKK 06/14 clinical trial phase II aims at assessing the safety, tolerability, efficacy and immunogenicity of intravesical instillations of VPM1002BC in patients with recurrent NMIBC after transurethral resection of the bladder (TURB) and standard *M. bovis* BCG therapy. The trial is "open label" and currently recruits patients in clinical trial sites in Switzerland and Germany.

The Netherlands Cancer Institute in Amsterdam will be involved in the phase II clinical trial as a clinical trial site in the Netherlands. Here, a license is being applied for the following procedures that will be performed at the site: receipt, storage, reconstitution, administration of VPM1002BC (bladder instillation), observation of patients, collection and processing of biological samples, waste disposal.
The study medication will be supplied to sites as a freeze dried cake in sealed glass vials, containing one dose of VPM1002BC. The investigational product will be shipped under control of a continuous temperature-monitoring device. After receipt of the study medication, the site pharmacist will immediately inspect all vials for damage. Any deviations or problems identified will be documented and promptly discussed with the sponsor in order to determine the usability of the supplies or if replacements must be sent. The site will be responsible for ensuring that the study products are stored in a locked refrigerator with limited access and in accordance with the instructions on the study

medication labels. Storage of the lyophilized powder and diluents shall be done at +2°C to +8°C, protected from direct sunlight. For application, VPM1002BC will be reconstituted with the provided diluent, using a closed drug transfer system. After reconstitution, VPM1002BC can be stored at room temperature and will be used within 3 hours. Before the actual application of VPM1002BC will take place, blood and urine samples will be taken from the patient. Thereafter, VPM1002BC will be applied into the bladder of the patient by intravesical instillation with a catheter.

Any waste material will be disposed of in accordance with local requirements (see A4.9). After the end of the study the unused study medication will be destroyed as bio-hazardous waste, in accordance with the current local practice, but only after inspection and approval by the monitor.

A1.5. Intended start and end date:

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

Expected period of release of VPM1002BC is from the date of approval of this study in the Netherlands until 31/03/2024.

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

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

No

Details of applicant

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

A1.7. Name of legal entity:

Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek Ziekenhuis

A1.8. Address of legal entity:

Plesmanlaan 121

A1.9. Postal code and town/city of location of legal entity:

1066CX, Amsterdam

A1.10. At which locations will the intended work take place?

Since the work applied for may only be carried out under the direct control of the license holder, it is only possible to carry out work at several locations if the license holder has full control of the way in which the work being applied for is carried out at all locations, in such a way that the license conditions are complied with. In that case you must state for each location what work will be carried out at which address and in which building. To clarify: you must state for all activities at which location they will be carried out. Apart from the location for the clinical activities with the GMO, you must also state the location or locations of laboratories in which activities with the GMO are carried out under the terms of this license application, such as procedures with patient samples.
In cases where central control is not possible, such as with a multi-centre study, a separate application must be submitted for each location.

Patient screening, treatment and follow-up, Study procedures and Blood sampling:

NKI-AVL (Netherlands Cancer Institute Antoni van Leeuwenhoek)
Onderzoek- en Behandel Centrum/Out Patient Clinic
Plesmanlaan 121
1066CX, Amsterdam, the Netherlands

Medication receipt, storage and disposal:

NKI-AVL
Onderzoek- en Behandel Centrum/Out Patient Clinic
Plesmanlaan 121
1066CX, Amsterdam, the Netherlands

Receipt will be done by pharmacist, storage and disposal will be done by authorized personnel in the Out Patient Clinic.

Medication preparation:

NKI-AVL
Onderzoek- en Behandel Centrum/Out Patient Clinic
Plesmanlaan 121
1066CX, Amsterdam, the Netherlands

Radiology assesments:

NKI-AVL
Afdeling Radiologie/Department of Radiology
Plesmanlaan 121
1066CX, Amsterdam, the Netherlands

Laboratory assessments

NKI-AVL
Centraal Klinisch Chemisch lab/Central Clinical Chemistry Laboratory

Plesmanlaan 121
1066 CX, Amsterdam, the Netherlands
Please note that only regular laboratory assessments will be done in the NKI-AVL.

Storage of urine samples will be done in the Out Patient Clinic and shipped afterwards to external parties.

A2. Construction and composition of the GMO

Bacterial strain from which the genetically modified organism was derived

A2.1. To which bacterial species does the strain belong that was used as the original strain in the construction of the GMO?

Provide, where applicable, the full scientific name, a trivial name (e.g. the commercial name), the sub-species and collection numbers.

To construct VPM1002BC *Mycobacterium bovis (M. bovis)* Bacille Calmette Guérin (BCG) was used as a recipient. Listeriolysin gene from *Listeria monocytogenes (L. monocytogenes)* was introduced into the genome of *M. bovis* BCG.

1. scientific name:

a) *Mycobacterium bovis (M. bovis)* Bacille Calmette Guérin (BCG)

b) *Listeria monocytogenes (L. monocytogenes)*

2. taxonomy:

a) *Bacteria; Actinobacteria; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium; M. bovis*; Strain BCG subtype Prague

b) *Bacteria*; *Firmicutes; Bacilli; Bacillales; Listeriaceae; Listeria; L. monocytogenes* renamed by Pirie 1940

3. other names (usual name, strain name, etc.):

a) BCG; BCG subtype Prague

b) *Listeria monocytogenes* EGD

A2.2. Is the original strain a GMO?

If so, provide a detailed description of the genetic modification. If the strain was developed or used in the Netherlands, then provide the number or numbers of the permits under which the work was carried out.

The original strain used to construct VPM1002BC is not a GMO.

A2.3. Describe the method of identification of the original strain.

The properties of the original organism form the basis for the environmental risk assessment. Confirmation of the identity is therefore important. Describe the characteristics that determine the identity of the original strain. This may be based on bio-information analyses, such as sequence analysis of a characteristic part of the genome, alignments or phylogenetic analysis. Information on the origins of the original strain, such as a micro-organism originating from the American Type Culture Collection (ATCC), may also be submitted.

After the generation of the original *M. bovis* BCG strain by consecutive sub-culturing (see section A2.5 for details), the number of *M. bovis* BCG sub-strains increased in the following decades. However, all these strains have in common, that they are highly attenuated forms of *Mycobacterium bovis*. This can be foremost attributed to the loss of a genetic region, RD1, encoding for important virulence factors (see section A2.5 for more details).

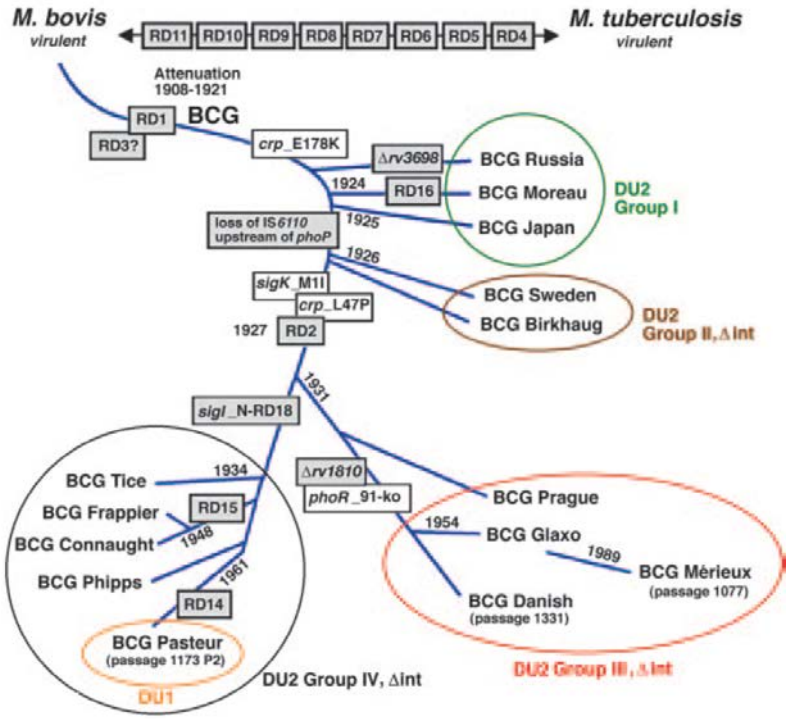

Figure 1: Genealogy of BCG strains according Brosch, *et al. M. bovis* BCG Prague, the parental strain of VPM1002BC, belongs to DU2 Group III. This is characterized by loss of RD1 and RD2, separating it from DU1 (e.g. BCG Pasteur) by still encoding RD14 and from DU2 Group I (e.g. BCG Russia) by still encoding RD16.

The parental strain of VPM1002BC was a *M. bovis* BCG strain, sub-strain Prague. This strain is characterized by loss of RD1 (common to all *M. bovis* BCG) and RD2, while still containing RD14 and RD16, separating it from *M. bovis* BCG Pasteur and *M. bovis* BCG Russia respectively (Figure 1). This places it in DU2 Group III, according to Brosch, et al. (9).

Development of VPM1002BC started at the Max Planck Institute for Infection Biology in Berlin. Introduction of listeriolysin into the *ureC* gene was initially tested in a *M. bovis* BCG sub-strain Pasteur background and further evaluated in the *M. bovis* BCG Danish sub-strain, before finally being assessed in *M. bovis* BCG sub-strain Prague. While the listeriolysin insertion and *ureC* deficiency are key to the mode of action, the genetic background was shown to be not important in all of these settings. Insertion of listeriolysin into *M. bovis* BCG sub-strain Prague, gave rise to VPM1002, a tuberculosis vaccine candidate that has been extensively characterized and developed in pre-clinical and clinical studies. VPM1002 initially contained a hygromycin-resistance mediating cassette. This has been removed (see A.2.9) to create VPM1002BC.

Characteristics that determine the identity of the parental *M. bovis* BCG strain used in the construction of VPM1002BC were confirmed by polymerase chain reaction (PCR) at the WHO reference centre FZ Borstel, Germany, where strains are defined by DNA fingerprinting in accordance with definitions by the WHO. Furthermore, whole genome sequencing was performed and the next generation sequencing analysis confirmed that the parental *M. bovis* BCG strain and VPM1002BC are related to *M. bovis* BCG Prague. This analysis was performed by Microsynth AG, Switzerland. Here, the whole genome sequences of the parental strain and that of VPM1002BC were compared to a reference sequence for *M. bovis* BCG Prague.

A2.4. What is the natural niche of the bacterial strain?

Describe the niche in which the original strain naturally occurs. For pathogenic or commensal bacteria, indicate the hosts in which they occur, and, for pathogens, the hosts that may serve as carriers.

No *M. bovis* BCG niche in nature has ever been documented (an extensive literature search did not find any reports). The *M. bovis* BCG, the parental strain of VPM1002BC, is a pure laboratory strain generated through continuous passages *in vitro* and is not viable in the environment.

A2.5. Provide relevant data on pathogenicity and possible attenuation and biological restrictions of the original strain.

If this concerns a pathogen-derived attenuated strain, describe the grounds on which the strain has been given a lower pathogenicity classification.

M. bovis BCG is an attenuated *M. bovis* strain, which has been generated through continuous passages *in vitro.* BCG was isolated after 230 serial passages of *M. bovis* on glycerol-potato-bile medium during a period of 13 years (10). It has been shown by comparison to the virulent *M. bovis* strain and *M. tuberculosis*, that all *M. bovis* BCG strains lack a genomic sequence called Region of Difference (RD) 1 (11). Deletion of RD1 is the initial genetic attenuation factor resulting in *M. bovis* BCG (11).. In addition, the *M. bovis* BCG Prague strain is lacking RD2 and all BCG sub-strains are lacking the RD3 sequence regions, which are intact in virulent members of the *M. tuberculosis* complex (11).

After initial production of *M. bovis* BCG, individual strains were sub-cultured independently over the last approx. 100 years. It has been reported that single nucleotide polymorphisms (SNPs) occurred, resulting in development of different BCG sub-strains with variable degrees of attenuation (9)(see Fig.1).

A2.6. Provide information about reproduction and survival of the bacterial strain in natural hosts.

Provide information about generation time under natural circumstances in natural hosts, and about possible survival or transmission structures formed by the original strain.

Not applicable because there are no natural habitats or reservoirs known for the pure laboratory strain *M. bovis* BCG, and therefore for VPM1002BC. An extensive literature research found no reports of natural hosts, habitats or reservoirs.

A2.7. What are the possibilities of survival, reproduction and transmission under environmental circumstances other than in natural hosts?

Provide all the observed and assumed transmission routes of the original strain, and indicate the effectiveness of the transmission and the role of the aforementioned transmission or survival structures.

There is no information that *M. bovis* BCG or VPM1002BC might be more resistant, or might better survive in the natural environment than wildtype *M. bovis*. An extensive literature search revealed no natural hosts for the pure laboratory strain *M. bovis* BCG. It is unlikely that the introduction of the *L. monocytogenes* listeriolysin into VPM1002BC has changed its non-resistance in the natural environment. Therefore, a similar survival time of VPM1002BC compared to *M. bovis* BCG and the wild type *M. bovis* can be predicted if it should be dispersed into the natural environment. Moreover, long time spreading of the vaccine strain *M. bovis* BCG into the environment has never been detected, even though it is in use for almost 100 years. An extensive literature search did not turn up any reports on spreading of the highly attenuated *M. bovis* BCG strain. One report by Young, et al. (12) shows that *M. bovis* BCG (Pasteur) deteriorates quickly in soil within 60 days. Another study revealed that orally *M. bovis* BCG-vaccinated brushtail possums (*Tichosurus vulpecula*) showed no *M. bovis* BCG in their faeces after 1 week (13). Both reports show that BCG is not viable in the environment. Furthermore, no natural habitats of *M. bovis* BCG are known.

