Methodology for environmental risk assessments in medical and veterinary biotechnology

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Voorwoord

De COGEM heeft jarenlange ervaring met de milieurisicobeoordeling van genetisch gemodificeerde organismen (ggo's). Op welke wijze de COGEM deze risicobeoordeling voor toepassingen in de medische en veterinaire biotechnologie (zoals werkzaamheden met gg-virussen, gg-bacteriën en gentherapieonderzoek) uitvoert, is echter nooit integraal vastgelegd. Om aanvragers, stakeholders en andere geïnteresseerden inzicht te geven in de gebruikte methodologie heeft de COGEM aan het bureau Perseus opdracht gegeven de door de COGEM opgebouwde risicoanalysemethodologie op het gebied van de medische en veterinaire toepassingen vast te leggen. Dit zowel voor ingeperkt gebruik als voor introductie in het milieu. Daarnaast heeft het rapport tot doel toekomstige COGEM leden een leidraad te bieden voor de wijze waarop de COGEM bij een milieurisicoanalyse te werk gaat.

Mevr. dr. ir. G. Smets en dr. P.L.J. Rüdelsheim hebben namens Perseus voor dit project vele adviezen geanalyseerd die de COGEM op medisch en veterinair gebied heeft uitgebracht. Daaruit is naar voren gekomen dat de COGEM een algemeen kader voor haar werkwijze hanteert, maar eveneens dat feitelijk iedere milieurisicoanalyse uniek is en specifiek voor de betreffende studie. Het algemene kader berust op nationale en internationale wet- en regelgeving. Bij de uiteindelijke risicobeoordeling van een studie spelen veel variabelen mee, zoals het type en het gebruik van het ggo, het donor- en acceptor-organisme, de aard van de genetische modificaties, etc. Gebrek aan kwantitatieve data over risico's en de blootstelling daaraan speelt eveneens een rol. Hierdoor is veel technische achtergrondkennis vereist om zorgvuldig te kunnen adviseren over iedere studie.

In nauw overleg met de begeleidingscommissie is er voor gekozen niet al te diep in te gaan op de technische details van de vele milieurisicoanalyses die de COGEM de afgelopen jaren heeft gedaan, maar primair een algemene methodologie te destilleren. Hierdoor is het rapport veel breder toepasbaar en ondanks de complexiteit van deze materie met een beperkte kennis van ggo's toch goed toegankelijk. In het kort komt het erop neer dat de milieurisicoanalyse onderverdeeld is in zes verschillende stappen. Deze stappen zijn: 1. identificatie van mogelijke nadelige effecten, 2. de ernst van deze effecten, 3. de kans dat deze effecten optreden, 4. bepaling van het risico van ieder effect door combinatie van de ernst en de kans van het effect, 5. mogelijke maatregelen om risico's te beperken, en 6. vaststellen van het uiteindelijke risico van de geplande werkzaamheden. Om de werkwijze van de COGEM te illustreren, is voor vier verschillende adviezen aangegeven hoe deze stappen ingevuld worden. Dit is weergegeven in de Bijlage van het rapport. Het betreft adviezen over ingeperkt gebruik en introductie in het milieu van zowel gg-bacteriën als gg-virussen.

Zoals algemeen bekend, kunnen werkzaamheden met ggo's milieurisico's inhouden. Het is van belang en wettelijk vereist deze risico's voor aanvang van de werkzaamheden in kaart te brengen en zonodig door middel van risicomanagement-maatregelen te minimaliseren. Dit rapport biedt een duidelijk overzicht van de elementen die de COGEM bij haar milieurisicobeoordeling van dergelijke werkzaamheden betrekt. Hopelijk geeft dit overzicht aanvragers en ander stakeholders inzicht in de werkwijze die de COGEM in de loop der jaren ontwikkeld heeft en ook voldoende houvast om mogelijke milieurisico's op een correcte wijze te identificeren en te beheersen.

Met vriendelijke groeten,

Voorzitter van de begeleidingscommissie
Dr. T.G. Kimman, CVI-Lelystad

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Summary

Aiming to provide a high level of protection of human health and the environment, European and Dutch legislation require that an environmental risk assessment (ERA) is conducted preceding any activity with genetically modified organisms (GMOs). The ERA is the basis for deciding on an adequate risk management strategy, if required to reduce potential adverse effects. The '*Commissie genetische modificatie*' (COGEM) as the advisory body to the government, examines the ERA conducted by the applicant for correctness and completeness. Subsequently the COGEM advices on the admissibility of the planned activities and accompanying risk management procedures.

Over time, the COGEM has composed and reviewed a large number of ERAs relating to human and veterinary medicine research and development. While each case has been evaluated on the basis of its specific characteristics, a general methodology evolved. This review presents a synopsis of the COGEM methodology based on a thorough analysis of its advices, as well as legal and scientific instruments underlying the formulation of an advice to the competent authority. As ERA concepts – in particular related to GMOs- continue to evolve, this report must be considered as state-of-the-art review and subject to further refinement.

The report is intended to provide applicants and assessors (*e.g.* new COGEM members) with a framework for conducting and evaluating future ERAs on biotechnology applications in human and veterinary medicine research and development. Importantly it also demonstrates COGEM's commitment on transparency of its approach.

Activities with GMOs are assessed on a case-by-case basis. They are either classified as 'contained use' or 'deliberate release' depending on the intended activities and the required specific measures to prevent exposure of the public and the environment to the GMO. Although an ERA for a contained use is treated differently from one for a deliberate release, the underlying methodology is basically the same. In a contained use assessment emphasis is put on containment, *i.e.* the procedures and facilities intended to prevent exposure of the public at large and the environment in general to the GMOs. In deliberate release exposure is to some extent assumed and therefore the consequences for the environment and human and animal health are assessed more broadly and in more detail.

The stepwise ERA evaluates in six steps direct or indirect effects as well as immediate or delayed effects, related to the intended activity with a GMO. In the first step the properties of the GMO and the proposed activities are described to identify potential hazards. Next the severity and the likelihood of occurrence of the hazards are assessed. Both these components will lead to the risk characterisation. When the risks are clearly defined management measures can be designed to prevent or mitigate the remaining risks. Finally, an overall assessment will indicate whether risks for human health and the environment are negligible or not. Four selected case studies of COGEM advices are provided in this report as examples of the process.

In the Netherlands a classification system has been developed to streamline ERAs for standard 'contained use' activities. The COGEM has established a systematic approach guiding users through a set of criteria to be checked for their planned activity in order to assign the activity to a containment level. Complying with the corresponding containment requirements adequately mitigates risks. The principle of the case-by-case assessment is preserved, while allowing for quick evaluation. Only in cases where not enough information is available or some aspects need specific consideration, the COGEM is requested to give advice. For 'deliberate release' submissions the COGEM is involved in every case evaluation, as - due to the wide range of experiments and applications - standardisation is hardly possible.

One of the difficulties encountered in conducting an ERA is the quantification of risks or the lack thereof. In many cases the ERA is qualitative or a ranking system is used with indications like “high”, “moderate”, “low”, and “negligible” for estimates of the magnitude, likelihood, and risk of adverse effects and their consequences. Yet, in many cases, quantification may not be possible or required, and the costs of gaining more precise information may not be proportionate to the gain in certainty of the ERA. Alternatives such as estimates, indications of uncertainty, worst-case scenarios, risk hypotheses and the tiered approach for testing are deployed where possible.

Reflecting a cautious attitude, many ERAs take into account hypothetical cases because it cannot be concluded that the risk is negligible. In some cases, this results in imposing protective measures that subsequently may prove to have been excessive. Fully appreciating this weakness on some ERAs, the COGEM prefers to err on the side of caution until more information is available to fine-tune the ERA.

In this report the state-of-the-art stepwise ERA methodology, based on European directives ad guidance documents, was used as reference, demonstrating that the same logical approach is embedded in the COGEM advices. The stepwise methodology can be used in the future as an additional verification to ensure that all relevant aspects of the ERA are taken into account. The large experience that the COGEM has accumulated over the years is good basis for the safe use of GMOs in contained use and deliberate release, both at the level of R&D as well as at the commercial scale.

Samenvatting

Om een hoge mate van bescherming te bieden aan mens en milieu vraagt de Europese en Nederlandse wetgever dat er een milieurisicobeoordeling (MRB) wordt uitgevoerd voordat handelingen met genetisch gemodificeerde organismen (GGOs) worden gestart. De MRB is het uitgangspunt voor een doeltreffende risicobeheersingsstrategie, als die nodig mocht zijn, om potentieel schadelijke effecten te verminderen. De Commissie Genetische Modificatie (COGEM), het adviesorgaan voor de Nederlandse overheid, beoordeelt de MRB zoals deze door de aanvrager is opgesteld op juistheid en volledigheid, en adviseert de overheid over de toelaatbaarheid van de voorgenomen activiteiten en de maatregelen om eventuele risico's te beperken en beheersen.

Gedurende haar werkzaamheden heeft de COGEM een groot aantal MRB-en met betrekking tot onderzoek naar en ontwikkeling van biotechnologische toepassingen in de humane en veterinaire medische sector geëvalueerd. Terwijl elk voorstel beoordeeld werd op basis van zijn eigen specifieke kenmerken (de 'case-by-case' benadering), evolueerde de algemene methode voor het uitvoeren van een MRB voor een GGO. In deze studie wordt de door de COGEM gehanteerde methodologie beschreven en geanalyseerd. Als basis daarvoor dienen de COGEM adviezen evenals de wettelijke eisen en wetenschappelijke inzichten die aan de basis liggen voor advisering aan de overheid. Aangezien MRB concepten – zeker met betrekking tot GGOs - nog steeds evolueren, dient dit rapport beschouwd te worden als een momentopname en onderhevig aan verdere actualisatie.

Het rapport is bedoeld om zowel aanvragers als beoordeelaars (bijv. nieuwe COGEM leden) een kader aan te reiken voor het uitvoeren van nieuwe MRB-en voor biotechnologische toepassingen op het gebied van medisch en veterinair onderzoek en ontwikkeling. Het rapport vormt ook een bevestiging van de transparante houding van de COGEM over zijn werkwijze en overwegingen.

Werkzaamheden met GGOs worden 'case-by-case' beoordeeld. Ze worden ingedeeld als 'ingeperkt gebruik' of 'introductie in het milieu' afhankelijk van de beoogde activiteiten en de keuze van specifieke maatregelen om blootstelling van de bevolking en het milieu aan het GGO te voorkomen. Hoewel een MRB voor ingeperkt gebruik verschilt van die voor introductie in het milieu, is de onderliggende werkwijze toch dezelfde. Bij de evaluatie van ingeperkt gebruik ligt de nadruk op inperking van het GGO, nl. de werkwijzen en inrichtingen bedoeld om te verhinderen dat mens en milieu worden blootgesteld aan het GGO. Een introductie in het milieu veronderstelt al een zekere mate van blootstelling. Daarom worden juist de gevolgen voor het milieu en de gezondheid van mens en dier uitgebreider en gedetailleerder onderzocht.

De stapsgewijze MRB evalueert in zes stappen zowel directe en indirecte effecten alsook onmiddellijk of vertraagd optredende effecten, die mogelijk kunnen optreden door de voorgenomen GGO-activiteit. In een eerste stap worden de kenmerken van het GGO en de voorgenomen werkzaamheden beschreven om mogelijk schadelijke effecten te identificeren. Vervolgens worden de mogelijke gevolgen van elk schadelijk effect en de waarschijnlijkheid van het optreden geëvalueerd. Beide componenten maken een inschatting van het risico mogelijk. Wanneer de risico's duidelijk zijn omschreven kunnen beheersmaatregelen worden opgesteld om de resterende risico's te voorkomen of te beperken. Als laatste wordt bepaald of het algehele risico voor de menselijke gezondheid en het milieu verwaarloosbaar klein is of niet. Deze stapsgewijze aanpak is verwerkt in de COGEM-adviezen. Om het proces te illustreren zijn er in deze studie vier COGEM adviezen uitgekozen en beschreven.

In Nederland is er een inschalingssysteem ontwikkeld om de MRB-en van standaard 'ingeperkt gebruik'-activiteiten te stroomlijnen. De COGEM heeft een systematische aanpak uitgewerkt die het gebruikers d.m.v. een reeks criteria mogelijk maakt hun voorgenomen werkzaamheden in te delen in een bepaald inperkingsniveau. Wanneer dan wordt voldaan aan de overeenkomstige

inperkingsvereisten, worden risico's op een adequate manier bedwongen of geminimaliseerd. Het principe om 'case-by-case' te beoordelen blijft behouden, terwijl het toch mogelijk is om snel te werken. Alleen in die gevallen waar niet genoeg informatie voorhanden is of bepaalde aspecten speciale aandacht vragen, wordt de COGEM om advies gevraagd. Bij kennisgevingen van introducties in het milieu wordt de COGEM altijd betrokken. Het is immers bijna onmogelijk om voor een breed scala aan experimenten en toepassingen één standaard uit te werken.

Eén van de moeilijkheden die men ondervindt bij het opstellen van een MRB is het kwantificeren van risico's of het gebrek aan kwantitatieve onderbouwing. In veel gevallen gebruikt men kwalitatieve termen of een systeem met aanduidingen, zoals "groot", "matig", "gering" en "verwaarloosbaar", voor schattingen van de ernst, de waarschijnlijkheid, en de risico's van schadelijke effecten en hun gevolgen. In veel gevallen zal kwantificering niet mogelijk en vereist zijn, en zal de vereiste inspanning verbonden aan het verkrijgen van preciezere informatie niet in evenredig zijn met de resulterende verhoogde zekerheid voor de MRB. Alternatieve benaderingen, zoals het werken met schattingen, aanduiding van de mate van onzekerheid, 'worst-case' scenario's, risicohypothesen en een stapsgewijze aanpak om experimenten uit te voeren, worden waar mogelijk aangewend.

Voorzichtigheidshalve wordt in vele MRB-en rekening gehouden met hypothetische aannames als niet zonder meer kan worden uitgesloten dat de risico's verwaarloosbaar klein zijn. Dit leidt soms tot het opleggen van inperkingsmaatregelen in gevallen die in eerste instantie bovenmatig lijken. In die gevallen blijft de COGEM liever aan de veilige kant zolang niet meer informatie voorhanden is om de MRB te verfijnen en nader te onderbouwen.

In dit rapport wordt de huidige stapsgewijze MRB-methode, die gebaseerd is op de Europese richtlijn en 'guidance' documenten, gebruikt als referentie en wordt aangetoond dat dezelfde logica de basis vormt voor de COGEM-adviezen. Deze stapsgewijze methodologie kan ook in de toekomst worden aangewend om alle relevante aspecten van een MRB in kaart te brengen. De uitgebreide ervaring die de COGEM in vele jaren heeft opgedaan vormt een stevige basis voor het veilig gebruik van GGOs in ingeperkt gebruik en bij introducties in het milieu, zowel in de onderzoeks- en ontwikkelingsfase als bij commercieel gebruik.

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List of abbreviations

AAV	Adeno-associated virus
BSC	Biosafety cabinet
COGEM	Commissie genetische modificatie (NL)
CT	Clinical trial
CU	Contained use
DR	Deliberate release
EC	European Community
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	Environmental Protection Agency (USA)
ERA	Environmental risk assessment
GGO	Genetisch gemodificeerd organisme
GM	Genetically modified
GMO	Genetically modified organism
HIV	Human immunodeficiency virus
IenM	Infrastructuur en Milieu
MRB	Milieurisicobeoordeling
MTB	*Mycobacterium tuberculosis*
PPE	Personal protective equipment
RQ	Risk quotient
SACGM	Scientific Advisory Committee on Genetic Modification (UK)
SARS	Severe Acute Respiratory Syndrome
SIN	self-inactivating

1 Introduction

According to European legislation the production and application of genetically modified organisms (GMOs) requires an assessment of the risks for human health and the environment. The environmental risk assessment (ERA) must be conducted in advance of any activity with a GMO. It provides the basis for identifying the need for risk management measures to reduce potential adverse effects, for designing risk management measures if needed, and for evaluating the remaining risk in case these measures are implemented.

The '*Commissie genetische modificatie*' (COGEM), as the advisory body to the Dutch government, has a broad experience with ERAs of GMOs relating to human and veterinary medicine research and development. While each case has been evaluated on the basis of its specific characteristics, a general methodology evolved. This review presents a synopsis of the COGEM methodology that has been gradually established. It can assist applicants in the preparation of future applications by indicating elements that COGEM has deemed essential in the ERA and may introduce new COGEM members in the approach. The authors belief that by making the report publicly available, the COGEM furthermore confirms its commitment to a transparent way of performing ERAs and formulating advices, all essential elements in the GMO decision-making process.

Perseus BVBA was commissioned to perform this ERA review based on previous COGEM advices, communications ('*signaleringen*') and reports. Also documents by the European Medicines Agency (EMA) or other authorities were used. The areas of interest include the medical and veterinary applications of GMOs (GM viruses, GM bacteria, gene therapy etc.) in contained use as well as under deliberate release. As the ERA of GMOs is performed on a case-by-case basis, this document was not expected to provide indications on specific data requirements, rather to reflect the logically structured and systematic ERA process. ERA concepts – in particular related to GMOs- continue to evolve. Consequently, this report must be considered as state-of-the-art review and subject to further refinement.

In the first part the underlying legislation, both at the European as well as at the national level, is summarised. Next the ERA procedure is explained based on existing guidelines, relevant literature on the subject, and COGEM experience, including information on data requirements. In the last chapter aspects of the methodology, including quantification of risks, are discussed. Finally, four advices that COGEM has issued in the past were used to illustrate the ERA process.

2 The legal framework for medical and veterinary applications of GMOs

2.1 European Community legislation

The requirement and the procedures for performing an ERA are laid down in Directive 2009/41/EC and in Commission Decision 2000/608/EC for contained use (CU) of GMOs, and in Directive 2001/18/EC and in Commission Decision 2002/623/EC for deliberate release (DR).

In this context, an ERA encompasses the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the GMOs may pose. Within the context of the GMO legislation, the scope is limited to effects in the environment. The effect on patients enrolled in a clinical trial is covered by other legislation and falls outside of the GMO ERA. Similarly the effect on healthcare workers exposed to the GMO is covered by legislation on worker's protection and is outside of the scope. However, the effect on humans (*e.g*. family members, visitors, and the public at large) must to be evaluated.

Similarly, economic impacts are outside the scope of the GMO evaluation. Nevertheless, it is recognized that the economic consequences of quarantine measures and trade restrictions may play a role in the classification of pathogenic organisms, in particular of animal diseases. *E.g.* an evaluation of a GM foot-and-mouth disease virus will attract particular concern, as an unintended release would result in drastic control measures.

Risk as a concept has two components, one related to the possibility of bad thing(s) happening, and the other related to the consequences if those bad thing(s) happen (Hill, 2005). Risk is therefore defined as the hazard combined with the likelihood that the hazard will occur:

Risk = Hazard & Likelihood

Commission Decision 2002/623/EC further elaborates that:

- 'Direct effects' refer to primary effects on human health or the environment, which are a result of the GMO itself and which do not occur through a causal chain of events.
- 'Indirect effects' refer to effects on human health or the environment occurring through a causal chain of events, through mechanisms such as interactions with other organisms, transfer of genetic material, or changes in use or management; observations of indirect effects are likely to be delayed.
- 'Immediate effects' refer to effects on human health or the environment, which are observed during the period of the release of the GMO. Immediate effects may be direct or indirect.
- 'Delayed effects' refer to effects on human health or the environment, which may not be observed during the period of the release of the GMO but become apparent as a direct or indirect effect either at a later stage or after termination of the release.

The ERA needs to take into consideration human health as well as the environment (protection goals in general). In the case of *e.g*. clinical trials (CTs) the patient to whom the medicinal product will be administered falls outside the scope of "human health and the environment" (EMA, 2005).

Commission Decision 2002/623/EC further elaborates that:

- Identified characteristics of the GMO and its use which have the potential to cause adverse effects should be compared to those presented by the non-modified organism from which it is derived and its use under corresponding situations.
- The ERA should be carried out in a scientifically sound and transparent manner based on available scientific and technical data.
- The ERA should be carried out on a case-by-case basis, meaning that the required information may vary depending on the type of the GMOs concerned, their intended use and the potential receiving environment, taking into account, inter alia, GMOs already in the environment.
- An analysis of the 'cumulative long-term effects' relevant to the release and the placing on the market is to be carried out.

2.2 GMO legislation in the Netherlands

EC Directives have to be implemented via national legislation. In the Netherlands the '*Besluit genetisch gemodificeerde organismen milieubeheer*' ('*Besluit*') of January 25, 1990 (BWBR0004703, Stb. 1990, 53) and amendments deal with both CU and DR[1]. Art. 5.1a and Art. 24 of the '*Besluit*' require an applicant to perform a risk analysis respectively before starting CU activities and before a DR in conformity with the European Directives.

The Decree implementing the law '*Regeling genetisch gemodificeerde organismen*' ('*Regeling*') of May 28, 1998 (BWBR0009653, Stcrt. 1998, 108) and amendments were last adapted in 2010 by the '*Wijzigingsregeling Regeling genetisch gemodificeerde organismen (herziening bijlage 1 en actualisering indeling handelingen in procesinstallaties*)' (BWBR0028026, Stcrt. 2010, 12420).

As the European CU Directive and the accompanying guidance documents only provide guidance for an ERA in general terms and make no link between specific types of GMOs and containment classes, the Ministry of Environment commissioned the COGEM and its predecessor to formulate such guidance in more specific terms. In order to standardise and also to simplify the assessment, rules were made for each type of GMO fulfilling a set of criteria and the corresponding containment level. The criteria have been devised in such a way that the containment level is adequate in the large majority of cases. This does not mean a deviation from the case-by-case approach. The rules are only to serve cases where the risk assessment is straightforward.

The '*Regeling*' comprises these rules for risk classification of GMOs in Annex 5 combining information on the type of recipient organism (Annex 1), the vector that is used (Annex 2.1) and the type of insert and its function (Annex 2.2) (Art. 7). The accompanying containment measures (equipment, facilities, working procedures) are listed in Annex 4. If biosafety is not adequately guaranteed using these standard classification rules, more precise and adjusted containment requirements will be formulated in the permit (Art. 7.2). In these cases the COGEM is to assess the risks in detail and give advice on (extra) containment measures.

For DR the '*Regeling*' refers to Commission Decision 2002/623/EC in Art. 13.2 concerning the ERA.

For activities with so-called naked DNA that are not part of the European Community legislation, the COGEM has formulated advices for permit procedures based on a general ERA (CGM/041223-02; CGM/101026-06). The COGEM justifies the evaluation of experiments with naked DNA on the basis that naked DNA may lead to the formation of GMOs by uptake and integration in somatic cells, germ line, bacteria, viruses etc.

[1] http://bggo.rivm.nl/Paginas/doc-reg.htm

2.3 Regulatory guidance documents

As both national authorities and authorities of the European Community (EC) are responsible for authorisations, the first for the research and development phase, the latter for market introduction, guidance for conducting an ERA can be found at both levels. Examples of relevant guidance documents on the European level are:

- 'Guideline on Scientific requirements for the environmental risk assessment of gene therapy medicinal products (EMEA/CHMP/GTWP/125491/2006)';
- 'Notice to applicants: guidance on environmental risk assessment for veterinary medicinal products consisting of or containing genetically modified organisms (GMOs) as or in products' (March 2006).

Since the basic principles of an ERA are generally applicable, also guidance not specifically addressing GMOs relating to medical and veterinary research and applications, might be relevant; *e.g*. the document 'EFSA Panel on Genetically Modified Organisms (GMO); Guidance on the environmental risk assessment of genetically modified plants' (EFSA, 2010).

During clinical development, national competent authorities are responsible for regulating investigational medicinal products consisting of GMOs. National law and the application of directives for CTs differ in the EU member states. However, the information necessary for an ERA should be identical (EMA, 2007).