In case of bladder cancer instillation, BCG in the urine of patients after intravesical treatment with standard BCG was detectable for approx. 1 week (14). Data from phase I part of the clinical trial SAKK 06/14 shows that VPM1002BC is even less persistent in urine of patients. Already after 24 hours after instillation no CFU were detectable (see section A2.16).

M. bovis BCG strains are known to be highly ultraviolet (UV) sensitive. As *M. bovis* BCG lacks a natural host, it can only persist in special culture media. The same can be assumed for VPM1002BC.

A2.8. Is the strain able to exchange material with other organisms?

Provide information about the strain's self-transmissible elements, mobilizing plasmids, transposons or other sequences involved in the transmission of DNA. Provide information about the incompatibility class and the host range of these elements.

Brosch et al. (15) analysed the whole genome of several mycobacteria and observed no evidence of any horizontal gene transfer. This was confirmed also for a genetically modified *M. bovis* BCG (16) with a genetic modification of plasmidic origin. There are no indigenous vectors known for mycobacteria, especially for *M. bovis* BCG. Additionally, none of the genetic material donated to VPM1002BC contains indigenous vectors.
In mycobacteria belonging to the *M. tuberculosis* complex, e.g. *M. bovis* BCG, such a horizontal gene transfer has been observed only in ancestor strains of the current strains of *M. bovis* (including BCG) and *M. tuberculosis* (17-22). This has not been observed in any experimental setting (16).
This issue was recently discussed at the 2nd Geneva Consensus Meeting of Live TB Vaccine Development at WHO (April 6-8, 2009). The consensus paper (23) states the following: "Comparative genomics of different *M. tuberculosis* complex lineages has revealed regions of difference (RD regions) that are now well characterised and known to form the basis for the attenuation of *M. bovis* BCG relative to the parent *M. bovis* or *M. tuberculosis*. A more detailed analysis of the sequence conservation around the junction of the RD regions has shown that there is a high degree of sequence conservation at the junction regions which are at sites within genes. These data support the contention that the RD regions almost certainly are consequent to deletion events (not genetic insertions) and the presence of such conserved deletions amongst the *M. tuberculosis* complex indicate deletion events in an early progenitor strain. The availability of detailed sequence data from numerous lineages has also provided further information that confirms that the *M. tuberculosis* complex overall is a highly clonal population with a perfect correlation between RD regions/junctions and SNPs and this provides significant evidence that there has been no horizontal gene transfer across lineages or even within lineages."

The genetically modified bacterium

A2.9. Has a vector been used in the genetic modification?

Is the vector fully or partly present in the GMO? Is the vector self-transmissible or could it become mobilized, or are these sequences involved in the transmission of DNA?

To generate VPM1002BC, an *Escherichia coli* (*E. coli*) pVEP2003 plasmid was used as a shuttle vector. Such vectors from apathogenic *E. coli* strains are commonly used for cloning. The plasmid carries an *E. coli* origin of replication but no mycobacterial origin of replication, without which it cannot replicate in mycobacteria. The plasmid carries no transposable elements. Identity of the vector pVEP2003 was analyzed by sequencing. The sequence is available in annex 1**.**
Only the following vector components are present in the genome of VPM1002BC after homologues recombination: *ureC* homologous region 5´, heat shock protein (*hsp)60* promoter, antigen (*Ag)85B-hly* fused sequence, sequence containing 1 $\gamma\delta$ resolvase-recognition site, and *ureC* homologous region 3´. Absence of other vector fragments in the GMO was confirmed by VPM1002BC genome sequencing. Whole genome sequencing (WGS) was performed using next generation sequencing technology and

the whole genome sequence of the parental *M. bovis* BCG, VPM1002BC and *M. bovis* BCG Prague were compared.

A2.10. Provide a description of the method of production of the genetically modified bacterium from the original strain and the original vectors.

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

A diagram describing the various steps used for construction of VPM1002BC is shown in Figure 2.

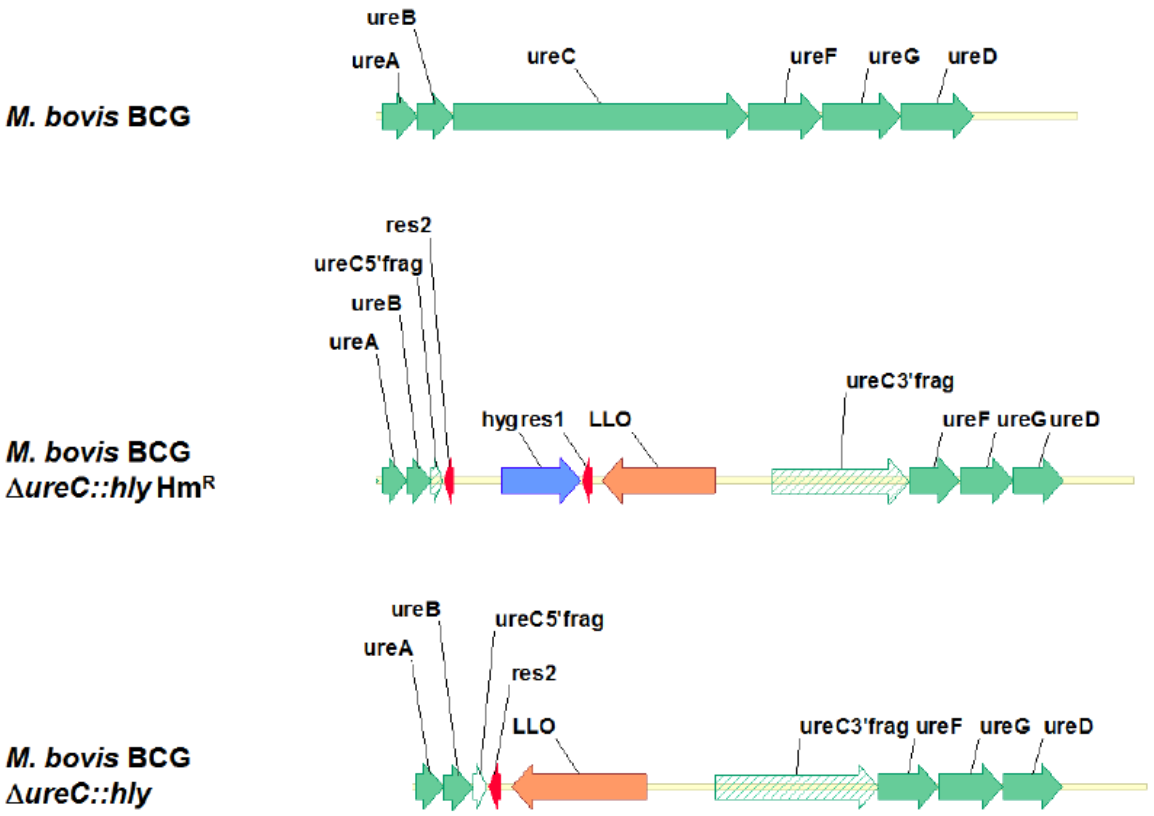

Figure 2: Schematic overview of genetic manipulation that resulted in VPM1002BC. Top: Urease C gene (*ureC*) in BCG wildtype background. Middle: Disruption of the *ureC* locus by insertion of listeriolysin O-expressing cassette (LLO) and hygromycin resistance marker (hyg). The latter is flanked by γδ-resolvase recognition sites (res1, res2). Bottom: removal of the resistance marker leaves the res2 site behind.

To generate VPM1002BC, a recombinant DNA vector pVEP2003 (Figure given in section A2.13) carrying the expression cassette containing a listeriolysin gene was introduced into *M. bovis* BCG. The plasmid pVEP2003 was based on the pJSC284 vector. The pJSC284 vector is a cosmid vector for cloning allelic exchange substrates. It contains an *E. coli* origin of replication, a single-cos site, a Pac I site for introduction into shuttle plasmid, and a hygromycin-resistance gene flanked by γδ resolvase recognition sites (24).
The pJSC284 vector was used as a backbone for the construction of the *Ag85B-hly*-coding cassette under the control of the *hsp60* promoter (25) and flanked by mycobacterial *ureC* sequences; resulting in the pVEP2003 vector.

The final cassette construct containing the target gene in VPM1002BC was achieved in 2 main steps:

- Homologous recombination-mediated insertion of the expression cassette containing the target gene and a hygromycin resistance sequence into the BCG genome

- γδ resolvase-mediated excision of the hygromycin-coding sequence out of the BCG genome

The initial VPM1002 contained a hygromycin resistance (Hyg+) encoding sequence flanked by 2 directly repeated homologous sequences containing γδ resolvase recognition sites. The two γδ resolvase recognition sites were used to excise the hygromycin out of VPM1002BC upon extra chromosomal expression of the resolvase. The pWM19 vector (26) was introduced transiently to VPM1002BC in order to allow for expression of resolvase (please see Figure 3). The plasmid encodes for two antibiotic resistance markers (gentamycin and hygromycin B), a temperature-sensitive origin of replication for mycobacteria (ts ori myco), as well as a *sacB* coding sequence. The latter two features render the vector under culturing conditions of 39 °C and high sucrose concentrations self-eliminating. The optimal replication conditions for the vector are 32 °C (above this it cannot replicate). Furthermore, the expression of *sacB* is toxic for mycobacterial cells under high glucose concentrations (26).
In addition, the plasmid encodes for γδ resolvase, a recombination enzyme, which catalyzes site-specific recombination resulting in 2 final products (27):

1. The excised DNA sequence between directly repeated sequences containing the resolvase recognition sites (circular DNA containing the hygromycin gene)
2. The rest of the target DNA containing one of the directly repeated sequences with the resolvase-recognition site (VPM1002BC)

Colonies with PCR-confirmed excision of hygromycin B gene from genomic DNA were cultured at 39°C on 7H11/sucrose agar dishes to eliminate the pWM19 vector via its temperature- and sucrose-sensitivity. Colonies from this plate were split in two pieces and used to inoculate complete 7H9 medium either with or without (w/o) hygromycin B. The growth patterns confirmed both absence of the genomically integrated hygromycin B resistance cassette as well as successful curing of the vector pWM19.

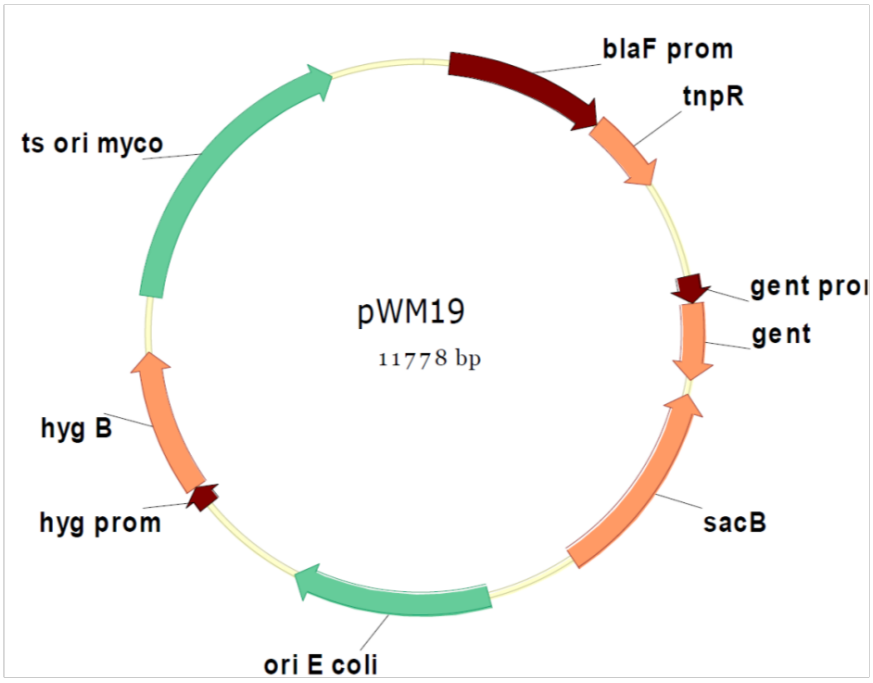

Figure 3: Schematic map of the pWM19 plasmid. The tnpR gene encodes for the γδ-resolvase. Two antibiotic resistance markers (gentamycin and hygromycin B) and the sacB suicide cassette of *Bacillus subtilis* are further features of pWM19. Replication of the plasmid in mycobacteria is temperature controlled (26).

The plasmid pVEP2003 was introduced to the live bacteria *via* electroporation.

Transfected mycobacterial cells were cultivated on hygromycin-containing agar. Growing colonies were picked and analysed by PCR. The goal was to elucidate the presence of the insertion of *hly* gene within the *ureC* gene.
The vector contained a *hly*-expressing cassette under the regulation of the mycobacterial promoter sequence from the *hsp60* fused to the leading sequences of the *Ag85B* to allow for secretion of the *hly*. A hygromycin resistance cassette was as well included in the vector for selection reasons. The plasmid contained approx. 800 bp *ureC* flanking regions cloned up- and downstream the *hly* cassette sequences, homologous to the *ureC* region to be deleted. Homologous recombination occurred autonomously, without further applications. Double homologous recombination led to replacement of the *ureC* genomic sequences (contained between P1-P2 and P3-P4, see Figure 4 and 5) with the listeriolysin and hygromycin resistance sequences, i.e. leading to the knock-out of the *ureC* gene and the knock-in of the hygromycin resistance and listeriolysin-coding cassette. In this step the first approx. 800 bp of *ureC* open reading frame (ORF) was deleted and replaced with the listeriolysin and hygromycin-coding casette. The hygromycin sequence was flanked by 2 γδ resolvase recognition sites. γδ resolvase recognition sites have been used to excise the hygromycin out of the VPM1002BC upon temperature-controlled extrachromosomal expression of the γδ resolvase and homologous recombination followed by activation of normal endogenous repair mechanism of the recipient.