For the Netherlands some guidance can be found in the application forms as explicatory notes for each of the questions about information that is required:

- '*Ingeperkt gebruik regulier: micro-organismen*';
- '*Ingeperkt gebruik regulier: micro-organismen, animale cellen en/of dieren*';
- '*Introductie in het milieu: veterinaire toepassingen van genetisch gemodificeerde organismen*';
- 'Assessment of clinical research involving gene therapeutics in the Netherlands'.

Furthermore COGEM and RIVM have published reports on risk assessment. Examples are:

- '*Integratie en verspreiding naakt DNA*' (CGM/041223-02);
- 'Recombinant and chimeric viruses: Evaluation of risks associated with changes in tropism' (CGM2005-04);
- '*Classificatie van dierpathogene virussen. Criteria en inperkingsmaatregelen voor pathogeniteitsklassen van dierpathogene virussen*' (CGM/060420-04);
- 'Environmental risk assessment of replication competent viral vectors in gene therapy trials' (RIVM Report 601850001/2008);
- '*Inschaling laboratoriumwerkzaamheden met lentivirale vectoren*' (CGM/090331-03).

The general principals to conduct an ERA are the same for CU or DR. However, the emphasis in both situations is different. In the CU procedure the focus is on achieving containment and avoiding exposure. For DR it is assumed that exposure will occur and therefore the effect on the environment is examined broadly and in more detail. Given this difference in emphasis, the approaches are treated separately in this study after the general explanation.

2.4 Stepwise ERA

The general scheme for an ERA (Figure 1) presents different steps. Each step is further elaborated in this section.

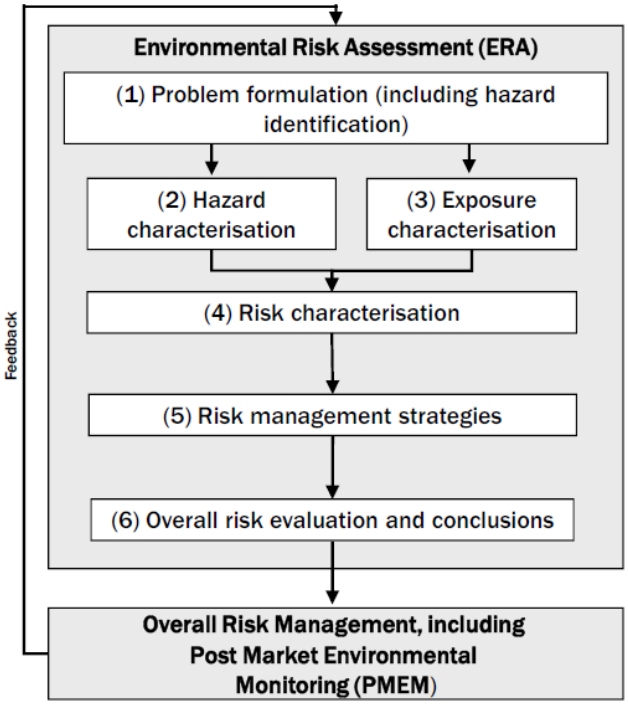

Fig. 1 Six steps within the environmental risk assessment (ERA) and relationship to risk management, including monitoring, according to Directive 2001/18/EC and Regulation (EC) No. 1829/2003 (source: EFSA, 2010)

Step 1: Problem formulation/ Hazard identification

A risk assessment starts with problem formulation in which the most important questions that merit detailed risk characterisation are identified. Problem formulation makes the risk assessment process transparent by explicitly stating the assumptions underlying the risk assessment. Problem formulation starts with the identification of hazards of the GMO, its production or use.

In hazard identification, potential adverse effects (hazards) are identified on the basis of knowledge about the characteristics of the recipient organism, knowledge about the function that the introduced traits have in the donor organism, knowledge about the way the newly acquired traits interact with the physiology of the recipient organism, and the anticipated interaction of the GMO with the receiving environment.

In problem formulation, the purpose for the assessment is spelled out, *i.e.* the problem is defined, and a plan for analysing and characterizing risk is determined. Problem formulation depends on the quality of these three products: assessment endpoints, conceptual models, and an analysis plan (EPA, 1998).

The broad protection goals of the EC legislation are the protection of human health and the environment (biodiversity, ecological functions). This should be translated in more precise objectives, so-called assessment endpoints that are measurable and that can be assessed through hypothesis testing. An assessment endpoint in this context is an object of value in the environment and a measure to evaluate harm to it, *e.g.* population size of a beneficial soil organism.

Then a conceptual model is built on how the GMO might interfere (cause harm to) with the protection goal. The conceptual model describes the pathway for analysis and establishes the proposed relationships between exposure and effect (Wolt *et al*., 2010). The analysis plan to evaluate the magnitude of harm then describes what to measure and the kind of studies that are needed. For each measurement endpoint, the level of environmental protection to be preserved is expressed through the setting of 'limits of concern'. Limits of concern can be defined by *e.g.* literature data, modelling, existing knowledge and policy goals (EFSA, 2010).

An ERA is performed not to demonstrate that an activity with a certain GMO is safe or not, but to demonstrate that a risk of harm is negligible or not (Raybould, 2010), hence the need for testing risk hypotheses and setting limits of concern. Effective risk assessment must select problems based on prior definitions of harm, rather than conducting research to describe possible changes or catalogue differences (Raybould, 2010), as changes are not *per se* causing harm.

In general, risk hypotheses are formulated by considering pathways or scenarios by which the activity under evaluation will cause harm (Raybould, 2010). The scenarios facilitate the interpretation of information and help to focus the ERA on what ultimately matters (*e.g.* if shedding does occur, it is not of primary importance to know how much shedding occurs, but whether it causes harm). Similarly if one or more steps in a chain of events leading to a negative effect can be ruled out, then the potential for realizing the negative impact would be considered negligible.

In an ERA, a range of scenarios should be examined from worst-case, where exposure and impact are expected to be highest, to more realistic situations (EFSA, 2010).

Step 2: Hazard characterisation

Hazard characterisation is about the evaluation of the potential consequences (harm), either as direct, or indirect, immediate and delayed effects. It involves the qualitative or, whenever possible and useful, quantitative description of the nature of the hazards and their respective accompanying uncertainties. It may also be described as determining the potential severity of adverse effects following exposure to a hazard, or the stressor-response assessment, or dose-response assessment when in relation to human health (Hill, 2005).

The estimation of the severity of the harmful effects is performed independently of the possibility of the harmful effect occurring (Commission Decision 2000/608/EC, Annex, 3.3). Often the effect caused by the GMO is compared with the effects introduced by its non-GMO counterpart (baseline principle).

Consequences may be the direct result of the introduced gene and its expressed product, or may be related to unintended effects that might be generated by *e.g*. the location of the insertion or other interaction with the organism's metabolism.

Elements to consider would be:

- Health status (healthy versus immunocompromised persons, infants, elderly people);
- Type of disease, prophylaxis, therapies;
- Genetic transfer capability of the GMO;
- Effects on target and non-target organisms (including personnel that are involved in the study and in the treatment);
- Effects on soil, water, air, individual ecosystems.

Evaluation of the potential consequences preferably is made quantitative, but most often the 'magnitude' is described qualitatively; 'severity' can be 'high', 'moderate', 'low' or 'negligible'

('ordered categorical description', EFSA, 2010). Commission Decision 2002/623/EC further clarifies the descriptions for hazards to the environment:

- **High level consequences** might be significant changes in the numbers of one or more species of other organisms, including endangered and beneficial species in the short or long term. Such changes might include a reduction in or complete eradication of a species leading to a negative effect on the functioning of the ecosystem and/or other connected ecosystems. Such changes would probably not be readily reversible and any recovery of the ecosystem that did take place would probably be slow.
- **Moderate consequences** might be significant changes in population densities of other organisms, but not a change, which could result in the total eradication of a species or any significant effect on endangered or beneficial species. Transient and substantial changes in populations might be included if likely to be reversible. There could be long-term effects, provided there are no serious negative effects on the functioning of the ecosystem.
- **Low level consequences** might be non-significant changes in population densities of other organisms, which do not result in the total eradication of any population or species of other organisms and have no negative effects on functioning of the ecosystem. The only organisms that might be affected would be non-endangered, non-beneficial species in the short or long term.
- **Negligible consequences** would mean that no significant changes occur in any of the populations in the environment or in any ecosystem.

When collecting documentation a tiered approach should be deployed. *E.g.* when determining the eco-toxicological properties of a gene product, first a desktop study (including *in silico* searches) and a basic set of biochemical characterisations are performed. If there are reasons for concern, controlled laboratory tests may be required to study the effect on organisms representative of the intended environment. These tests would typically be designed as a worst-case by directly exposing the test organism to a high dose of the gene product. Finally, if in these worst-case tests a harmful effect was noticed, then further safety tests under more realistic conditions could be justified.

Step 3: Exposure characterisation

Parallel to the hazard characterisation an assessment of the likelihood of occurrence of particular adverse outcomes or chance or probability of a harm being realized is performed. Although quantification of the exposure is preferable as a relative measure of probability (from zero to one, where zero represents impossibility and one certainty), this is not always possible. Again, an ordered categorical description (such as "high", "moderate", "low" or "negligible") can be used. However, an indication should be given of the range, within a numeric scale of 0 to 1, to which the term is intended to refer (link with probability). For example, "the likelihood of exposure of a healthcare worker to the investigational medicine when administering to the patient was estimated to be moderate, where 'moderate' in this context means within the range 0.1 to 0.4".

Some elements to consider are (not exhaustive):

- Exposure of staff and the broader human population (general public routes of biological dispersal or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact etc.);
- Likelihood of escape (number of GMOs proposed to be released with each use and the frequency of usage);
- Specifics of the ecosystem where dissemination could occur;
- Likelihood of excessive population increase (persistence, invasiveness, competitive advantage etc.); and
- Likelihood of post-release shifts in biological interactions or in host range.

It is important not to confuse exposure with hazard. *E.g.* shedding should be seen as a route of exposure and transmission, and is not a hazard *per se*. Shedding studies may be performed in animal models and CTs as a first step. Analytical methods should be able to distinguish between biological material capable of triggering an effect (*e.g*. causing an infection, replication, transmission, …) and other material (*e.g.* dead cells).

Data from non-clinical shedding studies are useful in guiding the design of clinical shedding studies, particularly as to sample types, sampling frequency, and duration; but are never a substitute. The difficulty is to choose an animal species in relation to permissiveness, immunity etc. As an example, the use of a tumour-bearing model might be appropriate to support the replication of oncolytic viral products. The impact of immunity to the virus / vector should be taken into consideration as this can affect the rate of viral/vector clearance and therefore shedding and transmission.

Samples most commonly examined include urine and faeces, but also buccal swabs, nasal swabs, saliva, and bronchial lavage. Samples should be taken long enough to detect a secondary peak for replication competent virus in case of persistence in certain tissues. Latency or reactivation may be a concern.

Step 4: Risk characterisation

Risk characterisation is the qualitative or quantitative estimate of the probability of occurrence and severity of adverse effect(s) or event(s) under defined conditions based on hazard identification, hazard characterisation and exposure assessment, including the attendant uncertainties (SSC, 2000). If it is certain that an event cannot occur, it is given a probability of 0; if it is certain that it will occur, it is given a probability of 1.

An estimate of the risk of adverse effects should be made for each hazard identified in the first step. The assessment of the level of risk posed by each identified hazard obtained by combining the magnitude of the consequence of each identified potential adverse effect with the likelihood of its occurrence may be presented in a matrix (Table 1).

Table 1 Example of how the magnitude and likelihood for a specific adverse effect may be combined to yield relative estimates of risk. Outcomes not presented in bold present are provided for illustrative purposes only and should be evaluated on a case-by-case basis. (Source: EMA, 2005)

	Likelihood of occurrence of adverse effect			
Magnitude of adverse effect	**High**	**Moderate**	**Low**	**Negligible**
High	**High**	High	Moderate	Negligible
Moderate	High	Moderate	Low	Negligible
Low	Moderate	Low	Low	Negligible
Negligible	Negligible	Negligible	Negligible	**Negligible**

Whenever the magnitude of an adverse effect is negligible and the likelihood of it happening is negligible, the resulting risk is negligible. Likewise, a high likelihood of occurrence of a highly adverse effect means a high risk. However, situations may occur where the outcome is not that clear and a case-by-case judgement may then be required; *e.g*. when a possible adverse effect of an GMO is irreversible, the risk level may not be negligible even if it is estimated to happen very seldom. The result will depend on the circumstances of the case and the weighting of certain factors. These cases therefore require an expert judgement and are often propounded for advice. Also, the overall uncertainty for each identified risk needs to be described.

Often it is hard to get a full overview of and characterise precisely all risks that are involved. In performing the ERA several levels of uncertainty may arise. Scientific uncertainty may find its origin in the tests that are conducted, the samples that are taken, the concepts and models that are used etc. Also, it may be a consequence of controversy on existing data or lack of some relevant data. Uncertainty may exist on qualitative or quantitative elements of the analysis (Commission Decision 2002/623/EC). However, attempts should be made to indicate the level of uncertainty. In a GMO application the discussion on this point may be about assumptions and extrapolations made at various levels in the ERA, different scientific assessments and viewpoints, the known limits of mitigation measures, and the limitations of the conclusion that can be derived from the data.

As uncertainty may arise at any step in the ERA, the estimate of uncertainty, once discovered, logically carries through the subsequent steps to the determination of the overall risk associated with the activities of the GMO. On the other hand, only those uncertainties should be highlighted that have a real impact on the ERA. *E.g*. the exact frequency of a recombination event may not be critical, if it can be concluded that the recombination does not result in a negative impact.

Assessing worst-case scenarios is another way to deal with uncertainty. In cases where insufficient data are available and the effort and cost for supplemental studies would be disproportionate, a scenario analysis based on theoretical assumptions may be used. In a worst-case scenario the assumptions for which no quantitative data are available, for example assumptions on exposure, are maximized. If the worst-case approach does not lead to a conclusion that the risk is not negligible, then a 'less than worst-case' would also not lead to such a conclusion (EMA, 2008a). If, for instance, infection of people is assumed, but no disease can develop due to vaccination, the effect will be negligible. Then no information on shedding or the likelihood of shedding leading to infection would be needed.

In an environmental risk characterisation, it is not allowed to balance the potential benefits of a GMO application against the risks its use may present. Benefits may be taken into account at a later step (conclusion of the risk assessment and/or decision-making).

Step 5: Risk management

Risk management should result in controlling identified risks and addressing remaining uncertainties. Management measures should be proportionate to the level of risk or uncertainty.

Control measures will only serve their purpose when their efficacy has been demonstrated. Working procedures, decontamination procedures, equipment etc. need to be validated for their protective and containing ability. In contained facilities, the biosafety cabinet (BSC) is the most studied equipment (CGM/2008-02).

Although all EC Member States have national legislation based on the same EC Directives the containment measures as required by the authorities may be different from one country to

another. Pauwels *et al*. (2009) provide an example by comparing conditions for activities with lentiviral vectors.

Step 6: Overall risk evaluation and conclusion

The final step is the overall conclusion concerning risks on the proposed activities. It is important to note that the acceptability of a risk is a decision to be made by the competent authorities, not of the risk assessors. Information on the potential benefits of a GMO may be important when evaluating the acceptability of a risk.

Monitoring

Delayed effects in particular may be difficult to determine, especially if they become apparent only in the long term. Appropriate measures such as monitoring can help in detecting these effects. Also, uncertainties or assumptions made in the ERA may require monitoring to improve knowledge. The ERA provides the basis for monitoring plans that focus on adverse effects on human health and the environment.

3 COGEM experience

The COGEM in its advisory role has published several advices and reports that include indications on the ERA process in medical and veterinary biotechnology. Either of a general nature or addressing very specific cases, all are based on the European ERA guidance. Some are specific for CU or DR. Figure 2 provides a schematic representation of the evolution of the number of advices per year (see also References for detailed listing).

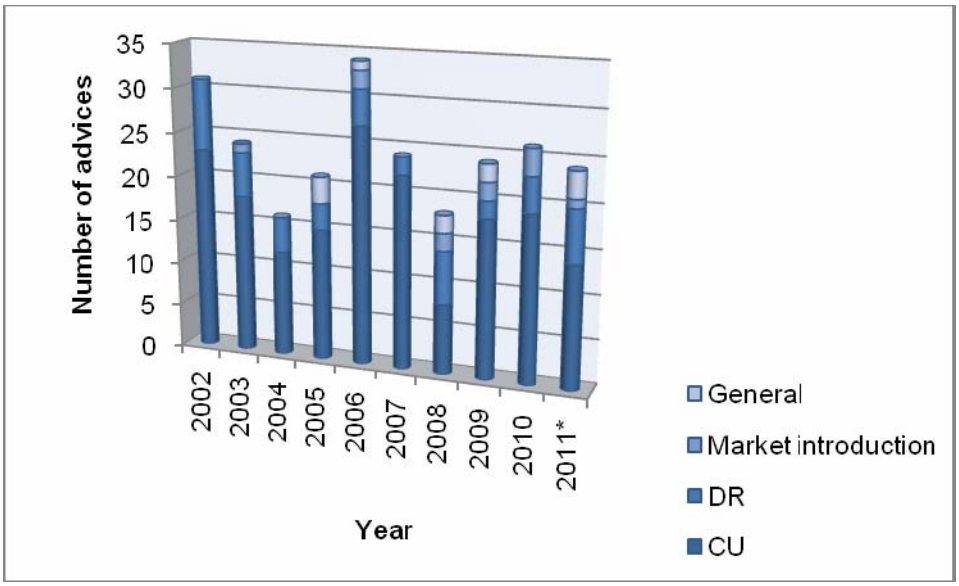

Fig. 2 Evolution of COGEM advices relevant for ERA in medical and veterinary biotechnology (* up to and including October)**.**

In this section the indications provided in these advices are elaborated based on the stepwise ERA approach presented before.

3.1 ERA for Contained use

During early stages in biotechnology research and development, many of the characteristics of a new GMO may not yet have been fully characterized and are considered 'unknowns'. As a precautionary measure, they may require containment to protect the workers, the environment and the wider public. In other cases, *e.g.* when working with specific genetically modified pathogens, the hazards may be fully understood and containment is a prerequisite for safe handling.

In 'contained use' the GMOs are contained at various levels by safe working practices (safe microbiological practice and good occupational safety and hygiene), biological containment and physical containment (equipment, lab facilities). The characteristics of the organism and the actual working procedures are taken into account in the risk analysis.

The ultimate result of the risk assessment according to Annex III Sections A and B of Directive 2009/41/EC following all 4 steps (hazard identification, characterisation, exposure assessment and risk assessment) leads to a classification of the activity in one of the 4 risk classes:

- **Class 1** Activities of no or negligible risk, that is to say activities for which level 1 containment is appropriate to protect human health and the environment.
- **Class 2** Activities of low risk, that is to say activities for which level 2 containment is appropriate to protect human health and the environment.
- **Class 3** Activities of moderate risk, that is to say activities for which level 3 containment is appropriate to protect human health and the environment.
- **Class 4** Activities of high risk, that is to say activities for which level 4 containment is appropriate to protect human health and the environment.

According to Directive 2009/41/EC (Annex III) the following ERA elements have to be determined:

- Identification of any potentially harmful effects of the organism: recipient – donor organism and vector – insertions;
- characteristics of the activity;
- the severity of the potentially harmful effects;
- the likelihood of the potentially harmful effects being realised.

This leads to the identification of the level of risk associated with the GMO. On this basis the selection of containment and other protective measures is made taking into account the environment likely to be exposed, the activity and any non-standard operations. This results in the assignment of the activity to one of the above classes. The final classification of the contained use should be confirmed by reviewing the completed assessment.

As mentioned before, Annex 5 '*Inschaling van activiteiten met genetisch gemodificeerde organismen' of the 'Regeling genetisch gemodificeerde organismen*[2] already combines information on the GMO with the measures to take in order to manage the risks. While the hazards (step 1), their severity (step 2), the probability of occurrence (step 3) and the related risks (step 4) are not explicitly mentioned, the assignment of the level of protection measures (step 5) for each type of GMO is based on the experience the COGEM has accumulated over many years.

While the classification may be applied to most standard activities, each application submitted for advice is reviewed case-by-case and supplementary measures may be imposed (Art.7.2 of the 'Regeling'). Indeed, the type or scale of the activity is not included in the schematic risk assessment of Annex 5. As a result the exposure assessment is not fully covered and the final risk class of the activity still needs to be evaluated. This may lead to a higher or lower protection level for certain activities.

Activities ('*handelingen*') can mean culturing, storing, putting at someone's disposal, applying, having available, transporting, disposing of or destroying GMOs (§1 of the '*Regeling*'), and, of course, performing experiments. It is obvious that the likelihood of exposure of an employee to a well-packaged and stored GMO is much smaller compared to the likelihood of exposure in case the culture medium of a culture of the same organism is refreshed. Therefore also the risk of storage is lower and so is the required safety level.

The basic set of containment measures (requirements for facilities and equipment, working procedures) for the different protection levels are listed in Annex 4 of the '*Regeling*'. As a rule-of-thumb GMOs of risk class 1 are handled in ML-I and DM-I, risk class 2 organisms in ML-II and DM-II, etc. However, depending on the outcome of the ERA, the COGEM advice may deviate from this general rule. The requirements as described in Annex 4, though, are focussing on the protection of human health and the environment. Storage and transport of GMOs are dealt with in Annex 7 and 9 resp.

[2] http://bggo.rivm.nl/Paginas/doc-reg.htm

Often the description of the GMO and its characteristics is put in relation to the unmodified organism. Therefore the ERA starts with information on the recipient organism. For those cases where classification of the unmodified organisms is not yet performed, Directive 2000/54/EC or other international or national classification schemes should be used. Since classifying microorganisms in pathogenicity classes is not within the scope of this report, it will not be further elaborated.

The combination of the characteristics of the recipient and donor organisms, the type of the inserted material, and the vector that is used determine the final properties of the GMO. Not only changes as a result of the genetic modification or any other change in the properties of the organism are considered, but also the properties unrelated to the modification must be taken into account.

The potential harmful effects (hazards) that are envisioned are (Directive 2009/41/EC, Annex III):

- disease to humans, including allergenic or toxic effects;
- disease to animals or plants;
- deleterious effects due to the impossibility of treating a disease or providing an effective prophylaxis;
- deleterious effects due to establishment or dissemination in the environment;
- deleterious effects due to the natural transfer of inserted genetic material to other organisms.

3.1.1 Step one: Hazard identification

The properties of the GMO (based on information of the recipient & donor, vector, insert and result of the genetic modification) and the activities with the GMO (type and scale) can be used to develop an inventory of hazards.

1. Pathogenicity of the recipient organism
   The following considerations have been included in COGEM evaluations:
   - **Pathogenicity class**
     Organisms are classified in 4 pathogenicity classes. Class 1 is not pathogenic. A list of these organisms regarding pathogenicity in relation to humans is included as Annex 1 to the '*Regeling*'. Class 1 organisms may be handled at containment level I (ML-I) if combined with safe vectors and insert (see below). *E.g.* most of the *E. coli* lab strains belong to Class 1. The list *'Pathogene (micro) organismen en agentia*'[3], linked to the '*Regeling*' contains class 2 to 4 organisms. For animal pathogens the COGEM has elaborated the classification and containment requirements based on the dissemination route, survivability, infectiousness, mortality etc. (CGM/060420-04; CGM/100712-05).

   - **Attenuation**
     Apart from the standard list of organisms, modifications may be made to attenuate the organism. The mechanism may be known (*e.g.* via deletions) or the nature of the attenuation may not be well understood. Furthermore, if the recipient organism is an attenuated form of a pathogenic organism, then environmental factors may cause reversion under selection pressure. Hence, the stability of the attenuation should be shown.

   - **History of safe use**
     Moreover, the wild-type or parental organism may have a history of safe use, *e.g.* virus strains that are used for vaccination.