Only one copy of the modifying DNA sequences is left in the genome of VPM1002BC. One of the γδ resolvase recognition sites is still encoded in the genome.

A2.11. Provide a full description of the genetic material inserted into the bacterium.

The description should contain an elaboration of the following aspects, in particular:

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

The listeriolysin gene from *Listeria monocytogenes (L. monocytogenes)*, EGD strain has been integrated into the genome of *M. bovis* BCG. Listeriolysin is a pore forming protein whose activity is restricted to the phagosomal compartment (28). Listeriolysin is active in the phagosome after endocytic uptake of the bacterium, enabling the bacterium to move into the cytosol. Listeriolysin only operates under a very close pH-optimum of 5.5-6 (i.e. at the acidic pH inside the phagosome) and is inactive at physiological pH 7.4 (29). Thus, listeriolysin is able to mediate phagosomal escape of the bacterium without host cell toxicity (30). Once it reaches the cytoplasmic environment (pH = 7), the activity of listeriolysin O (LLO) is abrogated (31). In addition, a PEST (P, Proline; E, Glutamic acid; S, Serine; T, Threonine)-like sequence, present in the N-terminus of the mature peptide sequence of the LLO protein, allows for its targeted degradation by proteasomes (32). Hess, et al. 1998 used the vector pMV306 containing *hly* to stably express LLO in *M. bovis* BCG by chromosomal integration. This rBCG pMVA306:*Hly* showed impaired persistence in THP-1 and J774A.1 macrophage cell lines, already 1 day post infection when compared to the parental *M. bovis* BCG. Of note, 12 days post infection even sterile elimination was observable, at least in the presence of extracellular gentamicin. Furthermore, lactate dehydrogenase assay (LDH) assays with J774A.1 macrophages demonstrated no increased cytotoxic activity for rBCG pMVA306:*Hly* (25). In conclusion, incorporation of listeriolysin into the BCG genome decreases the persistence and does not affect the low cytotoxicity of the *M. bovis* BCG.
To generate VPM1002BC, only the DNA sequences encoding for the mature peptide, flanked by *E. coli* hemolysin fragments, were inserted into the genome of the parental *M. bovis* BCG. This refers to the region 1564-3075 bp of the coding sequence (CDS) according to the GenBank entry for listeriolysin O (Accession Number: M24199; **see annex 2**). This was confirmed using the basic local alignment search

tool (BLAST) algorithm (33) to align the above mentioned CDS of LLO with the reference sequence of VPM1002 Hyg-. The BLAST analysis (**annex 3.1**) shows that the region 1560-3077 bp of the LLO CDS is found in the genome of VPM1002BC (respective locus of the WGS) with 100 % sequence identity.
To allow for secretion of listeriolysin, its coding sequence was fused to the gene encoding for *Ag85B* (34). The vector contained a listeriolysin (*hly*) expressing cassette under the regulation of the mycobacterial promoter sequence from the heat shock protein 60 (*hsp60*) fused to the leading sequences of the *Ag85B* to allow for secretion of the *hly* (35). A hygromycin resistance cassette was as well included in the vector for selection purposes. This cassette confers resistance by coding for the kinase hygromycin B phosphotransferase from *Streptomyces hygroscopicus*, which was first identified in *Escherichia coli* (36;37).
For homologous recombination reasons ca. 800 bp *ureC* flanking regions were cloned up- and downstream the *hly* cassette. Homologous recombination of these regions in the genome of *M. bovis* BCG led to the replacement of the *ureC* coding region with the recombinant expression cassette. The gene *ureC* encodes for the alpha subunit of the heterotrimeric hydrolase urease in *Mycobacteria*. Insertion of new genetic features into this genes renders the whole enzyme non-functional (38). This was also achieved by the introduction of LLO with the pVEP2003 vector. Sequence data of the respective locus in VPM1002BC shows that the original *ureC* gene is separated into two parts by the introduced *hly*-encoding cassette (**see annex 3.4**) (33). The 3'-part of the gene cannot be transcribed due to the lack of promoter region and initiation of transcription start. The *ureC* 5' fragment in VPM1002BC is only 151 bp long and does not contain any important features (39). Also, uniprot.org does not list any relevant functions for this short sequence.

The hygromycin resistance cassette was flanked by 2 γδ resolvase recognition sites, originally belonging to the prokaryotic transposable element Tn3 found in *E. coli* (40;41), which have been used to excise the hygromycin out of the VPM1002BC upon extrachromosomal expression of the resolvase (42). *Hly* is encoded as a single copy in the genome of the VPM1002BC. It is expressed under the constitutively expressed promoter of the *hsp60*.

A2.12. Provide a molecular characterization of the genetically modified bacterium.

A sequence analysis must be supplied for all inserted or deleted sequences, so that the precise location of the modification in the GMO and, in case of insertion, the number of copies present and the orientation of the insertion cassette can be determined. The sequence to be submitted must contain the region of the intended modification as well as the recombination sequences used, also including the flanking sequences of the genome of the original organism. In the case of biological unrestricted GMOs, newly created fusion ORFs must be described and subjected to a bio-information analysis.
The complete genome of the genetically modified bacterium must be characterized on a molecular level. This may be done according to a sequence analysis or a Southern blot analysis. Observed anomalies in the genome compared with the expected sequences, such as unexpected deletions, mutations or recombinations, must be described and interpreted. Phenotypic analysis or bio-information data may be used to support the data on the molecular characterization. If the GMO is to be based on a non-pathogenic bacterium or on a pathogenic bacterium from which the virulence factors have been removed, an elaborate analysis of the genome regarding deletions and recombinations is not necessary.
The sequence of the complete genome does not need to be submitted. A bio-information analysis must be carried out for the sequences, and the annotated results must be presented. For more information, please refer to the COGEM advisory report CGM/130227-05.

VPM1002BC contains a listeriolysin-coding insert integrated into the *ureC* locus of the genome. This site-directed integration (described in section 2.11) renders the *ureC* gene not functional. The integration of the insert has been confirmed by NGS of VPM1002BC, which also serves as a reference sequence for batch releases. The sequence of this locus taken from the WGS can be found in annex 4. This sequence data also shows that the cassette was introduced into the genome in an antisense direction relative to the *ureC* gene locus. By analysis of the WGS of VPM1002BC it could be shown that the insert was only introduced once into the genome and only at the intended site.

The inserted cassette consists of listeriolysin, the signal sequence of *Ag85B*, the promotor of *hsp60* and 1 resolvase site left over from the excision of the hygromycin resistance-mediating hygromycin B phosphotransferase.

Bioinformatic analyses of the sequence data from WGS of VPM1002BC also confirms that the introduced DNA sequence coding for the mature peptide of listeriolysin, flanked by hemolysin sequences from *E. coli*, shows 100 % identity to the corresponding database reference sequence M24199.1 (mature peptide range 1564-3075). The alignment of the sequences can be found in annex 3.1.

The *Ag85B* signal sequence 5' of listeriolysin was also compared against its database reference sequence Y207396.1. As expected, it shows sequence identity with the start of the reference sequence. Although three base pairs were not identical with the reference sequence, this has no effect since translation of both sequences yields the same amino acid sequence due to the wobble effect. The alignment of the DNA sequences as well as a comparison of their translation can be found in annex 3.2.

The third important feature of the insert cassette, the *hsp60* promotor, was also characterized. The respective sequence from the WGS of VPM1002BC shows 100 % identity with the database reference sequence for the vector pSL718 (KJ865750.1); a vector commonly used for the expression of foreign proteins in mycobacteria (43). An alignment of the sequences can be found in annex 3.3.
Analysis of the *ureC* gene at the genomic locus of the insert shows that the insert has been properly introduced into the gene, therefore disrupting it. The remaining 5' *ureC* fragment is 147 bp long and shows 100 % sequence identity with the corresponding data base reference sequence L41141.1:1054-2787. The 3' fragment of the *ureC* gene is 917 bp long and has 3 misaligned bases when compared to the sequence in the database. These minor mutations will have no effect on VPM1002BC, since the expression of the 3' fragment of the *ureC* gene is not possible due to the its disrupted nature. For alignments, please refer to annex 3.4.

No likelihood of post-release selection leading to the expression of unexpected and /or undesirable traits in the modified organism exists. During the scientific development, several passages of the strain VPM1002BC were performed and genetic instability was never observed in terms of modifications in the inserted recombinant construct. The insertion fragment was analyzed by PCR and sequencing. To the contrary, it was proven that all strains, starting with parental seed up to the GMP material of VPM1002BC are identical with regards to the inserted sequence. Sequencing data are available for various batch releases of the product, which reveal high stability of the genetic construct. In fact, four consecutive clinical GMP batches of VPM1002BC showed 100 % identity to the reference sequence (WGS of VPM1002BC) with their insert sequences. Alignments can be found in annex 4. No deviations from the expected sequence have been found.

A2.13. Summarize the data under A2.10 to A2.12 in a diagram ('map') of the genetically modified organism.

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

To generate VPM1002BC, an *E. coli* pVEP2003 plasmid was used as a shuttle vector (Figure 4). The plasmid carries an *E. coli* origin of replication but no mycobacterial origin of replication, which does not allow replication of this plasmid in mycobacteria. In addition, the plasmid contains an expression cassette carrying listeriolysin and an expression cassette carrying the hygromycin resistance sequence. These sequences are located between *ureC* P1-P2 and *ureC* P3-P4 region of the plasmid and have been integrated into the *M. bovis* BCG genome in the first step of the genetic manipulation. In this step double homologous recombination led to the replacement of the *ureC* genomic sequences (contained between P1-P2 and P3-P4, see Figure 4) with the listeriolysin and hygromycin resistance sequences.

Hence, knock-out of the *ureC* gene and the knock-in of the hygromycin resistance and listeriolysin-coding cassette has been accomplished. The plasmid carries no transposable elements.

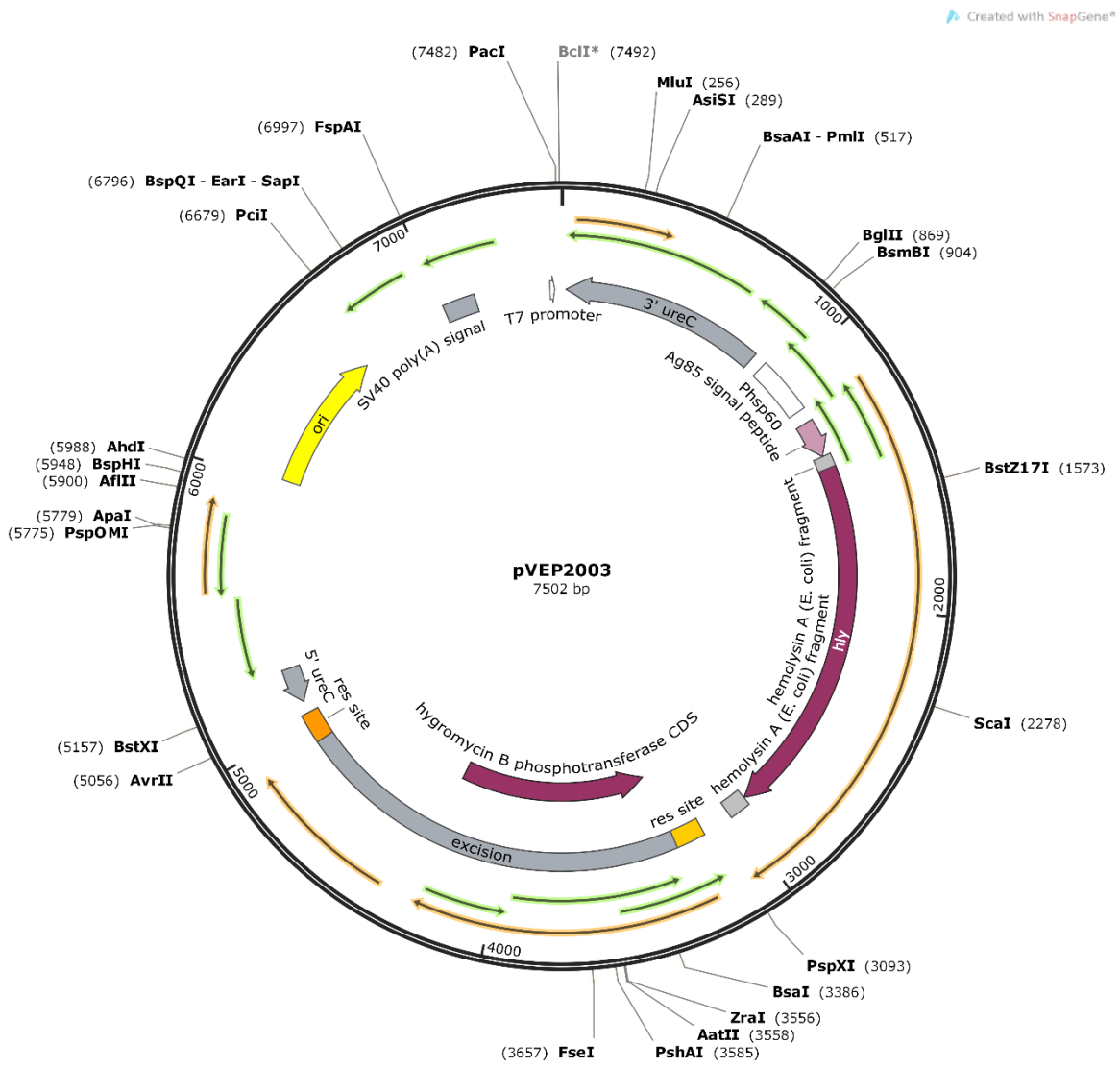

Figure 4: pVEP2003, the plasmid used to generate the VPM1002BC is an *E. coli* shuttle vector, based on the pJSC284.

The whole, annotated sequence of pVEP2003 vector is given in annex 1. An overview of pVEP2003 vector sequences encoding for a specific component of the recombinant construct inserted into VPM1002BC hyg+ precursor is given in Table 1.

Table 1: Summary of base pair positions in pVEP2003 vector used for construction of VPM1002BC hyg- precursor.

Encoding feature	Start [bp]	End [bp]	Length [bp]
ureC P1-P2 BCG 5' *ureC* homologuous sequence	5237	5091	147
Region containing γδ resolvase-recognition site	4914	5037	123
Hygromycin	4294	3296	999
Region containing γδ resolvase-recognition site	3270	3148	123

Encoding feature	Start [bp]	End [bp]	Length [bp]
Hly	1418	2929	1512
Ag85B signal sequence	1208	1363	156
hsp60 promotor	912	1151	240
ureC P3-P4 BCG 3' *ureC* homologuous sequence	858	19	840

An annotated illustration of the insert-containing genome locus of VPM1002 taken from the WGS of the reference sequence can be found in Figure 5. The corresponding sequence data can be found in annex 5. Overview of sequences encoding for a specific component of the recombinant construct inserted into VPM1002BC is given in Table 2.

Table 2: Summary of base pair positions in VPM1002BC genome sequence based on analysis of VPM1002BC genome fragment containing recombinant cassette with flanking regions.

Encoding feature	Start [bp]	End [bp]	Length [bp]
5' *ureC*	3731	3877	147
Region containing γδ resolvase-recognition site	3925	4047	123
Hly	4265	5776	1512
Ag85B signal sequence	5831	5986	156
hsp60 promotor	6043	6281	239
3' *ureC*	6335	7251	917

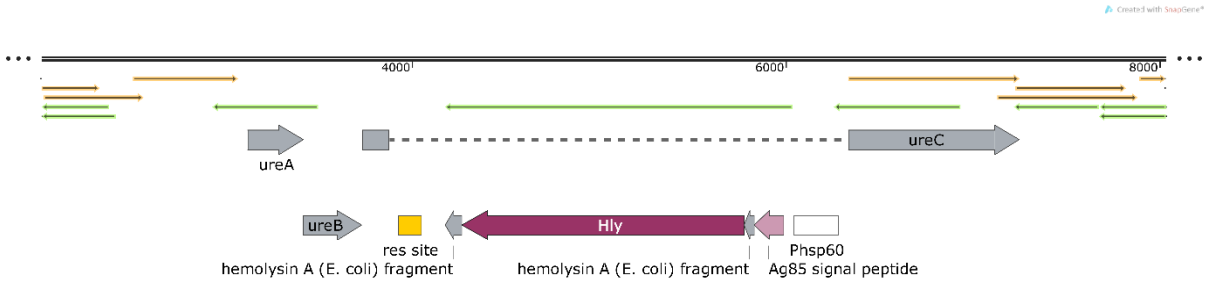

Figure 5: Annotated illustration of the genome locus containing the insert cassette in VPM1002BC.

A2.14. What physiological (including pathogenic) effects may be caused by the genetically modified bacterium; and what are the available treatments?

Indicate what physiological processes may occur following the application of the GMO in the host, and to which phenotypes this could lead. In addition, describe the degree to which the genetic modification will affect the pathogenic properties of the GMO. A comparison must be made between the possible pathogenic properties of the GMO and those of the original strain. In particular, the pathogenic properties that may be created specifically by the genetic modification must be considered.

The transmission of diseases, i.e. tuberculosis to humans can be ruled out, because VPM1002BC derives from the highly attenuated and long time in use *M. bovis* BCG. The necessary RD1 gene regions, encoding for virulence genes, are already inexistent in the original organism *M. bovis* BCG as well in VPM1002BC.

There is no information that *M. bovis* BCG or VPM1002BC might be more resistant, or might better survive in the natural environment than wild type *M. bovis*. It is unlikely that the introduction of the *L. monocytogenes* listeriolysin into VPM1002BC has changed its non-resistance in the natural environment. Due to incorporation of listeriolysin, VPM1002BC shows less persistence in macrophages (25). This is due to the fact that listeriolysin is sufficient to induce autophagy in macrophages, therefore

limiting its sole niche and reducing persistence (44). Any other influence of LLO expression on persistence in the natural environment is highly unlikely. Deletion of *ureC* from *M bovis* BCG has been shown not to result in altered persistence (45). Therefore, it can be concluded that the genetic modifications that resulted in the generation of VPM1002BC did not change its non-resistance in the natural environment when compared to parental *M bovis* BCG. A similar short survival time of VPM1002BC compared to the wild type *M. bovis* and *M. bovis* BCG can be predicted if it should be dispersed into the natural environment.

Several M. bovis BCG sub-strains are marketed, authorised medicinal products (e.g. *M. bovis* BCG Tice, Onco TICE; *M. bovis* BCG Connaught, ImmuCyst, *M. bovis* BCG RIVM, BCG-medac) with a well-known safety profile (e.g. information derives from the Summary of Product Characteristics of BCG-medac, national version: 08/2014; annex 8). *M. bovis* BCG is apathogenic and has been proven not to be virulent according to European Pharmacopeia (Ph. Eur.) as part of release testing of all clinical batches. Furthermore, *M. bovis* BCG is not known for inducing allergic reactions. To the contrary, BCG is known of preventing immunised individuals of gaining allergies (46;47)**.** *M. bovis* BCG does not carry any pathogens.

While deletion of the urease C gene has been shown to not significantly affect the persistence of *M. bovis* BCG (45), the introduction of listeriolysin further reduces the ability to persist of the already attenuated *M. bovis* BCG. Grode, *et al.* (8) have shown that expression of listeriolysin leads to induction of apoptosis, which is not only beneficial for a more pronounced and specific immune response, but also limiting its own niche, the macrophages. Furthermore, it has been shown that listeriolysin leads to the induction of autophagy (44). This has been specifically shown for VPM1002 by Saiga, et al. (48). This again is not only beneficial for the induction of favourable immune responses, but also strongly decreases the persistence of VPM1002BC.

The applicant (represented by Leander Grode) was also part of the Second Geneva Consensus Meeting for the recommendations for novel live TB vaccines at the WHO headquarters. There, non-clinical and clinical research requirements for upcoming *M. bovis* BCG TB vaccines were discussed and defined (23). The non-clinical development of VPM1002 as a TB vaccine was the blueprint for these recommendations. The comprehensive analysis of its safety, tolerability and efficacy in a wide array of animal models can also be transferred to VPM1002BC, since both derive from the same strain and master cell bank, and only differ in the dosage. A list of non-clinical studies performed on VPM1002 can be found in annex 6.1.

Furthermore, several nonclinical studies have been performed to assess VPM1002BC's safety, organ distribution and excretion after intravenous bolus injection or intravesical instillation. In a preclinical study on mice, repeated intravenous dosing of VPM1002BC led to less systemic spreading when compared with the marketed product BCG-medac. While considerable CFU counts were found in the BCG-medac control group, drastically less or even none (in spleen) were found in the VPM1002BC group. A summary of the data of these experiments can be found in annex 6.2.

A study with severely immune-deficient C.B-17-SCID mice showed that subcutaneous application of VPM1002BC or its hygromycin-resistant predecessor strongly increased survival when compared to standard *M. bovis* BCG SSI. These findings highlight the increased safety of VPM1002BC. A summary of the data from this study can be found in annex 6.3.

Furthermore, two toxicological studies of intravesical instillations of VPM1002BC and the comparator BCG-medac were performed in CD® rats to assess the candidate product's toxicity in the target organ. In the first study a single intravesical instillation with either the human equivalent dose (scaled down to the test animal) or the human target dose (the actual dose for administration in humans) was performed. In the second study, 6 weekly instillations of the above described VPM1002BC and BCG-medac doses (as comparator) were applied to the rats by intravesical instillations. Both studies found no test item related mortalities and no change in behaviour, external appearance or condition of the faeces was noted during the test period. No test-item related CFU counts were found in examined organs and blood, and VPM1002BC was rapidly cleared from the site of instillation. A summary of these two studies can be found in annex 6.4 and annex 6.5, respectively.

Taken together, the non-clinical data shows that VPM1002BC is safer than comparable *M. bovis* BCG strains and systemic spreading after intravenous bolus injection is drastically reduced when compared to the comparator *M. bovis BC*G strain. Of note, no systemic spreading was observed at all when VPM1002BC was applied by intravesical instillations.

Additionally, VPM1002BC has been characterized by testing for drugs commonly used in the treatment of mycobacterial infections and other antibiotics, used in the treatment for bladder infections. Table 3 provides an overview of the tested substances and the results.

Table 3: Results of antibiotic sensitivity testing for VPM1002BC.

Substance	Sensitivity
0.1 µg/ml isoniazid	sensitive
5.0 µg/ml ethanbutol	sensitive
1.0 µg/ml rifampizin	sensitive
1.0 µg/ml streptomycin	sensitive
4 µg/ml azithromycin	sensitive
0.125 µg/ml clarithromycin	sensitive
32 µg/ml gentamicin	sensitive
4 µg/ml doxycyclin	sensitive
16 µg/ml sulfamethoxazole	sensitive
0.25 µg/ml ofloxacin	sensitive
16 µg/ml nitrofurantoin	sensitive
50 µg/ml hygromycin B	sensitive

A2.15. Indicate the degree to which the virulence of the genetically modified bacterium is or may be altered, compared with that of the original strain.

When answering this question, provide an argument that elaborates on the virulence of the genetically modified bacterium, compared with that of the original strain. Also consider any modifications that were made in order to achieve the original strain.

In comparison to the virulent *M. bovis* strain and *M. tuberculosis*, all *M. bovis* BCG strains lack the genomic region called RD1 (11). Deletion of RD1 in virulent *M. bovis* and *M. tuberculosis* has been identified as the initial genetic attenuation factor resulting in *M. bovis* BCG (11). As genetic manipulations that led to construction of VPM1002BC were performed in the *ureC* region of *M. bovis* BCG, lack of virulence due to loss of function of the RD1 region remained conserved in VPM1002BC. In addition, enhanced apoptosis of and autophagy in VPM1002BC containing cells, as a limiting factor for persistence of VPM1002BC, is pointing to a higher degree of attenuation of VPM1002BC in comparison to parental *M. bovis* BCG. For example, SCID (severe combined immunodeficiency) mice, characterized by absence of adaptive immune response, were treated with VPM1002BC or BCG subcutaneously and followed by a recovery period of up to 500 days. This study showed that mean survival time of the animals vaccinated with VPM1002BC was significantly prolonged when compared to the experimental group that received *M. bovis* BCG. Two subsequent studies analysing the effects of single and repeated intravesical instillations of VPM1002BC in rats showed no mortality and morbidity in the tested animals. For detail refer to section A2.14.

L. monocytogenes listeriolysin is not active under normal physiological conditions (pH 7). It is unlikely that the introduction of the *L. monocytogenes* listeriolysin into VPM1002BC has changed its non-resistance in the natural environment. Therefore, a similar survival time of VPM1002BC if it should be

dispersed into the natural environment compared to the wild type *M. bovis* and *M. bovis* BCG can be predicted. Spreading and persistence of the vaccine strain *M. bovis* BCG into the environment has never been detected. No natural habitats of *M. bovis* BCG are known. For details see section 2.7.

A2.16. Indicate the possible transmission routes of the genetically modified bacterium.

Make a comparison with the original strain. Argue whether or not and how the genetic modification may affect the aforementioned aspects regarding host range and transmission routes.

M. bovis BCG is a bacterial strain which has been generated through continuous passages *in vitro*. Spread of the vaccine strain *M. bovis* BCG into the environment has never been detected (an extensive literature search could not find any evidence of spreading of *M. bovis* BCG into the environment). No natural habitats of *M. bovis* BCG are known. It is unlikely that the introduction of the *L. monocytogenes* listeriolysin into VPM1002BC has changed its inability to survive in the natural environment.

To gain data on possible routes of transmission of VPM1002BC into the environment, urine samples were collected before (hour 0) and 2, 4, 8 and 24 hours after the instillation in the phase I part of the SAKK06/14 clinical trial and analysed by CFU counts and PCR. The performed real-time MTB-PCR has been recently published. In this publication the used in-house method uses the multi-copy insertion element IS6110 for the sensitive and specific detection of MTB. It was compared to an established method (Abbot RealTime MTB test), and was shown to have 100 % sensitivity, 99.2 % specificity for tested samples, and a detection limit of 10 CFU (49).Taken together the implemented surveillance tests allowed for collection of data and subsequent evaluation of the potential risks for dispersion of the VPM1002BC into the environment and for human-to-human transmission.