   - **Biological containment**

[3] http://bggo.rivm.nl/Paginas/doc-ig.htm

The recipient organism might be biologically contained. Auxotrophs are typical examples of microorganisms that can only survive in media with certain supplements. Also animal cells cultured *in vitro* will not survive outside the culture flask or the bioreactor.

- **Purity**
  For primary cells, cell lines, lines that are established, immortalised, inactivated etc. a description on how these cells were obtained and checked for presence of other organisms, specifically pathogens and organisms that may interact with the GMO, should be considered. Validation of the analytical methods should be provided. Also the purity and correct identity of the GMO are highly relevant (*e.g.* purity of bacterial strains).

2. Vector

The vector is the vehicle that is equipped to deliver 'foreign' genetic material to a cell. Vectors already judged to be safe are listed in Annex 2.1 of the '*Regeling*'. These vectors allow activities on the lowest containment level (ML-I).

Vectors often consist of a plasmid. Vector plasmid maps should be included in an application dossier with details on genetic elements (*ori*, genes, viral sequences, promoters, enhancers, markers, …), restriction sites, sizes etc.

Vectors may be derived from pathogenic organisms such as a virus. These viral vectors use the viral transfer mechanism but are (usually) not, or no longer, able to spread or cause disease by themselves. For viral vectors traits to be checked are:

- **Integration competence**
  Although integration is often envisioned in developing vector systems, some viruses, *e.g.* retroviruses, might be oncogenic or causing malignant disease, either by insertional mutagenesis of the host chromosomes, the transactivation of neighbouring genome sequences or as a result of having acquired host oncogenes.

- **Replication competence**
  If replication is no longer possible this should be demonstrated (*e.g.* in a permissive cell system). Virus vectors derived from oncolytic viruses may be intended to propagate selectively in certain target tissues, but would cause harm in other cells. Information on how selectivity is obtained and tested may therefore be required.

- **Mobilisation competence**
  Once integrated in the host's genome virus vectors may be mobilised due to complementation by a wild-type or related virus.

3. Insert and its product(s)

Annex 2.2 of the '*Regeling*' lists the sequences that are not suitable for the creation of GMOs of group I.

Ideally the function of the insert is well known. Gene functions and products must be discussed. If the insert is not well characterised, the characteristics of the donor organisms are important. The reasoning is that deleterious products may be produced from inserts originating from pathogenic organisms. In assessing the safety of the uncharacterised insert, the pathogenicity class of the donor will be deciding. Mutagenesis, on the contrary, most often leads to a decrease in fitness and/or virulence.

Sometimes not enough information is available. For example, when chimeric viruses are used with sequences interchanged between related viruses, the resulting pathogenicity may not always be

predictable (see also case study in Annex). Indeed, recombination between viruses in nature is a way to increase virulence and change transmission routes.

On the other hand insertions may be used to interrupt a gene function, *e.g.* a toxin producing or a virulence gene. In such cases, genetic instability or recombination is important as it may result in a reversion to the wild-type organism.

For inserts known to produce a harmful substance data on toxicity (LD_{50}) and allergenicity should be provided, if possible. How the lethal dose is determined should be explained. Toxicity classes are described in the '*Regeling*' Art.1. Otherwise, a description that would allow for a classification should be given.

4. Result of the genetic modification

Characteristics of the GMO will be evaluated in comparison with the parental or non-GMO. The resulting GM viruses (virus vectors) or bacteria may show:

- **Altered pathogenicity**
  Pathogenicity is influenced by different characteristics of an organism. Any change that could lead to a change in pathogenicity may require adapting the classification of the organism. This is however not always possible as the effect of the change may be hard to predict.

- **Replication/reproduction capacity**
  Usually virus vectors are made replication incompetent. However, for certain applications replication is a requirement, even if only conditionally.

- **Genetic and phenotypic (in)stability**
  Especially attenuating mutations that might revert need attention. Data on mutation frequency, number of generations/cycles without change as demonstrated by molecular means may support stability statements.

- **Altered susceptibility to prophylaxis and therapy**
  Deletion of functions or point mutations may lead to resistance to antibiotics or antivirals. *In vitro* studies to show efficacy of existing therapeutics may provide clarity on this potential hazard.

- **Unintended (gene) transfer**
  Gene transfer is not a harmful process by itself. Whether this presents a hazard is influenced by i) the conditions, *e.g*. scale and dose, of the release, ii) the availability of susceptible hosts for the GMO, iii) ability of the GMO to replicate and iv) the potential for recombination of the genetic material within the GMO, as will be explained further on. In this respect, it should be noted that for CU, in general, no self-transmissible vectors are allowed at het ML-I containment level.

- **Dissemination, survival and colonisation**
  In addition to direct exposure, dissemination may lead to spreading beyond the location of application, establishment in the environment and subsequent exposure of susceptible targets (human or animal).

  To be able to spread in the environment or to infect humans or animals several functions are needed. Viruses are often restricted to certain host species or cell types in their ability to multiply (biological containment). A summary on properties and built-in biosafety features of the derived virus vector needs to be given. An example may be preventing the building of the protective protein layer around the viral particle that would severely reduce survival outside the host cells and consequently dissemination.

Successful dissemination and establishment will largely depend on the ability of the organism to survive, compete and replicate. An evaluation of the capacity to survive and colonize is based on a thorough understanding of the limiting factors and the characteristics of the GMO. *E.g.* animal and human cell cultures already are vulnerable to changes in their environment. They are biologically contained and do not survive outside the laboratory. Genetic modification most often does not change this. On the other hand, certain antibiotic tolerance genes may contribute to the ability to compete with other organisms, possibly leading to colonisation in a new environment.

5. Type and scale of activities
   Working in closed systems will obviously give less chance for contact with operators compared to 'open' handlings where inhalation or direct contact with skin or mucus is harder to avoid. This will eventually lead to different containment requirements.

   Research activities are usually small scale with small quantities of GMOs. Commercial production of vaccines may be large scale possibly resulting in large losses of virus particles in case of leakage. Bioreactors therefore may require large collecting tanks to prevent spread into the environment.

   Human clinical studies may be conducted in a hospital setting where patients remain in an isolation room with specific containment measures. Veterinary studies require a very different setting and can pose challenges in relation to handling manure and other waste materials.

   Non-routine activities trigger a more detailed assessment. Examples are cell sorting, flow cytometry and freeze-drying of samples containing GMOs, and PET-scan, CT-scan or MRI-scan of animals. Connecting tubes in bioreactors may induce aerosol formation. In microscopy when vessels need to be opened and infectious particles are still present, the operator may inhale aerosols carrying the pathogen. The description of the type and scale of use often already contains a proposal for the conditions with regard to biosafety. Efficacy of the containment measures needs to be demonstrated.

   Waste treatment is another activity potentially causing harm; *i.e*. if not completely inactivated GMOs may spread into the environment, or infect people and/or animals. Again, one has to validate the chosen inactivation method.

3.1.2 Step two: Hazard characterisation

The assessment of the severity of harm a GMO could cause, whether it would happen or not, leads to the allocation of the GMO to an initial containment class. The nature of the activities with the GMO will affect human and environmental exposure and therefore the possibility of harm occurring. A detailed description and analysis may be needed if procedures are non-routine.

The severity of a potential hazard 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'). Hazards may become apparent as direct or indirect, immediate or delayed effects. Direct effects are those that can be attributed to the GMO itself, indirect effects are the result of subsequent events.

Each hazard identified in the first step needs to be further characterised. Some examples of characterizations found in COGEM advices:

- **Altered pathogenicity**
  Different cases of alteration of pathogenicity have been put forward:

– Change in transcriptional control systems	A gene expressed in another host, other tissue, other developmental stage, at other concentrations may have different effects.
– Genomic instability	Point mutations might revert to the original sequence or even induce a higher fitness. When inserted sequences enlarge a viral genome to a size where it is not packaged efficiently, the genome can be prone to rearrangements.
– Altered immunogenicity profile, allergenicity	A decrease in immune response from the host may result in an organism causing disease. It is noted that in applications of gene therapy this may be a positive trait.
– Altered susceptibility to the immune system	Loss of immune evasion function may be a positive thing, but it might also induce increased inflammation. Also, genes encoding immune modulatory functions that are not native to the parental organism might affect pathogenesis.
– Altered host or cell tropism	Tropism refers to specific host species, or specific cell types within those species that are targets for infection by a virus or a pathogen in general. A change in tropism could redirect or enlarge the scope of hosts/cell types that may be infected and thereby change the pathogenic characteristics of an organism. Change of cell or host tropism might be intentional or unintentional, new or extended. It can be the result of changes in receptor binding, but also in changes in susceptibility to host response mechanisms. The consequences of a change in host tropism may be far reaching. Successful jumps from one species to another happen only occasionally in nature, indicating that it is generally an unlikely event. However, once it has occurred it may have severe consequences, as is the case for SARS, HIV and avian influenza.
– Insert producing a toxin	Toxin genes are sometimes intentionally introduced to destroy tumour cells. Depending on the characteristics of the vector and the type of the toxin, this may present a major hazard or not.

- **Replication/reproduction capacity**
  In case a (theoretical) possibility exists that a recombination renders a replication incompetent virus the capacity to replicate, the characteristics of that new organism will determine whether and how serious the adverse effects will be.

3.1.3 Step three: Exposure assessment

The concentration of a GMO and the scale (volume, frequency) of the cultures that are worked with influence the likelihood of exposure and should be considered. Also culture conditions (equipment, physical, chemical and biological containment) will influence the probability of exposure. Finally the receiving environment is of importance (presence of susceptible species, possibility to support survival of the GMO, shift in properties, balance). Waste treatment needs special attention as, if not properly managed, waste will be a major factor in exposing humans and the environment to the GMO.

Risk hypotheses attempt to clarify whether a specified harmful outcome is likely, whereas research hypotheses seek precise quantitative predictions. In risk assessment, one is usually testing a hypothesis that a particular phenomenon is unlikely, which is the equivalent of testing the hypothesis that *e.g.* the gene product has no effect on soil organisms. For hypotheses of this kind, laboratory studies are the most rigorous tests. Indeed if the effect is not seen under worst-case circumstances in the laboratory, it is even less likely to be seen in the field (Raybould, 2010).

Each identified hazard will be assessed for the possibility to become reality. Several factors influence the probability of exposure or likelihood of a hazard to happen. Proposed containment measures can already be taken into consideration in estimating the likelihood. The following considerations have been included in COGEM advices:

- **Loss of containment**
  Accidental exposure may be the result of an incident like breakage of containers, leakage from a bioreactor inherent to the physical construction or manipulations during culture.

  Single-use bioreactors are made from plastic instead of the traditional stainless steel constructs. Integrity depends on the strength of the material and the way sensors and peddles are fixed. Integrity tests are therefore desired. Starting a culture, taking samples and harvesting may introduce the danger for spillage.

- **Shedding**
  Exposure via shedding by treated humans or animals in containment depends on the type of vector, the dose, the application mode (intravenous, in tumours, via mouth or nose) and bio-distribution (presence in blood, faeces, urine, …).

- **Dissemination vector**
  The presence or absence of the organism responsible for the transmission of the GMO or spread into the environment (dissemination vector) influences the probability of further exposure.

- **Mode of transmission**
  This is an important element in estimating the probability of a hazard to occur. Potential infection via blood-blood contact may lead to extra measures to prevent, for example, needle stick injuries or to prevent contact with small wounds on gloved hands. Transmission via aerosol formation will require protection from inhalation. Absence of this transmission route may lead to a lower level of biosafety containment.

  Another example is infection by prions that does not occur via aerosols, but via food/feed or injuries. Infecting lab animals via injection therefore needs special attention to avoid needle stick injuries, scratching and biting. Analysis of prion-infected tissue, again, using sharps is considered very risky.

- **Mutation**
  The frequency of mutations that revert to, for example, a fully infectious virus must be assessed. Often literature on specific organisms is available, but studies may be needed to estimate the frequency of this kind of mutations.

- **Recombination**
  Homologous recombination between viruses only happens when they are present in the same cell and have overlapping sequences. The likelihood of this happening will define whether such recombination needs further investigation.