Data gathered during the phase I part of the SAKK 06/14 clinical trial on the excretion profile showed that VPM1002BC is rapidly cleared from the urine of the patients. After 24 hours no CFU were detectable in neither of the 6 patients' urine (Figure 5). This was confirmed by the performed real-time MTB-PCR (Figure 6). This rapid clearing of VPM1002BC is considerably faster than what has been shown for standard *M. bovis* BCG. Durek, et al. (14) reported presence of *M. bovis* BCG in 27.1 % of the samples one week after the instillation.

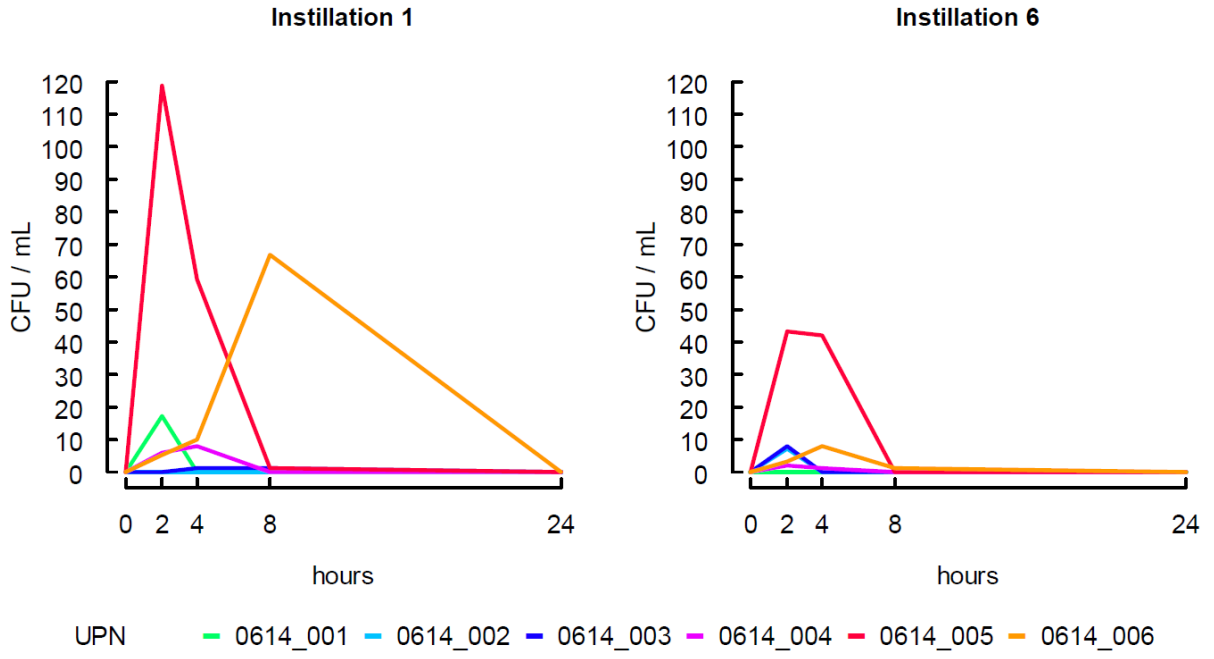

Figure 5: VPM1002BC CFU counts per ml urine (individual development)

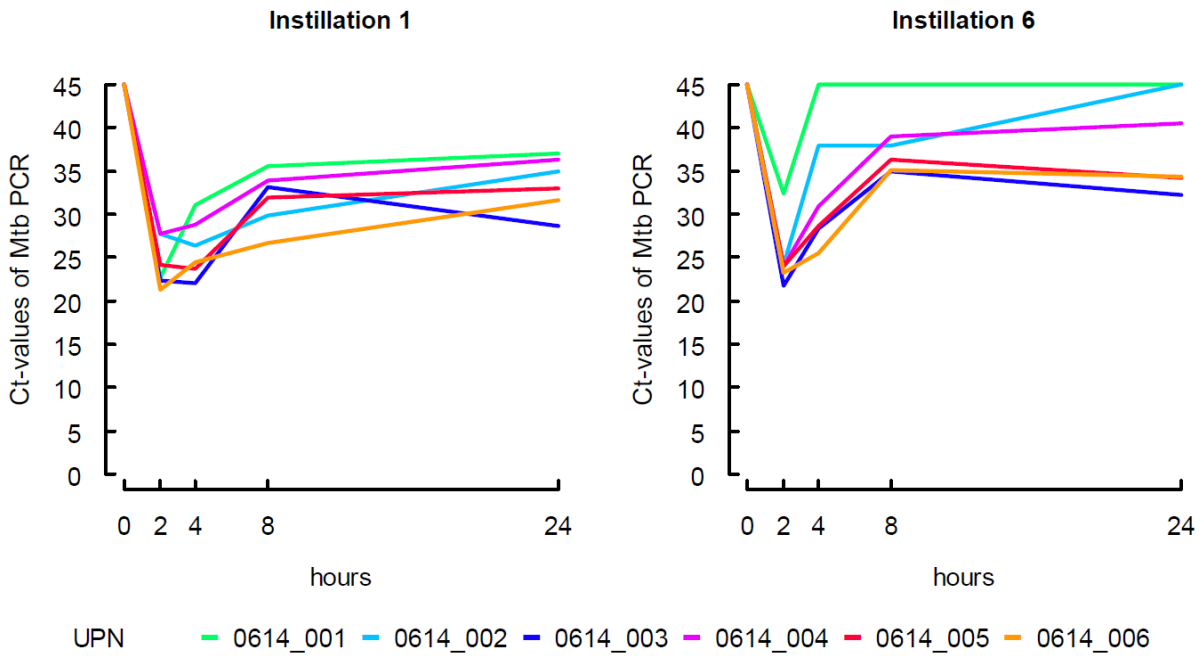

Figure 6: Ct values from real-time MTB-PCR (individual development)

At no point during the assessment (before and 24 hours after the instillation) was VPM1002BC found in the sputum or blood of the patients.

A3. Production of the GMO

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

Production will be under the responsibility of third parties.

VPM1002BC is produced by Serum Institute of India Pvt. Ltd. (SIIPL) and distributed through their subsidiary Bilthoven Biologicals, Bilthoven, the Netherlands, who is responsible for the Qualified person (QP)- release of VPM1002BC. The Netherlands Commission on Genetic Modification (COGEM) classified rBCG:ΔureC::*hly*+ (VPM1002(Hyg+)) as an GMO that can be handled at the ML-I / DM-I level without endangering the safety of humans or the environment according to the Dutch law (CGM/070402-05).

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

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

VPM1002BC and its hygromycin-resistant predecessor VPM1002 have been thoroughly characterized and a table detailing the tested parameters, the methods used, the specifications and the rationales can be found in annex 7.

The manufacture of VPM1002BC is a controlled, continuous process. No intermediate holding step is performed. The drug substance is obtained after several pre-culture steps and fermentation. After each pre-culture step the optical density at 600 nm (OD_{600}), the amount of colony forming units by plating on Löwenstein-Jensen (LJ) plates are determined, as well as a microbial examination by acid fast staining is performed. During fermentation, pH and dissolved oxygen are monitored, CFU (LJ plating), OD_{600}, and microbial examination by acid fast staining are performed. The fermentation harvest is collected and the total amount of bacteria is determined as in-process control (by plating on LJ plates). In addition, pH measurement, visual examination, OD_{600} and microbiological examination

are performed. The bacterial culture concentrate is formulated into the final bulk. The following tests are performed on the final bulk: OD_{600}, CFU and microbiological examination. Final bulk is distributed into vials, lyophilized and the vials are sealed. Tests on the final bulk and final lot are performed according to release specifications (see section A3.3 and annex 7).
Media fills were performed, to aseptically simulate the production of VPM1002BC batches. All of the performed media fills passed.

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

State which criteria are used to reject a batch.

Two PCR analyses confirm the identity of VPM1002 by the disruption of the ureC locus with in introduction of the listeriolysin encoding cassette. This is further confirmed by Sanger sequencing of the insert region to confirm the proper insertion of the cassette and rule out mutations. This sequences are compared against a reference sequence taken from the whole genome sequence of VPM1002BC. Furthermore, Every batch is tested in accordance with Ph. Eur. for the absence of virulent mycobacteria.
Identification and identity test are performed on each lot and the following specifications have to be satisfied. A detailed table with tests, their rationales and specification can be found in annex 7.

VPM1002BC-specific PCR
A PCR test should result in primer specific fragment size (1415 bp) for *hly* gene, confirming the absence of the hygromycin gene and presence of listeriolysin gene and its relative position of *hly* gene in the 5´-region of *ureC* gene. Another PCR using primers located in the 5' and 3' parts of the *ureC* gene produces a larger 2863 bp fragment if the listeriolysin-containing cassette is present in the genome, and a 1076 bp fragment if it is absent. These absence of the smaller fragment confirms that no other M. bovis BCG is present in the final product. In both tests nuclease free water is used as a negative control. Being qualitative tests, detection limits are not evaluated.

Sequencing of insert region
For each batch, the region of the insert in VPM1002BC, produced by the aforementioned PCR is sequenced and compared to a reference sequence taken from the whole genome sequence of VPM1002BC. This sequence was obtained by next generation sequencing.
The release criteria are 100 % sequence identity of the listeriolysin mature peptide coding sequence and the *Ag85B* signal sequence in comparison to the reference sequence on amino acid level, 99 % identity for the *hsp60* promoter and 95 % sequence identity for the flanking *ureC* fragments. Being a qualitative test, the detection limit is not evaluated.

Furthermore, the following tests are required for the release of the IMP.

Visual inspection
A vial of rBCG for immunotherapy is taken and visually observed for colour and appearance. VPM1002BC is reconstituted with the recommended volume of diluents and observed by transferring the content in a test tube with help of a syringe. A white lyophilized cake in 4 ml amber colour vial having mauve colour flip off aluminum seal should yield whitish turbid suspension after reconstitution.

Test for the presence of bacterial or fungal contamination
Newly produced VPM1002BC batches have to comply with the tests for sterility except for the presence of mycobacteria. The method is defined in the European Pharmacopoeia and validated for *M. bovis* BCG, and performed in accordance with the guideline.

Test for the absence of virulent mycobacteria

In this test, guinea pigs are injected subcutaneously with VPM1002BC and the animals are observed during a period of 42 days. The product complies with the test if none of the guinea pig shows signs of tuberculosis and if not more than 1 animal dies during the observation period. The method is defined in the European Pharmacopoeia, validated for *M. bovis* BCG, and performed in accordance with the guideline.

Determination of residual moisture
The residual moisture of the final lot is determined by Karl-Fischer titration in accordance with Ph. Eur. Karl Fischer titration is a classic titration method in analytical chemistry that uses coulometric or volumetric titration to determine trace amounts of water in a sample. The determined residual moisture should not exceed 10 %; otherwise, this would lead to the rejection of the batch. The method is defined in the European Pharmacopoeia and validated for *M. bovis* BCG, and performed in accordance with the guideline.

Container / closure integrity
The vial is visually inspected for its integrity. No evidence of collapse/dissolution of cake and/or presence of water in the product container should be observed.

Test for viability
The product has to contain 1-19.2 x 10E8 CFU/vial. This is determined by plating on LJ plates in accordance with Ph. Eur. The method is defined in the European Pharmacopoeia and validated for *M. bovis* BCG, and performed in accordance with the guideline.

Non-compliance with any of the above-mentioned criteria would lead to the rejection of the batch.

A4. Description of the research

Administration

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

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

This license is being applied for handling and application procedures regarding VPM1002BC in the phase II clinical trial in the Netherlands, where the Netherlands Cancer Institute in Amsterdam will be involved as a clinical trial site and treat a maximum of 39 patients.

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

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

VPM1002BC and the diluent will be provided by SIIPL, through their subsidiary Bilthoven Biologics, Bilthoven, the Netherlands. The trial drug will be supplied as freeze dried cake containing 1 dose of VPM1002BC, live, 1-19.2 x 10E8 CFU, in a brown glass bottle with bromobutyl stopper and aluminum

crimp cap. The 50 ml diluent for re-suspending the cake is supplied in a separate brown glass bottle with bromobutyl stopper and aluminum crimp cap. 1 vial of VPM1002BC and 1 vial of diluent are provided in one secondary packaging. The lyophilized powder VPM1002BC and the diluents are stored at the trial site in a locked refrigerator with limited access and in accordance with the instructions on the study medication labels (2-8°C, protected from direct sun light). The study medication is reconstituted by the use of a closed drug transfer system and the instillation must be performed within 3 hours. The BD PhaSeal™ Closed Drug Transfer system will be used (see BD PhaSeal™ System Basic Instructions for details, annex 9).
The treatment room of the 'Onderzoek- en Behandel Centrum' has a washstand with basin that will be used during the preparation of the trial drug in combination with the Transfer System. Disinfection will be performed with a disinfectant proven to be active on mycobacteria.
Transport from Bilthoven Biologics to the Netherlands Cancer Institute as well as transport within the institute will be done according to Appendix 1 of the "Regeling GGO 2013".

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

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

Strict hygiene procedures will be followed. The principal investigator will instruct all medical staff accordingly.
The administration site of the GMO is the bladder of the treated patients. VPM1002BC is applied into the bladder, according to the protocol mentioned below, in a treatment room of the outpatient clinic (Onderzoek- en Behandel Centrum).

1. A large absorbent cellulose underpad (with impermeable backing) is placed on the table
2. The patient lies down on the table
3. A catheter is hung up using adhesive strips
4. The three-way valve of the syringe with medication (GMO preparation) is connected to a syringe filled with physiological saline (NaCl 0.9%)
5. The materials are placed on a second cellulose pad
6. The pad carrying the materials is placed between the patient's legs
7. The patient's genital area is cleaned using cotton swabs
8. A sterile tray is placed between the patient's legs
9. The catheter is removed from the packaging and inserted into the patient
10. The urine flows into the tray
11. The administration set is connected to the catheter
12. The GMO preparation is slowly administered, after which the three-way valve is closed
13. The three-way valve is opened towards the NaCl and 5 mL is injected in order to administer the full quantity of medication still remaining in the catheter. The three-way valve is closed
14. The cellulose pad is folded around the catheter before it is withdrawn from the patient in order to prevent splashing/spill
15. The medication must remain inside the bladder for at least 1 hour (please note turning is not required)
16. Patients are provided with incontinence material during the bladder instillation (which will be treated as contaminated material if urine is spilled after the bladder instillation)
17. After 1 hour the patient will void in a urinal bottle containing two Javel-Tabs. After 15 min the urine will be disposed by flushing down the toilette.
18. All materials will be disposed of in the biohazard waste containers.
19. All surfaces in the treatment room of the outpatient clinic (Onderzoek- en Behandel Centrum) are smooth and easy to clean and disinfect. They will be disinfected with a disinfectant proven to be active on mycobacteria. The room will be closed during administration.

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

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

During the phase I part, the recommended phase 2 dose (RP2D) was determined. Two dose levels were foreseen (see table below). No dose de-escalation was necessary and dose level 1 was established as RP2D.

Dosage group	Foreseen dose levels (for dose finding in Phase I)
Dose level 1	1-19.2 x10E8 CFUs (colony forming units)/50ml
Dose level -1	1-19.2 x10E7 CFUs/46.4ml

Each patient treated in phase II will receive a maximum number of 15 doses of VPM1002BC administered by intravesical instillation at dose level 1. The dose level 1 (1-19.2 x 10E8 CFUs) is the maximum dose to be administered into patients. As a batch release criterion the vials of VPM1002BC have to contain 1-19.2 x 10E8 CFU. This is determined by CFU plating on LJ plates. The broad CFU range results from the fact that mycobacteria are clumping due to a glycolipid called cord factor and the exact amount cannot be precisely determined. However, since each batch is determined to contain maximally 19.2 x 10E8 CFU of VPM1002BC per vial, this is the maximum applied dose.

VPM1002BC will be administered once per week as intravesical instillations as follows:

Induction:
- 6 instillations at weekly intervals. First instillation has to be done within 14 days after registration and corresponds to day 1 of the trial treatment schedule (= treatment start).

Maintenance:
- 3 instillations at weekly intervals starting at week 13 from day 1
- 3 instillations at weekly intervals starting at week 25 from day 1
- 3 instillations at weekly intervals starting at week 49 from day 1

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

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

Not applicable.

Sampling

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

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

In phase II, urine and blood samples will be collected at various time points, but always before the intravesical instillation of VPM1002BC.

The pharmacokinetic excretion profile of VPM1002BC was investigated in the phase I trial on 6 patients. To gain knowledge on routes of transmission of VPM1002BC into the environment urine,

blood and sputum samples were collected and analyzed by the CFU counts and PCR. The PCR was designed to detect *M. tuberculosis*-complex DNA based on the multi-copy target IS6110. The detection limit of the PCR assay applied is 10 CFU. The PCR and its detection limit has been recently published by Hinic, *et al.* (49). See section A2.16. Maximal dose of VPM1002BC to be administered into patients is 19.2 x 10E8 CFUs. Taking into account the aforementioned, the applied methodology allows detection of as few as 5.2x10E-7% of the maximal administered dose. The data assessed the persistence of VPM1002BC in clinical samples (urine, blood, sputum) after intravesical urinary bladder instillation treatment and demonstrated no systemic spread of VPM1002BC after intravesical application in patients. Furthermore, VPM1002BC was not detectable after 24 hour in the urine of the tested patients (for details see section A2.16).
Based on preclinical data described in section A5.1, it is expected that persistence of VPM1002BC is lower than in case of *M. bovis* BCG.
Data on persistence of *M. bovis* BCG in different samples after intravesical instillations from several clinical studies is available. For example, Durek, *et al*. (14) analyzed blood, urine, sputum and bladder biopsy samples from 49 patients treated with 6 weekly instillations with BCG. Urine samples were collected immediately before, 2, 4 and 24 hours after instillation, and once a day during the following 6 days. Sputum was collected 2 hours after instillation. Blood samples were collected before and 2 hours after instillation. This study reported no viable *M. bovis* BCG in blood or in 127/128 sputum samples before and 2 hours after instillation. In urine samples *M. bovis* CG was detected in 96.4% of the specimens after 2 hours and in 67.9% after 24 hours after instillation. The number of positive specimens decreased and it was 27.1% on day 7 (before the next instillation). Interestingly, in case of biopsy samples *M. bovis* BCG positive PCR results were observed up to 24 months in between 4.2% and 37.5% of the investigated bladder biopsies.
Siatelis *et al.* (50) assessed persistence of *M. bovis* BCG in urine, blood and bladder biopsy samples of 10 patients receiving *M. bovis* BCG intravesical induction therapy (initial 6 weekly instillations). Blood and urine samples were collected on weekly intervals for the first 3 instillations and 6 weeks after the last instillation. Bladder biopsies were collected 6 weeks after the last instillation. *M. bovis* BCG DNA was detected by real-time PCR. All urine samples taken 24 h after the instillations were positive, as well as 24% of the specimens collected 7 days after the instillations. Furthermore, *M. bovis* BCG DNA was detected in a single urine specimen taken 6 weeks after the last instillation. In the presented study *M. bovis* BCG was detected in 5 blood samples (out of 60 samples in total) taken 24 h after each instillation (8.3%). Finally, 1 out of the 10 bladder biopsy samples was positive, indicating persistence of BCG in the bladder up to 6 weeks.
Based on the excretion data from the phase I part of the SAKK 06/14 clinical, it can be assumed that VPM1002BC has a much lower persistence than standard *M. bovis* BCG.

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

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

During the phase II of the trial in the Netherlands, the following measurements will be performed: blood assessments and urine assessments. Blood samples will be analyzed for hematological values, hepatic function and renal function. Urine assessments to be performed are: dipstick analyzing nitrite and urine culture at the discretion of the treating urologist. Blood and urine samples for regular assessments in the induction phase of the phase II trial treatment will be collected prior to the instillation 1, 3 and 6. As part of the maintenance phase, blood and urine samples for regular assessments will be collected prior to the instillation 1 and 3 of each of the maintenance course. In addition, bladder biopsy samples and cytology bladder wash samples will be collected at weeks 12, 24, 36, 48 and at the end of treatment (week 60).

The collection of blood and urine samples at the time points indicated above will take place before the administration of the GMO into the bladder. The time interval between 2 instillations is 1 week. Data from phase I showed that already 24 hours after the instillation, VPM1002BC is no longer present in the urine of the patients. Therefore, biological samples collected before the next instillation (7 days after previous instillation) are not at risk of containing the GMO.

A4.8. 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.

Assessments of excretion of VPM1002BC in urine, blood and sputum were performed on samples collected during the phase I of the trial. No further assessment of excretion of VPM1002BC will be performed in the phase II of the trial.

Measurement was performed during induction phase for instillation 1 and 6 for the following samples collected in phase I:

- Urine (30-50 mL) collected before instillation and at 2, 4, 8 and 24 hours after instillation.
- Blood (1.5 ml for PCR + 3-5 ml for culture) collected before instillation and at 24 hours after instillation.
- Sputum collected before instillation and at 24 hours after instillation

Please see Chapter A4.7 of this document.

For the detection methods used, please refer to section A.4.6

Waste management

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

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

1. Type of waste generated:

VPM1002BC will be supplied as freeze dried cake containing 1 dose of VPM1002BC, live, 1-19.2 x 10E8 CFU, in an amber glass bottle with bromobutyl stopper and aluminum crimp cap and has to be reconstituted before application. The 50 ml diluent (0.9% sodium chloride, 0.025% Tween 80, in water for injections) for resuspending the cake is supplied in a separate amber glass bottle with bromobutyl stopper and aluminum crimp cap. Reconstitution to the desired dose will be performed in a disposable closed drug transfer system, i.e. glass bottles with rubber stoppers and syringes will be used in this process. Any partially or completely used VPM1002BC vials and all other equipment, packaging and materials exposed to the product are considered as biohazardous materials and will be disposed accordingly. Any material or tools used for handling accidental leakage, spill, break of IMP or cleaning the IMP reconstitution area (e.g. absorbent pads) is considered as biohazardous waste. The type of waste generated during application of the IMP normally would consist of liquid and solid waste (glass bottles containing the reconstituted VPM1002BC, syringes with needles, gloves and absorbent pads used for cleaning the IMP reconstitution area).

In case of urine spill or spread of urine of treated patients due to incontinence, all the materials that get in touch with urine should be treated as biohazardous materials and should be disposed accordingly. In case of surfaces coming in touch with GMO-containing urine, they should be cleaned according to local requirements, using a disinfectant proven to be active on mycobacteria.

Patients will be instructed by the Principal Investigator to follow strict hygiene rules.

2. Expected amount of waste:

The amount of waste expected is rather small comprising of approximately 1200 syringes with needle, ca. 600 small glass bottles (50 ml), ca. 600 small glass bottles (4 ml) containing liquid waste, gloves used by personnel during the IMP handling and material used for cleaning the IMP reconstitution area (e.g. absorbent pads).

3. Description of treatment envisaged:

VPM1002BC contains viable attenuated mycobacteria and should be handled as an infectious agent at all times. Any unused VPM1002BC vials must be stored for monitoring drug accountability.

VPM1002BC is classified as a genetically modified organism with Biological and Genetic Safety Level 1 (CGM/070402-05). Any partially or completely used VPM1002BC vials and all other equipment, packaging and materials exposed to the product contain viable attenuated mycobacteria and should be handled as an infectious agent at all times. Therefore, they should be immediately placed in a container for biohazardous materials and disposed of as biohazardous waste. Any waste material should be disposed of in accordance with local requirements. Leakage, spill and break should be disposed of in accordance with local requirements. VPM1002BC and potentially contaminated materials and samples will be transported in-house in a disinfectable, leak-proof and unbreakable container. Trained personnel will perform the transport.

A5. Environmental risk analysis

Environment-related information originating from earlier experiments

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

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

Preclinical:

Persistence of VPM1002, precursor of VPM1002BC was tested *in vitro*. VPM1002 showed lower persistence in infected macrophage cell lines THP-1 and J774A.1already 1 day post infection when compared to parental *M. bovis* BCG. Of note, 12 days post infection even sterile elimination was observable, at least in the presence of extracellular gentamicin (25). Based on these observations indicating lower intracellular persistence of VPM1002 in comparison to the parental *M. bovis* BCG strain, it is considered that the probability of dispersion of VPM1002 into the environment is lower than in case of parental *M. bovis* BCG (25;51).

Systemic spread of VPM1002BC was studied in female BL/6 mice by 6 intravesical instillations in the bladder at 1-week intervals and 2 intravenous injections into a tail vein at a 1-week interval. Experimental groups received VPM1002BC or commercially available *M. bovis* BCG or PBS (control). Different organs (liver, lungs, urinary bladder, spleen, ovaries, kidneys) and blood samples were analyzed for presence of respective mycobacteria (CFU counts) at 1, 3, 5 and 6 weeks after administration.

In case of results after intravenous administration of VPM1002BC or commercially available *M. bovis* BCG, considerable CFU counts were noted in liver, lungs and spleen of the animals treated with

commercially available *M. bovis* BCG, but drastically lower CFU counts or even none (in spleens) were noted in these organs for the treatment with VPM1002BC. Intravenous treatment with VPM1002BC appeared to lead to a less pronounced systemic spread of mycobacteria than intravenous treatment with the commercially available BCG used as a reference. See annex 6 for a summaries of the relevant non-clinical studies. A study with severely immune-deficient C.B-17-SCID with subcutaneous application of VPM1002BC or its hygromycin-resistant predecessor mice showed strongly increased survival when compared to standard *M. bovis* BCG SSI. These findings highlight the increased safety of VPM1002BC. A summary of the data from this study can be found in annex 6.3.

Furthermore, two toxicological studies of intravesical instillations of VPM1002BC and the comparator BCG-medac were performed in CD® rats to assess the candidate product's toxicity in the target organ. In the first study a single intravesical instillation with either the human equivalent dose (scaled down to the test animal) or the human target dose (the actual dose for administration in humans) was performed. In the second study, 6 weekly instillations of the above described VPM1002BC and BCG-medac doses (as comparator) were applied to the rats by intravesical instillations. Both studies found no test item related mortalities and no change in behaviour, external appearance or condition of the faeces was noted during the test period. No test-item related CFU counts were found in examined organs and blood, and VPM1002BC was rapidly cleared form from the site of instillation. A summary of these two studies can be found in annex 6.4 and annex 6.6, respectively.

Clinical:
Several clinical trials have been performed with VPM1002 and VPM1002BC. A summary of these trials can be found in Table 4.