  In virus vector production, helper cell lines are often used to complement for functions that are absent in the virus vector. Without overlapping sequences with the vector recombinations will be very unlikely. An example is the advanced generation lentiviral vector systems where the different virus functions are separated into at least 3 plasmids. The more recombination events are needed to reassemble the complete virus the less likely this will happen; indeed, this has not been reported to date, neither during vector production, nor during experimentation with laboratory animals (CGM/090331-03; Pauwels *et al*., 2009). Similarly, homologous recombination and the formation of replication competent viruses can be prevented by proving

that the related wild-type viruses are not present in cells that will be transduced by a replication deficient vector. Conversely, endogenous viral sequences potentially present in non-established cell cultures need special attention.

It is good practice to demonstrate that vector stocks are devoid of replication competent viruses. Testing for the absence of replication competence should be done as part of routine analysis of each batch (EMA, 2008a). This should be mandatory if the risk assessment outcome is based on the replication defective nature of the vector. Each method needs to be sensitive, precisely validated and detection limits must be provided. Direct plating of vector stocks onto permissive cell lines and monitoring for indications of viral replication (*e.g.* cytopathic effects or syncytia formation) may be used to detect replication competent virus particles; however such approaches do not always give a clear result. Specific detection methods such as immunostaining or PCR are good alternatives (EMA, 2008a).

The COGEM emphasises the importance of adequate tests for presence of replication competent viruses in mammalian cells after transduction. Lack of expression of the insert is not conclusive, as the insert may have been deleted from the vector in replication competent virus particles. ELISA and PCR detection methods for respectively the virus envelop protein and DNA/RNA may be valuable tests for replication competent viruses. These tests may be omitted when enough evidence is provided that no virus particles are present in the experimental setting, for example due to the short half-live of the virus or due to absence of transmission modes.

After unintended release, recombination with wild-type is, again, only possible when the wild-type is present in the receiving environment. Similarly, the probability of recombination of a GM virus with a wild-type virus in an animal may be strongly reduced when the wild-type virus is not present in the country or when animals are tested to be free from the relevant viruses.

Illegitimate recombination may occur between DNA sequences that contain only a few identical nucleotides. Although there is only a remote possibility of it happening, it may nevertheless be considered in an ERA when very severe consequences are expected.

- **Uptake**
  In its advices on naked DNA applications the COGEM assesses the risk of spread into the environment. This might be possible when sequences are present in the naked DNA that allow for interactions with viral sequences in for example lab animals resulting in new, potentially infectious viruses that may then spread into the environment via shedding. Therefore, the likelihood of uptake by the cell and nucleus, the integration in somatic cells and the germ line, and finally shedding is evaluated.

  If the possibility for gene transfer to non-target organisms exists, the next step should be considered: the potential for expression of inserted sequences (*e.g.* eukaryotic versus prokaryotic genes).

Statements that organisms are *e.g.* “non-viable” or “replication incompetent” should be supported by a description of the test and resulting figures indicating the limits of detection.

3.1.4 Step four: Risk characterisation

As mentioned earlier the risk of a certain activity with a certain GMO is determined by a combination of the magnitude of the hazard it presents and the probability of its occurrence. For each of the identified hazards this risk characterisation needs to be made. The risk characterisation will lead to the assignment of the risk class of the activity. Some examples are presented in the risk matrix of Table 2 and are explained below.

Table 2 Examples of risk characterisation (based on COGEM advices; explanation see text)

	Likelihood of occurrence of adverse effect			
Magnitude of adverse effect	**High**	**Moderate**	**Low**	**Negligible**
High	**High** GM foot-and-mouth disease virus	High 'Open' manipulations with 1st generation lentivirus	Moderate	Negligible Cloning Foot-and-mouth disease virus genes in *E. coli*
Moderate	High	Moderate	Low	Negligible
Low	Moderate	Low Eye infection with an adenovirus	Low Development of Influenza vaccine	Negligible Production of *Vibrio* vaccine
Negligible	Negligible Shedding measles vaccine -vaccinated employees	Negligible	Negligible	**Negligible** Leakage of animal cells from a bioreactor

- **High risk**
  Foot-and-mouth disease is one of the most infectious animal diseases (pathogenicity class 4, contact and air transmission). For young animals the disease is often deadly and economic consequences are enormous (high magnitude of the adverse effect). Due to the high transmissibility (high likelihood for dissemination *e.g.* when laboratory personnel would inadvertently release the virus into the environment), GM activities with fully virulent virus are considered very risky resulting in an overall risk class 4 (CGM/060711-01).

- **Moderate risk**
  A lentiviral vector system of the 1st generation (pathogenicity class 3) has a moderate probability to revert to wild-type lentivirus as (only) two recombination events are required to result in a replication competent virus. Secondly, a replication competent virus would be able to infect a person via open wounds and integrate in the genome. The severity of the hazard would be high, as infection will lead to the development of AIDS, which can be treated but for which no cure is available. The risks as a consequence would be high, *i.e.* risk class 3 (CGM/090331-03).

- **Low risk**
  Recombinant adenovirus having a broadened host range may induce eye infections especially at the time of production (moderate likelihood). Adenovirus eye infections are relatively unharmful for the eye (pathogenicity class 2), but the virus is very infectious and might easily spread to other persons (low effect, moderate probability). Treatment is not available, but good hygiene is usually sufficient for recovery (CGM/060516-01).

  Operators involved in the development of a vaccine against influenza H5N1 (pathogenicity class 2) would run the risk of becoming infected with the attenuated strain thereby giving it the opportunity to recombine with endogenous influenza strains to become a more pathogenic organism. The frequency of infection is estimated to be moderate, that of recombination and of

the generation of a more virulent strain is estimated to be low, but the effect would be rather serious. As a whole the risk would be classified as low (risk class 2)(CGM/060224-01).

- **Negligible risk**
  GM *Vibrio cholera* (pathogenicity class 2) vaccine reverting to wild-type would induce diarrhoea, and when left untreated, results in death in 60% of the patients. For this to happen both the GM and wild-type need to be co-present. However, wild-type *Vibrio* is not present in the Netherlands (negligible likelihood, risk class 1)(CGM/110712-01).

  Shedding of an experimental, low virulence, live measles vaccine is of less importance when all employees are vaccinated. Transmission of this infectious disease would be estimated high (aerosols) but the effect would be negligible (CGM/110509-01).

  Cloning individual genes from the Foot-and-mouth disease virus in *E. coli* is assigned risk class 1 as the virus is not able to replicate in prokaryotic systems and genes are not expressed in prokaryotic systems. The likelihood of generating a fully virulent virus that spreads into the environment is therefore negligible (CGM/060711-01).

  Biological containment already might reduce the likelihood of a hazard from happening. Leakage from a bioreactor with biologically contained animal cells will not result in a major risk for the environment (CGM/101028-03).

Overall the evaluation is driven by a cautious attitude[4]. Dealing with uncertainty will often result in a relatively high-risk classification. Whenever there are no clear quantitative indications, worst-case scenarios may be developed as a guiding tool for addressing extreme situations, even if these are only theoretically conceivable. Unknown, newly discovered organisms, possibly pathogenic or where the severity of pathogenicity is not known, need to be treated with caution. Similarly, if data are not sufficient to demonstrate attenuation of a GMO compared to the recipient organism, the risk level will remain the same as for the recipient organism. The same is true for viral vector systems for which no adequate data are available to ascertain that replication competent viruses are not formed.

3.1.5 Step five: Risk management

The risk characterisation will be the basis for identifying management measures to conduct the activities with a particular GMO in a safe way. Most often they aim to reduce the likelihood of an adverse effect to happen and thereby making the risk negligible. Reducing the magnitude of the adverse effect is a less frequent option. For example it may be decided to replace an element in the gene construct, if that particular element is of concern, as part of the risk management.

There is a cascade of protective measures, both collective and personal. Biological containment, safe microbiological techniques and physical containment features are the main protective measures for CU. The containment measures prescribed in Directive 2009/41/EC are part of the management of the risks. Standard containment requirements at the different biosafety levels including working procedures are listed in Annex 4 of the '*Regeling*'.

[4] In this document the word "cautious" is used to refer to the attitude of the COGEM when conducting the ERA. This attitude should not be confused with the "precautionary principle", which refers to the basis for the GMO legislation as well as the considerations that may prevail at decision-making. While the ERA evaluation is an operational element of the implementation of the "precautionary principle". This evaluation is conducted in a cautious manner.

Reliability, possible failure rates of equipment and correct use will have a significant impact on the effectiveness of any containment measure deployed. While not a COGEM responsibility, it should be noted that containment measures need to be validated case-by-case, *i.e*. substantiating that the measures provide an adequate protection for the specific organisms in the form that it is worked with (*e.g*. the validation of waste inactivation). Also regular maintenance, verification and/or on-site testing of equipment and facilities are often standard (*e.g*. BSCs according to EN12469; for HEPA filters: EN 1822-1).

For most standard operations the containment requirements and working conditions as mentioned in Annex 4 of the '*Regeling*' will be adequate to protect humans and the environment. The COGEM may advise for extra measures to be taken to tackle specific risks. Specific requirements have been indicated in individual advices, including:

- **Preventing recombination**
  To avoid recombination resulting in replication competent virus particles several measures can be taken:
  - Spatial and/or temporal isolation of activities with different vectors.
  - Checking for absence of wild-type or related viruses in cell cultures or lab animals, before taking the next step in a virus vector experiment. While presence of wild-type virus may be shown via disease symptoms, often a specific test is advised. Detection limits and validation methods need to be set. Alternatively, in some cases it is possible to use animals or animal cell cultures that are no natural host for that particular virus.
  - Exclusion of staff that are carrying wild-type or related viruses from experiments (showing disease symptoms).

- **Checking for stability**
  If biological containment of a vector depends on mutations in certain sequences the conservation of the change needs to be checked. More in general, when the ERA is based on presence or absence of certain features of the GMO, batch control should be performed to confirm this state and to check for contaminants.

- **Preventing transmission**
  - Depending on the transmission mode of the organism PPE should be worn, like protective clothing, including gloves to minimize contact or a facemask, goggles or safety spectacles to protect the lab worker from aerosol transmissible pathogens.
  - Vector batch control to demonstrate that vector stocks are devoid of replication competent viruses.
  - Washing of cell cultures after transduction to dilute the number of free virus particles that are still infectious. This procedure is often combined with a trypsin treatment to inactivate the virus (coat proteins are cleaved).

- **Vaccination**
  Vaccination of employees is often advised when working with highly pathogenic viruses. In this way the magnitude of an adverse effect is reduced.

- **Waste management**
  Waste streams are important routes for dissemination into the environment. Here emphasis is put on preventing or reducing the adverse effect, *i.e*. inactivating the material in order to prevent it from having an effect. General rules for waste treatment are described in Annex 4 of the '*Regeling*'. Annex 8 describes how to store waste awaiting inactivation or transport for incineration. Extra requirements may be included in the advice, such as:
  - Collection and inactivation of faeces for organisms that can survive the gastrointestinal tract.

- The use of kill tanks after a process run in a bioreactor and in situ disinfection of the bioreactor or inactivation of single-use bioreactors as a whole.
- Specific methods (*e.g.* with NaOH) to inactivate waste containing prions, as many prion types are heath stable and resistant to many disinfectants.

- **Alternative containment level**
  Although risk characterisation will lead to a certain containment level, sometimes experiments can be designed in a way allowing for a changed containment level. Some examples:
  - For activities with GM Foot-and-mouth disease virus an ML-III facility with extra requirements (shower after work, no contact with susceptible animal species for 3 days) may be as safe as working in a ML-IV lab in relation to the protection of the environment, especially herds of susceptible livestock.
  - With extra measures equipment may be used that is often shared with other research groups or programs and that is at a lower than required containment level. Examples are testing to exclude the presence of replication competent viruses for 'open' manipulations or inactivation of the material before microscopic examination.
  - Using containers that are permeable to water vapour but not to microorganisms in a lyophilisation process may reduce exposure significantly.
  - Whereas medical masks are still necessary for working with air transmissible viruses like influenza in a BSC class II, a BSC class III does not require extra protection of mouth and nose.
  - Exposure from bioreactors may be reduced working in a closed system using *e.g.* sterile connection devices to add a culture or to take samples.
  - Animals may return to a lower level of containment as soon as GMOs are shown to be no longer present in shed material.

In the specific case of lentiviral vectors risk management is covered in detail in the COGEM report '*Inschaling laboratoriumwerkzaamheden met lentivirale vectoren*' (CGM/090331-03) including decision trees for correct assignment of the containment level.

Dealing with uncertainty may lead to a higher risk class, as explained before, and therefore more stringent containment measures. *E.g.* applications or studies where genes and sequences are interchanged among virus strains might lead to a more virulent strain. The COGEM therefore advises to work with these chimeric viruses for example on an ML-III level, instead of ML-II that would have been required for the wild-type virus.

Likewise, unknown, newly discovered organisms, possibly pathogenic or where the severity of pathogenicity is not known, need to be treated with caution. Sometimes ML-III containment is recommended. Sometimes ML-II is sufficient, but, although related organisms may be moderately pathogenic, extra requirements like working in a BSC class II and wearing gloves to prevent transmission to the laboratory personnel may be proposed.

While the role of the COGEM is limited to advising on appropriate containment measures, the Minister of Infrastructure and the Environment (IenM) eventually decides on the measures that need to be implemented.

3.1.6 Step six: Overall risk evaluation and conclusion

In this step the overall risk is considered taking into account the risk management requirements. The final classification and containment measures are determined taking into consideration all potential harmful effects that were reviewed and the control measures following from the initial classification. Then the level of human and environmental exposure should be reassessed.

The COGEM then advises the competent authority, *i.e*. the Minister of IenM, on the outcome of the ERA, which will inform the decision-making process. Based on this general conclusion, the Minister may issue a permit with or without extra conditions or a denial.

3.1.7 Monitoring

The COGEM may advise for monitoring in relation to 4 different objectives:

- as confirmation that the imposed measures and conditions are adequately addressing the identified risks,
- as management tool to decide on reduction of containment measures (*e.g.* to determine whether studied animals may return to conditions without containment),
- as alert system to enable quick response in case an adverse effect would occur, or
- as source of scientific information that may be useful for future experiments (*e.g*. collecting shedding or bio-distribution data).

3.2 ERA for Deliberate release

Deliberate Release (DR) covers both experimental stages as well as placing on the market, the common factor being that there are no (or less) measures for limiting exposure of the public and the environment. Typically, in the course of the development of a GMO before and during the DR, information is gradually gathered about the characteristics of the GMO. The required containment may therefore become less stringent as one becomes more confident on the properties of the GMO in particular if it is confirmed that the potential of causing harm is very limited. Differences in the ERA may result from differences in existing data, time-scale and area of release (Commission Decision 2002/623/EC).

Whereas COGEM advises in CU dossiers only when one or more aspects do not allow standard classification as in Annex 5 of the '*Regeling*', for DR every submission is evaluated case-by-case.

The basic data to start from are not different from the requirements in case of CU of the organism. The information to be considered will merely cover the same as for the CU, but more 'unknowns' will need to be clarified in order to remove containment conditions. Consequently, the receiving environment becomes increasingly important and therefore more data on the sites where the GMO is to be released, the conditions and the scale of the release are needed. Information needs to be provided on:

- the recipient or parental organism(s);
- the genetic modification(s), be it inclusion or deletion of genetic material, and relevant information on the vector and the donor;
- the GMO;
- the intended experiment or use including its scale;
- the potential receiving environment; and
- the interaction between these.

The ERA for a DR follows the same steps as for CU activities and all the points presented before remain valid for the ERA. However, as release inevitably leads to some form of exposure the evaluation of the impact on human health and the environment is now emphasised. The safety of the patient (human or animal) in a CT, being covered by other legislation, is not addressed in the ERA. Likewise, no risk-benefit balancing in applying the GMO is made.

General information on the wild-type organism and the genetic modification will lead to the identification of potential hazards. The biological properties of the GMO are the starting point to define later management, in particular if some type of biological containment mechanism would limit possible effects and/or their occurrence. A list of items to be addressed can be found in the application forms and the guidance provided by the EU (Commission Decision 2002/623/EC; European Commission, 2006). Obviously, not all questions will be relevant in each case.

In case of virus vectors used in gene therapy the information should include: host-range, pathogenicity, bio-distribution and persistence, tissue tropism, cell lysis and lateral spreading, horizontal and vertical transmission, physical and genetic stability, availability of treatment of the parent organism etc. The genetic modification may change aspects of the viral life cycle (*e.g*. entry, transcription, translation, assembly, replication, release, cell tropism, transmission) and/or the interaction with the host (*e.g*. immunomodulation, apoptosis, extracellular and intracellular signalling, pathogenesis, recombination and adaptation) possibly leading to hazards for the environment (RIVM, 2008).

Often the characteristics of the GMO have already been described in support of the CU ERA when developing the material and experimenting in animal models. These items will not be repeated here (reference is made to section 3.1.1 of this report). Safety data on cell culture experiments, biosafety studies on shedding, persistence, etc., assays to demonstrate that the GMO is not capable of replication, dissemination or transmission, animal studies, information from comparable studies with related organisms and other observations may contribute to the ERA of CTs or veterinary studies.

As already put forward, the hazards possibly caused by a GMO are a consequence of the properties of the GMO. On the other hand, their magnitude is depending on several factors. If dissemination or spread into the environment leads to a negative impact, then evaluating the capacity to spread into the environment will take into account not only the biological fitness (*e.g.* viability of spores as survival forms), but also the conditions of the exposed environment like temperature, humidity, presence of susceptible organisms, presence of dissemination/transmission vectors, etc. For that reason, special attention will be given to the receiving environment in which the GMO is to be released (site-by-site assessment, region-by-region assessment, …).

For example in a CT setting, these consequences are influenced by the genetic constitution of the GMO, the exposed environment (*e.g.* hospital, stables), the health status of those likely to be exposed (*e.g.* caretakers, non-treated animals in the same herd), the method of administration and the frequency of use of the GMO. Consequences may arise directly (*e.g.* via infection of a family contact with virus shed from a target patient treated with a gene therapy product) or indirectly (*e.g.* via infection of an animal species by a novel organism originating from recombination of the GMO with a wild-type strain of the same species or genus), and the effects may be immediate (*e.g.* allergenicity) or delayed (*e.g.* oncogenicity) (EMA, 2005). Also cumulative long-term effects due to continuous or repeated introductions (as with commercialisation) have to be taken into account.

All possible, realistic scenarios of sequences of events are used to determine the points at which exposure occurs and hazards might be realized. For each of them the stepwise ERA is applied.

3.2.1 Step one: Hazard identification

The potential harmful effects that are envisaged are (Directive 2001/18/EC, Annex II):

- disease to humans including allergenic or toxic effects;
- disease to animals including allergenic or toxic effects;
- effects on the dynamics of populations of species in the receiving environment and the genetic diversity;
- altered susceptibility to pathogens facilitating the dissemination of infectious diseases and/or creating new reservoirs or vectors;
- compromising prophylactic or therapeutic medical, or veterinary treatments.

A distinction is made between harmful effects and mechanisms through which these may occur. Such mechanisms include:

- spread of the GMO(s) in the environment;
- transfer of the inserted genetic material to other organisms, or the same organism whether genetically modified or not;
- phenotypic and genetic instability; and
- interactions with other organisms.

The inventory of hazards can be developed on the basis of properties of the GMO (recipient, donor, vector, insert) and the activities with the GMO (type and scale). At this stage it is important not to discount any potential adverse effect on the basis that it is unlikely to occur.

1. Pathogenicity of the recipient organism
   As already mentioned for CU the recipient organism may be pathogenic or strongly attenuated, may have a history of safe use, the population may have built up immunity to it, etc.

   In describing the recipient organism for DR more focus is put on properties such as the natural host, environmental niche, dissemination, survival, natural reproduction, exchange of genetic material, etc.

2. Vector
   For vector plasmids or viruses it is important to characterise in detail the replication (in)competence, the possibility to recombine or revert to wild-type, and the mobilisation and integration competence. These properties determine for a large part the likelihood for hazards to occur. All vector features and their function are presented on a schematic map. Helper sequences, complementation potential and description of genes and function should be discussed.

   Some examples of routes for potential adverse effects mediated by the vector characteristics:
   - Virus vectors are usually made replication incompetent while the missing functions are complemented *in trans* on one or more helper plasmids. During the production process in cell lines recombination may generate replication competent viruses next to GM virus. To harvest the virus particles the cells are lysed and the lysate is then purified, but the presence of replication competent viruses cannot be excluded.
   - Non-human lentiviruses, incapable of infecting human cells, may become infectious by pseudotyping the viral particles and/or altering viral promoter sequences. In pseudotyped viruses, the natural envelope gene is replaced by one from an unrelated virus.
   - Conditionally replicating viral vectors may have several therapeutic advantages, as they only replicate in target cells and not or to a lesser extent in other cells. This restricted replication ability must be demonstrated.
   - Plasmid DNA as such may be used as a therapeutic. Plasmids can be replicating or contain integration enhancing elements or elements to improve recombination or uptake by microorganisms etc. In replicating plasmids the incompatibility category is important.

3. Insert
   The insert by itself may present a hazard when expressed beyond the intended aim.

   Examples:
   - An insert may encode a cytotoxic substance, as often is the case for trials in cancer research. Alternatively, the insert encodes an enzyme able to generate such a substance. If not properly controlled, this may affect cells in other persons.
   - An insert meant to induce an immune reaction against certain target cells, may, when spread to and internalise into non-target cells (in other persons), lead to an auto-immune response.
   - Antibiotic resistance genes may be transferred to commensal organisms or microorganisms in the environment, possibly outcompeting natural populations.

4. Result of the genetic modification
   New properties of the resulting GMO need to be discussed in as much as they may indicate a changed hazard, *e.g.* pathogenicity, genetic stability, host range, cell and tissue tropism, routes of dissemination, survival outside the host, persistency, invasiveness, selective advantage, competition, many microbiological, molecular biological, biological, physiological, pharmacological, and ADME (adsorption, distribution, metabolism, excretion) effects, efficacy of available therapies, interaction with other organisms, effects on soil, fish and aquatic organisms, etc.

Inserted sequences may not be stably integrated in the GMO. As part of the validation of the production process evidence should be provided that the GMO is stable throughout the manufacturing process to the finished product and that the integrated sequences have not undergone any rearrangements or mutations.

The resulting GMO may be attenuated in comparison to the wild-type organism. Based on the mechanism assumptions can be made on the stability of the attenuation. Mechanisms include:

- **Altered cell tropism** A virus may be modified in its coat protein in such a way that less cell types can be entered compared to the natural virus.
- **Survival dependency** Inserting the gene of interest in an essential gene (*e.g*. enzyme in a biosynthesis pathway) increases dependence of the GMO on laboratory growing conditions. The GMO cannot multiply in the natural environment and does not colonise (survival, dissemination, competition).
- **Altered multiplication** Tumour cells (autologous) that have been modified to elicit an immune response are irradiated to prevent multiplication in the patient's body.
- **Altered mobilisation** Self-inactivating (SIN) vectors lost their ability to mobilise from the genome they have integrated in.
- **Altered replication capacity** A conditionally replicative virus may replicate in a restricted number of cells types, resulting in a virus that is more attenuated than the parent virus

5. Type and scale of activities

A description of the intended release, the potential receiving environment and the interaction between the two, will also determine whether a hazard might be present. Experimental treatment in a hospital or ambulant, in animal housing or in the open (meadow) will define the receiving environment. The amount of GMO to be used, the frequency of use will influence the likelihood of an effect.

The number of patients in a CT (or animals in veterinary trials), mode, dose(s) and timing of application, may influence the recombination/replication probability, the bio-distribution, persistence and shedding of the experimental drug. Also activities as taking samples, their frequency and the type of tissue that is sampled possibly containing GMOs or not, will determine the exposure likelihood. The production process of the therapeutic and quality checks (batch control) will give an idea of the possibility of complementation and recombination. Also storage, transport and waste disposal (or disposal of excess material) should be considered properly for exposure potential.