Table 4: Clinical trials with VPM1002 and VPM1002BC and their status.

Study Code	Title	Status
VPM1002-GE-1.01TB (NCT00749034)	Phase I Open Label, Randomized, Controlled, Dose-Escalation Study to Evaluate Safety and Immunogenicity of VPM1002 in Comparison with BCG in Healthy Male Volunteers Stratified for History of BCG-Vaccination	completed
VPM1002-ZA-1.10TB (NCT01113281)	Phase Ib Open Label, Randomized, Controlled, Dose-Escalation Study to Evaluate Safety and Immunogenicity of VPM1002 in Comparison with BCG in Healthy Volunteers in South Africa	completed
VPM1002-ZA-2.12TB (NCT01479972)	Phase II open label, randomized, controlled study to evaluate safety and immunogenicity of VPM1002 in comparison with BCG in HIV-unexposed, BCG naive newborn infants in South Africa	completed
VPM1002-ZA-2.13TB (NCT02391415)	Phase II double-blind, randomized, controlled study to evaluate the safety and immunogenicity of VPM1002 in comparison with BCG in HIV-exposed and HIV-unexposed, BCG-naive newborn infants	recruitment completed
VPM1002-IN-3.01TBR (NCT03152903)	A multicenter phase II/III double-blind, randomized, placebo controlled study to evaluate the efficacy and safety of VPM1002 in the prevention of Tuberculosis (TB) Recurrence after successful TB treatment in India	CTA approved; not yet recruiting
SAKK 06/14 (NCT02371447)	A phase I/II open label clinical trial assessing safety and efficacy of intravesical instillation of VPM1002BC in patients with recurrent non-muscle invasive bladder cancer after standard BCG therapy	Phase I part completed; phase II ongoing

No transmission of *M. bovis* BCG has ever occurred (no reports have been found). Nevertheless, possible routes of transmission of vaccine VPM1002 administered intradermally were explored in phase I clinical study conducted on healthy volunteers in Germany and South Africa (52). This study reported no case of transmission of VPM1002 to other persons. Furthermore, to assess putative routes of transmission of VPM1002 into the environment after intradermal application, blood, urine, saliva and stool samples were collected from patients of the trial VPM1002-GE-1.01TB (NCT00749034) before vaccination, on day 1, day 11 and 6 months after vaccination. The samples were analyzed by PCR detecting unique genomic DNA regions of VPM1002 (*M. bovis* rBCGΔ*ureC*::Hly+::Hyg+). All PCR results were negative (52). The detection limit of this PCR was equivalent to about 3 to 30 CFU when purified DNA from VPM1002 was used as template.In the second clinical trial, VPM1002-ZA-1.10TB, 24 healthy male or female adults with pre-exposition to BCG were vaccinated in South Africa, predominantly from the indigenous African population. The study supports data from the first clinical trial showing that a single vaccination with VPM1002 is safe, well tolerated and elicits a profound immune response.

The third clinical trial was the first investigation of VPM1002 in newborn infants in a setting with a high burden of TB (South Africa), the population at highest risk of TB and which stands to benefit from a safe and effective new vaccine. The clinical trial, VPM1002-ZA-2.12TB, was conducted in Cape Town, South Africa. It followed an open label, randomized, controlled design to assess the safety and immunogenicity of a single dose of VPM1002 in comparison to the commercially available BCG vaccine. 48 HIV-unexposed, BCG-naïve neonates of both sexes were vaccinated with VPM1002 2.5 x 10E5 CFU (n=36) in comparison to BCG 2.5 x 10E5 CFU (n=12). Data from this study suggest that vaccination with VPM1002 is at least as safe, and possibly better tolerated, than that with an equivalent dose of BCG (1-4 x 10E5 CFU) in the target population.

A phase II double-blind, randomized controlled study to evaluate the safety and immunogenicity of VPM1002 in comparison with BCG in HIV-exposed and HIV-unexposed, BCG-naive newborn infants is currently ongoing in South Africa (VPM1002-ZA-2.13TB. Clinical Trials.gov Identifier: NCT02391415).

Putative routes of excretion of VPM1002BC in clinical setting after intravesical urinary bladder instillation have been analysed in 6 patients during the phase I part of the clinical trial SAKK 06/14. Urine, blood and sputum were collected and analysed by the CFU counts method and by mycobacterium tuberculosis (MTB) polymerase chain reaction (PCR). The performed real-time MTB-PCR has been recently published, and was shown to have a detection limit of 10 CFU (49).

Blood samples were collected before instillation and at 24 hours after instillation. Urine samples were collected before instillation and at 2, 4, 8 and 24 hours after instillation for instillation 1 and 6. Furthermore, sputum was collected before instillation and at 24 hours after instillation for instillations 1 and 6.All blood and sputum samples were negative for CFU and PCR for all patients at all assessed time points.
VPM1002BC was rapidly cleared from urine; no CFUs and a negative PCR were obtained 24 h post instillation. For more details see section A2.16 and Figures 5 and 6.

It has been shown in several animal experiments in mice and guinea pigs, that VPM1002 has no increased pathogenicity compared to the well-established *M. bovis* BCG vaccine (see annex 6.1 for table). The Netherlands Commission on Genetic Modification (COGEM) classified rBCG:Δ*ureC*::*hly*+ (VPM1002) as a GMO that can be handled at the ML-I / DM-I level without endangering the safety of humans or the environment according to § Dutch law (CGM/070402-05). Meanwhile, the toxicological preclinical animal study program as well as results from clinical development as TB vaccine (so far 3 trials completed, including one trial in newborn infants) confirmed that the safety of VPM1002 is at least as good as that of the well-established BCG.

VPM1002BC did not show any influence on erythrocyte count and haemoglobin values in a study in monkeys. An increase of free haemoglobin would have been observed theoretically if erythrocytes had

been lysed by listeriolysin, but this was not the case. Moreover, in reference to Directive 93/88/EEC (on the protection of workers from risks related to exposure to biological agents at work), VPM1002 has been classified as safety level 1, i.e. not pathogenic to humans by the Dutch authority COGEM.

Based on the reports presented in section A4.6 of this document (14;50) it is to be noted that after intravesical application *M. bovis* BCG is mostly released *via* urine. In rather rare cases standard *M. bovis* BCG was detected in blood and sputum samples. In addition, bladder tissue analyses demonstrated persistence of *M. bovis* BCG in this organ up to 24 months (14). Of note, in no study was this the case for VPM1002BC, which was rapidly cleared and did not spread systemically after intravesical instillations in non-clinical and clinical settings.

Risk analysis

This is the most important aspect of the whole application!

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

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

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

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

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

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

Side effects of intravesical treatment with *M. bovis* BCG are classified into: (i) local side effects such as hematuria (most common), severe dysuria and chemical or bacterial cystitis and (ii) systemic toxicity. Systemic toxicity can happen if *M. bovis* BCG is absorbed through the bladder wall into the system and it includes fever, skin rash, generalized malaise, systemic infection and septicemia. For some groups of patients with NMIBC it is recommended to avoid treatment with *M. bovis* BCG due to the risk of toxicity (53). In addition, *M. bovis* BCG should be used with caution in immunocompromised patients (immunosuppression, HIV infection) due to *M. bovis* BCG-related infectious complications that may occur (54).

Existing non-clinical and clinical data clearly suggests that the likelihood and severity of these side effects is strongly reduced with VPM1002BC (see section A5.1).

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

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

Potential ways in which dispersion from test subjects may occur are:

- Micturition poses a high risk for dispersing the GMO into the environment, since it will be instilled into the bladder and substantial amounts will be voided through urine. Data gained in phase I of the clinical trial shows that after 24 h VPM1002BC was no longer detectable in the urine of the patients (see section A2.16, and Figures 5 and 6).
- Semen and contact with genital area poses a potential risk for dispersing the GMO into the environment, since there is a likelihood that the GMO can be found there after administration.
- Bleeding is an unlikely risk for dispersing the GMO into the environment, since the GMO is unlikely to enter the blood stream of the patients. Data from the phase I part of the clinical trial SAKK 06/14 showed that VPM1002BC was not found in the blood of the patients at any of the analyzed time points.
- Coughing is an unlikely risk for dispersing the GMO into the environment; as mentioned above, it is highly unlikely for the GMO to spread systemically and to the lung of the patients. Data from the phase I part of the clinical trial SAKK 06/14 showed that VPM1002BC was not found in the sputum of the patients at any of the analyzed time points.

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

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

- Risks for the patients treated with VPM1002BC, their contact persons or the staff handling the VPM1002BC do not exceed the known risks of the well-established immunotherapeutic agent *M. bovis* BCG. There are two reports in literature that describe the nosocomial transmission of *M. bovis* BCG to patients and subsequently healthcare workers by contaminated chemotherapeutics (55;56). Investigations showed that contaminated biosafety cabinets were responsible for the transmission. As a result, strict hygiene measures were implemented (proper cleaning, gloves). Nosocomial transmission of VPM10002BC in this respect is not possible, since the reconstitution is taking place in a single-use, closed drug transfer system (annex 9).
- Chances for induction of severe side effects by VPM1002BC are smaller than in case of *M. bovis* BCG. VPM1002BC is a genetically modified *M. bovis* BCG containing *ureC* gene disrupted by the introduced listeriolysin-expressing cassette. The modifications introduced in parental *M. bovis* BCG resulting in VPM1002BC improved its safety properties, as demonstrated in non-clinical studies showing a better safety profile of VPM1002BC when compared to *M. bovis* BCG (toxicity test in SCID mice, toxicity studies after single and repeated intravesical instillation; VPM1002-BC-Pre.01, VPM1002BC-GE-Pre.02, VPM1002 BC-GE-Pre.03; see annex 6). For more information, see sections A2.14 and A5.1. Furthermore, in the SAKK 06/14 phase I study all patients completed the full course of VPM1002BC induction intravesical therapy. The treatment was generally very well-tolerated and no grade 3 or 4 adverse events occurred (grading of adverse events according to CTCAE).
- There is no risk of inducing health complications in immunocompromised patients due to administration of VPM1002BC in this study. *M. bovis* BCG should be used with caution in immunocompromised patients (immunosuppression, HIV infection) due to *M. bovis* BCG-related infectious complications that may occur (54). In addition, a preclinical safety study in SCID mice

demonstrated superior safety profile of VPM1002 in comparison to normal BCG. Nevertheless, VPM1002BC will not be applied in immunocompromised patients in this study.

- Due to the restricted access to the IMP, it is highly unlikely that unintended persons will be exposed to it.
- Chances for the patient´s partner to be exposed to VPM1002BC during sexual intercourse are minimal. Up-to-date no documented evidence of sexual transmission of BCG from patients receiving intravesical BCG instillations to their partners has been reported. The applicant conducted an extensive literature search on this topic.
- Transfer *via* excrement, blood and other bodily fluids is highly unlikely and has so far not been observed for *M. bovis* BCG. The applicant conducted an extensive literature search on this topic. Data from the phase I part of the trial also show that VPM1002BC is not found in the blood and sputum of the patients at any time during the tested time period. Moreover, VPM1002BC was not detectable after 24 hours in the urine of the tested patients (see section A2.15 and A5.1).
- Risk of systemic distribution of VPM1002BC after intravesical application into patients is minimal. This estimation is based on preclinical studies (VPM1002-BC-Pre.01, VPM1002BC-GE-Pre.02, VPM1002 BC-GE-Pre.03; see annex 6), in which systemic spread of VPM1002BC was assessed after 1 or up to six intravesical instillations of VPM1002BC to female BL/6 mice or CD® rats. No test-item related CFU counts were noted for the examined organs and the blood of the animals treated with VPM1002BC.

Even if VPM1002BC is dispersed into the environment, it is not probable that VPM1002BC will survive or spread its genetic material to other bacterial organisms. VPM1002BC is a laboratory strain and it does not have a natural niche, as well as its parental strain *M. bovis* BCG. It is not likely that genetic modifications introduced to VPM1002BC (deletion of *ureC* and introduction of listeriolysin-expressing cassette) improved its capability of survival in nature. VPM1002BC does not contain any antibiotic resistance-encoding sequence. Furthermore, horizontal gene transfer to other bacteria has never been observed for *M. bovis* BCG and other members of the *M. tuberculosis* family (15) Therefore, risks of harming the environment in terms of introduction of VPM1002BC genetic construct are minimal.

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

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

Even though the risk of spreading of the GMO is considered negligible due to the statements presented in section 5.4, certain risk management measures will be in place during the conduct of the clinical trial. The trial subjects/patients will be informed that they will be treated with genetically modified bacterium and that an inactivation of the urine as well as additional hygiene measures will be required as to minimize as much as possible the dispersion of the investigational medicinal product into the environment. The patients will be instructed to adhere to the following hygienic measures: urinate in sitting position, wash thoroughly their genital area (especially at the first voidings after the treatment with VPM1002BC), wash their hands with warm water and soap. In case of contamination of skin lesions (e.g. of the hands, the genital area) an appropriate washcloth should be used. This prevents an unintended transfer of the live mycobacterium to non-treated persons.

Treated patients will carefully be monitored for any adverse events and the intended therapeutic effect.

The patients will be instructed about the following behavioural procedures:

- Special precautions will be taken at the first voiding after each VPM1002BC instillation, as most of VPM1002BC is excreted in the urine at this time point. The first voiding will take place in the hospital.

1 hour after the instillation the patient will void in a urinal bottle containing two Javel-Tabs. After 15 min the urine will be disposed by flushing down the toilette as described in section A.4.3.

- Up to 1 week after instillation patients should void while seated to avoid splashing of urine. Urine voided during this time should be disinfected with chlorine tablets (e.g. Javel-Tabs containing Sodium-Dichlorisocyanurate dihydrate from Steinfels Cleaning Systems). The patients will be instructed to add two tablets into the toilet bowl **before** urinating, and after urination to wait 15 min until flushing down the toilette.

The management strategy for accidental self-injection of VPM1002BC by personnel at the site (during reconstitution of the VPM1002BC) is described in section A.5.9 of this document. Waste handling management is described in chapter A.4.9 of this document.

Risk management

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

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

No specific inclusion/exclusion criteria are necessary as preventive measures for environmental safety.

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

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

The patients will be treated with VPM1002BC at the clinical sites. Access to the treatment rooms is limited to authorized study personnel and the patients will only enter this area when accompanied by authorized study personnel. After the instillation patients will remain at the clinics for at least 4 hours. Patients will be asked to withhold urination for at least 1 hour after the instillation to allow sufficient contact time with the bladder wall. During this time, patients will be allowed to move freely around the clinics perimeter, as patients should be mobile to improve contact with the bladder wall.

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

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

Not applicable. Treatments with VPM1002BC will be performed at the clinical trial site.
Subjects will not be hospitalized, although they will be required to remain in the clinic for at least 4 hours following administration of VPM1002BC for observation for signs of adverse reactions.

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

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

Handling of VPM1002BC in preparation for application will be conducted in a closed drug transfer system. Hygiene procedures follow a daily general cleaning by authorised staff and using suitable cleaning agents and disinfectants. Working areas used for preparation of VPM1002BC for instillation are maintained on a routine daily basis using suitable disinfectants and disinfection procedures proven to be active onthe mycobacteria. General and study specific hygiene instructions will be followed for general cleaning and in case of contamination. Instructions for handling of GMOs and preventing its dispersion, prepared by the Out Patient Clinic, will be followed as described in sections A4.3 and A4.9.

In case of emergencies, response plans will be followed as described below.

Emergency response plans:

1. Methods and procedures for controlling the GMOs in case of unexpected dispersion:

PCR-methodology is available for detection of VPM1002BC in samples. In the highly improbable case of a detected human-to-human transmission leading to an unsustainable adverse drug reaction, a set of suitable rescue medication is available for treatment.

In case of accidental injection of a partial dose the same warnings and precautions, liver effects, systemic adverse reactions as for *M. bovis* BCG should be expected.

The sites will be instructed by the sponsor about safety precautions and behavioral practices in case of contamination caused by self-injection.

In case of accidental injection of the entire dose of 1-19.2x10E8 CFU, the effects are not generally considered to be more severe than for lower doses, as the immunological response is not proportional to the dose.

Instruction for the personnel not involved in the accident at the site:

Because of the risk of accidental injection into a blood vessel, the affected person should consult a member of the study team immediately via a 24 hour emergency telephone and consult the Principle Investigator (PI) of the site or if not present a sub-investigator (SI) participating in the trial and combination therapy with tuberculostatics should be considered.

The management of accidental self-injections with VPM1002BC should be handled similar to that of *M. bovis* BCG. Management of systemic infections or persistent local infections following vaccination with *M. bovis* BCG tuberculosis vaccine includes treatment with antimycobacterial drugs. The same measures should be taken in case of systemic or persistent local infections following accidental self-injection of VPM1002BC. VPM1002BC is sensitive to all common tuberculostatics.

Clean any blood-contaminated surfaces according to A4.9.(3) of this document. Collect any blood-contaminated waste and dispose it as biohazardous waste according to A4.9.(3) of this document.

Instructions that should be handed to the affected person:

- Cover the injection site with a suitable dressing immediately. This will further absorb any VPM1002BC that may leak out through the needle track.
- You may take off the dressing after 30 minutes.
- Just after you have covered the injection site, inform a physician as well as the PI or if not present a SI of the trial about the incidence.
- Avoid any direct contact with the injection site in order to prevent transmission of the IMP to other parts of the body, to other persons or objects.
- Use the additional supply of dressings provided to you in the event that the original dressing detaches.

- Discard the detached dressing(s) in the plastic bag you received and return to the clinical site for professional disposal.
- If possible the injection site should not be touched, scraped or rubbed within the first few days, especially if the site has started ulcerating, to prevent an infection of the injection site with ubiquitous agents and dispersing of the recombinant mycobacteria into the environment.
- In case of direct contact with the site of injection or with the material that covered it, wash your hands thoroughly using warm water and soap.
- Do not apply any ointments and creams to the injection site until it is completely healed.
- Keep the site of injection dry
- Minimize wetting of the area e.g. during bathing, until the injection site is completely healed.
- Contact the PI/SI of the trial if any notable local injection site reactions occur. Depending on the progression of the ulceration, subsequent appointments for ambulant visits may be arranged.

2. Methods for decontamination of the areas affected, for example eradication of the GMOs:

Working areas used for preparation of VPM1002BC for instillation are maintained on a routine daily basis using suitable disinfectants and disinfection procedures proven to be active on mycobacteria. Compare also chapter A4.9.(3) of this document.

Procedure in case of unexpected situations and serious incidences

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

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

The site will report to the Gene Therapy Office all adverse events that will occur at the site according to the requirements for GMO trials of the Gene Therapy Office.
In addition, **s**uspected unexpected serious adverse reactions (SUSARs) and serious adverse events (SAEs) occurring in the entire study will be reported by the sponsor to the authorities.

All sites participating in the trial (including sites in Switzerland and European Union (EU)) will report any urgent safety signals or new risks to the sponsor within one working day. The sponsor will assess all new risks and safety signals and inform the authorities according to the safety reporting requirements of the applicable European and national laws. In case changes in the risk management are required for medical reasons, the sponsor will take decisions for trial conduct and inform the sites participating in the trial and the authorities, if applicable.

In phase I, an emergency plan was applied during weekends and public holidays in case a local investigator notices a medically important issue that needs to be spread to the other sites as soon as possible.

An independent safety committee was consulted for the evaluation of safety data in phase I. They were assigned to review the safety data on an ongoing basis, give advice on dose de-escalation and recommend the dose for phase II.

Every reported death will be immediately evaluated by the medical advisor at SAKK and the centre will be informed if the sponsor considers that an autopsy is needed. The staff performing the autopsy will be informed to use precautions as for handling of infectious patients and to take measures for preventing the spreading of any biological waste into the environment. However, considering that VPM1002BC cannot replicate outside treated organisms, the risk for the environment is minimal.

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

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

In case of premature treatment termination, the patients will be transferred to the follow-up phase. The evaluations at the end of treatment will be performed as per protocol.
Follow-up phase starts after the last evaluation at the end of treatment or after last trial treatment in case of pre-mature treatment stop. The duration of follow-up phase without trial-specific investigations is 5 years after last patient last treatment (LPLT). The trial team may decide to shorten the duration of the 5 years follow-up. Shortening of the follow-up period will be performed only after approval by involved ethic committees (ECs) and the competent authority.
Treatment–related adverse events will be followed-up until resolution or stabilization. Survival status, recurrence and progression will be reported every 3 months in the first 2 years and thereafter every 6 months until 5 years.
VPM1002BCG is a recombinant *M. bovis* BCG. Data from preclinical studies and clinical phase I and phase II trials for TB vaccination suggest that vaccination with VPM1002 is as safe as, and possibly better tolerated than that with an equivalent dose of conventional *M. bovis* BCG. As the kind of side effects to be expected are supposed to be the same as for conventional *M. bovis* BCG, the management strategy for side effects associated with intravesical VPM1002BC will be the same as for those for conventional *M. bovis* BCG. This includes following aspects: (i) system of clearly defined adverse effects (AEs) and SAEs and corresponding reporting procedures compliant with good clinical practice (GCP) requirements, (ii) well established AEs reporting system during trial treatment and after end of trial treatment, (iii) reporting of individual SAEs, serious adverse drug reactions SADRs and SUSARs by the sponsor, (iv) reporting of urgent safety signals and (v) periodic reporting on safety to PIs.

Monitoring

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

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

For identification of VPM1002BC and differentiation from other organisms, the PCR method will be used, which serves as GMP-release-test for the investigational medicinal product.

1. Methods for tracing the GMOs, and for monitoring their effects:

We have specific techniques available to discriminate between environmental mycobacteria and VPM1002BC, in case needed.

In case of a known contact to the patient and possible transmission to other persons or objects, the subjects are asked to inform the clinical site about this event. The subjects are asked to report to the site any signs or symptoms reported by a contact person that resemble symptoms of an adverse drug reaction against the immunotherapy. A supervision of the contact persons reporting these symptoms by suitable medical assessments is intended; provided the person in question agrees to this procedure. If the medical supervision confirms the reported symptoms, a further analysis by suitable detection methods (PCR analysis) is intended.

2. Specificity, sensitivity and reliability of the monitoring techniques:

The established PCR to detect the deletion of *ureC* is highly sensitive and highly specific for VPM1002BC. It was performed in the first place by the German and WHO Reference Centre for Mycobacteria (Borstel, Germany). For GMP-purposes, it was also performed by SIIPL and Microsynth AG, Balgach, Switzerland. The method was developed by Fresenius/Wavre, Belgium. Data specific for the *in vivo*-situation in human is available from three clinical trials.

3. Techniques for detecting transfer of the donated genetic material to other organisms:

The donated genetic material will not be transferred from VPM1002BC to other organisms. Brosch et al. (15) analysed the whole genome of several mycobacteria and observed no evidence of any horizontal gene transfer. This was confirmed also for a genetically modified *M. bovis* BCG with a genetic modification of plasmidic origin (16). In VPM1002BC the genetic modification is of genomic and not of exosomal, plasmidic origin. Hence, the genetic stability of the modification is high. Furthermore, chromosomal integration of distinct genetic material to VPM1002BC or *M. bovis* BCG is a highly complicated lab-procedure and therefore cannot occur spontaneously.

4. Duration and frequency of the monitoring:

Monitoring is predicted to last 60 weeks per patient.

A6. Conclusions of the possible environmental effects

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

In summary, from all data obtained from preclinical and clinical trials, there are no risks for the treated patients or their contact persons or the staff handling the GMO that exceed the known risks (see section A5.6) of the well-established immunotherapeutic agent *M. bovis* BCG. The trial subjects/patients will be informed that they will be treated with genetically modified bacteria and that an inactivation of the urine as well as additional hygiene measures will be required as to minimize as much as possible the excretion of the investigational medicinal product into the environment. This prevents an unintended transfer of the live mycobacterium to non-treated persons. VPM1002BC, similar to the well-established *M. bovis* BCG, cannot replicate outside treated organisms or lab cultures. Hence, no risk for the environment is expected.

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

Considering the genetics of and the measures taken to ensure controlled handling of VPM1002BC, post-treatment monitoring and release processes listed in this document, we conclude that there is no risk of VPM1002BC to become persistent and invasive in the natural environment.

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

VPM1002BC is sensitive to all commonly used antibiotics in the treatment of mycobacterial infection, e.g. isoniazid, rifampicin and ethambutol. VPM1002BC contains no additional antibiotic selection marker. Therefore, there is no selective advantage of VPM1002BC in the environment compared to its parental strain. On the contrary, VPM1002BC is even more attenuated than its parental strain, the highly attenuated *M. bovis* BCG.

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

Based on the arguments listed in section A2.8 we conclude that there is no potential for gene transfer to other species.

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

The transmission of diseases, i.e. tuberculosis to humans can be ruled out, because the respective gene regions are already inexistent in the original organism *M. bovis* BCG and as well in VPM1002BC.
Interactions with other organisms can be ruled out in case of the VPM1002BC. Thus, no consequences are to be expected.
Adverse effects in the treated patients are expected to be less or similar to the conventional *M. bovis* BCG. The sponsor evaluates that the benefit-risk-balance of the study is acceptable.

4. Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the GMO and persons working with, coming into contact with or in the vicinity of the GMO release(s).

The transmission of diseases, i.e. tuberculosis to humans can be ruled out, because the respective gene regions are already inexistent in the original organism BCG and as well in VPM1002BC. There is no risk for transmission of any known disease to subjects, patients, close contacts or hospital staff.
The submitted clinical trial comprises the first application in bladder cancer patients. VPM1002 has already been applied in three clinical trials as a tuberculosis vaccine, including the application in newborns and demonstrated its safety. Treated patients will carefully be monitored for any adverse events and the intended immunogenicity effects. Management strategy regarding contact persons of treated patients is described in section A5.12 of this document. The management strategy for accidental self-injection of VPM1002BC by personnel at the site (during reconstitution of the VPM1002BC) is described in section A5.9 of this document**.**
For identification of VPM1002BC and differentiation from other organisms, the PCR method will be used, which serves as validated GMP-release-test for the investigational medicinal product.

5. Possible immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from consumption of the GMO and any product derived from it, if it is intended to be used as animal feed.

Not applicable as VPM1002BC is not intended to be used as animal feed.

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

VPM1002BC does not interact with environmental processes because its environmental life span is too short.

7. Possible change in the current medical practice.

VPM1002BC was developed with the objective of inducing improved immune response and better safety/tolerability profile compared to the parental BCG strain routinely used in clinical application. Therefore, it is expected that application of VPM1002BC will replace standard *M. bovis* BCG, finally providing a therapy for non-muscle invasive bladder cancer characterized by improved efficacy and safety.

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