Also, the immune status of the patients is relevant, or in general, inclusion and exclusion criteria, as well as criteria for patient discharge after the trial. Other treatments that are combined with the investigational medicine may have an effect and need to be taken into account (*e.g*. extra antibody to stimulate the immune response). Emergency procedures, *e.g.* in case unexpected surgery is urgently required, need to consider points of exposure. In such case the patient will have to leave his hospital room for the operation theatre possibly exposing staff and visitors to the GMO, for example via coughing or sneezing during transfer.

3.2.2 Step two: Hazard characterisation

As in CU applications all identified hazards, potentially leading to a direct or indirect, immediate or delayed effect, are further evaluated. The severity or magnitude of the identified adverse effect should be discussed whether it could become reality or not. Whenever consequences for human health are suspected, the impact will generally be considered serious. Many, such as lethality, infertility induction,

teratogenicity and oncogenicity, will inevitably be rated as of high magnitude. The level of uncertainty associated with each estimate of magnitude should be stated (EMA, 2005).

- **Medical research**
  Some examples from CTs may illustrate this step:
  - An investigational medicine developed for its therapeutic properties may have adverse effects when found in non-target tissues. Modified viruses designed to lyse tumour cells may have severe consequences if family members or caretakers become infected (direct effect, severity of high magnitude).
  - By mechanisms like recombination (in case of both DNA and RNA viruses) or re-assortment (in case of RNA viruses with a segmented genome) novel viral variants may arise that may have different properties compared to the applied vector (indirect effect, severity unknown).
  - An insert meant to induce an immune reaction against certain target cells, may, when spread to and internalise into non-target cells (in other persons), lead to an auto-immune response (immediate effect, severity of high magnitude).
  - Using growth factors to stimulate the generation of new blood vessels, might theoretically lead to uncontrolled growth of blood vessels when integrated in non-target cells (direct effect, severity of high magnitude).
  - Antibiotic resistance genes that are already abundantly present in the environment will not provide for a selective advantage and thus are not a hazard when released into the environment. If the antibiotic has no medical use, there is no conflict with healthcare (negligible effect).
  - When integration is envisioned, this might happen close to a gene resulting in either activation or, on the contrary, inactivation. In some cases this may have serious effects not only for the patient but also to other persons that were exposed. Integration near a gene encoding a growth factor, may lead to uncontrolled cell divisions finally causing tumour growth, *e.g.* leukaemia (delayed effect, severity of high magnitude).
  - Replication competent naked DNA may multiply and spread in the patient (human or animal) and is possibly shed. The naked DNA may then interact with viruses in the environment or be taken up by bacteria etc. The acquired traits, though, will usually not offer a competitive advantage to these organisms (delayed, indirect effect, severity unknown).

  The availability of prophylaxis or therapy against a pathogen may also influence the severity of an infection by that pathogen. However, this aspect is already included in the classification for pathogenicity.

- **Veterinary research**
  For veterinary studies the environment is very important not only for direct contact with the GMO, but also in relation to the potential transfer to other animals. For veterinary products and their effect on the environment it may be required to investigate:
  - fate and behaviour of the GMO in soil, water and dung,
  - its effects on aquatic organisms, and
  - its effects on other non-target organisms (including humans).

  For live veterinary vaccines safety studies should be performed for relevant species sharing the same ecosystem as vaccinated animals. They need to focus on species known to be susceptible to the vector, in particular the natural host species of the parental vector. Effects following transmission from vaccinated target animals to non-vaccinated target animals, to non-target animals and to humans should be investigated. Live attenuated vaccines may

recombine with wild-type. But if recombination results in wild-type pathogens that are already present in the environment, the use of the vaccine will not effect in a higher disease pressure.

Relating to the risks to human health, special attention is needed when dealing with GMOs that are or are derived from zoonotic agents.

3.2.3 Step three: Exposure assessment

In assessing the likelihood of the hazard to occur, *i.e.* the assessment of exposure to the hazard, one needs to consider routes of dissemination, stability and persistence profile of the GMO, characteristics of the receiving environment etc.

In some cases both the likelihood and the frequency should be addressed. Besides the hazard itself, the number of GMOs, the receiving environment and the conditions of the release are important for defining the likelihood. The Commission Decision 2002/623/EC further states that the probable number of gene transfer events or the extent to which transfer will occur should be considered, if gene transfer is likely. If the GMO has pathogenic or toxic characteristics, the proportion of target organisms in the environment likely to be affected should be assessed. The level of exposure should be made quantitative as much as possible. However, if it is known that exposure happens, to know the magnitude of the exposure may be not that important, only the magnitude of the adverse effect (Raybould, 2010).

Since the safety of the patient or treated animal is not considered in the ERA, the exposure of the patient or treated animal is not discussed here.

Procedures that might lead to exposure or adverse events happening in a CT as well as in veterinary applications, include (Figure 3):

- production and preparation of the GMO,
- administration, and
- waste disposal.

Routes by which GMO(s) come into contact with human beings or enter the environment may result from normal handling and use (*e.g.* wound leakage), accidental release and disposal of unused product, waste product and patient excreta (EMA, 2005).

Where veterinary products and trials are assessed, the following exposure routes might be important depending on animal species and husbandry conditions (EMA, 2007):

- Removal of material containing the product (manure, bedding material, dirty water, fish farm effluent);
- Excretion via faeces and urine (grazing animals);
- Spillage at external application and/or direct exposure outdoors;
- Leaching, run-off and drainage from manured land;
- Direct spillage and/or feed spillage;
- Direct excretion into water (pasture animals);
- Direct application in water (aquaculture);
- Direct discharge of water into surface water (indoor aquaculture); and
- Release from Sewage Treatment Plants (indoor aquaculture).

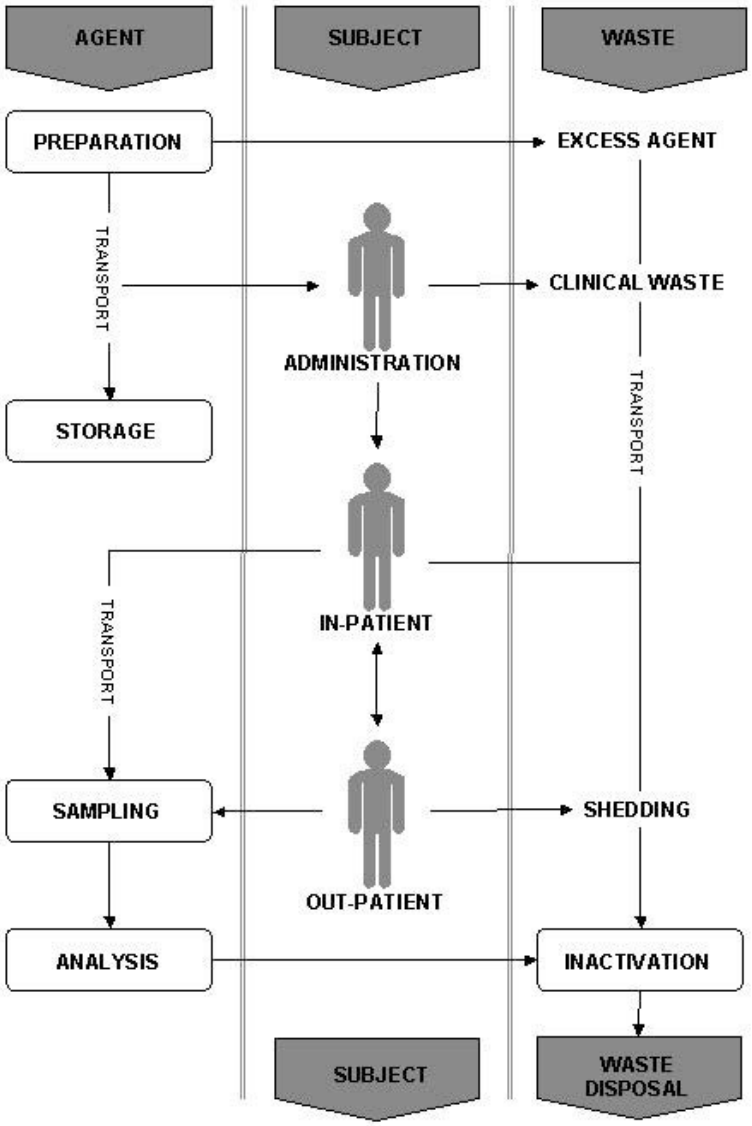

Fig. 3 Clinical trials: schematic checklist of areas of concern, ordered according to 3 pathways: the GMO itself, the administration to the patient and waste treatment (SACGM, 2007).

The measures that are already proposed in the application to reduce or limit the harmful effect determine the exposure.

1. Production and preparation

   Apart from accidental spills during production, the process itself may face unwanted events, such as the formation of replication competent viruses. Measures must be taken to reduce the possibility of this happening.

   Adapting the production process of a viral vector using several helper plasmids, on which the deleted sequences are distributed instead of using only one helper plasmid, will substantially reduce the generation of replication competent viruses. This can be confirmed by checking the

batch for absence of replication competent viruses (*e.g.* via an ELISA test). As a result the probability of replication competent viruses present in the investigational medicine and therefore in the shed liquids is extremely low.

Transduced cell lines are commonly washed several times and treated with trypsin to dilute and destroy free virus particles. Calculations can be made to measure the effect and the number of steps needed. Finally, culture procedures using the single-cell clone method will decrease the presence of free virus particles. Another point of concern is the presence of contaminants or unnoticed endogenous viral sequences that may be mobilised and co-packed.

Preferably data are quantified. The amount of free viruses particles present in a single dose may be calculated including detection limits. In its generic advice on lentiviral vectors, the COGEM presents a formula to calculate the free lentivirus reduction ratio in cell cultures (CGM/090331-03). The proposed formula takes into account the number of wash steps, the number of wash steps with trypsin or human serum, the time that has elapsed after transduction and the half-life of the virus. Rare events, *e.g*. the rates of mutation and frequencies of recombination during viral replication are open to quantitative analysis and are known and published for many viruses (EMA, 2008a).

Also, when the therapeutic is not delivered 'ready-to-use' and preparations need to be made at the hospital pharmacy, this is an extra step where accidental release might happen.

2. Administration and follow-up

The route of administration, frequency and dose will determine the likelihood of exposure. Leakage during administration and accidental spilling are the first possibilities for release. The likelihood of an effect will then depend on the amount of the therapeutic shed or spilled and the half-life.

Several aspects may need attention at this point:

- **Probability of recombination**

  The possibility of recombination with wild-type vector is reduced when patients will be excluded from the trial that have an acute virus infection of the relevant type. Alternatively the vector may be designed in such a way that recombination within wild-type infected persons is very unlikely because of the limited homology. Also, codon optimisation will lower the homology.

  For recombination between DNA/RNA containing viral sequences and wild-type viruses, presence of both in the same cell is required. An estimation of this possibility depends on the fate of the GMO in the patient's body and the infection route of the wild-type. The frequency of newly formed viruses or activation of latent viruses follows thereof.

  The use of sequences from non-human viruses may exclude or reduce the chance for recombination in human cells due to absence of homology with human viruses. In the opposite case, sequences of human viruses may prevent recombination with animal viruses in veterinary trials.

- **Frequency of integration in the genome**

  Cell culture studies or literature data may be available. Genome homology, protection from degradation in the cell will influence the probability. Again, calculations can be made. For example, when applied intramuscularly, less than 1% of a dose of naked DNA will be taken up. Once in the cell the half-life of naked DNA in the cytoplasm is about 50 to 90 minutes. In the cell the DNA then needs to be transported to the nucleus for an integration to take place.

The frequency of a potential mutagenic effect of integration is considered to be lower than the natural mutation frequency in humans. Improvements in the administration techniques may increase this frequency. For example lipids will facilitate uptake by the cell. Also, the presence of homologous sequences or viral sequences may enhance integration.

- **Activation of endogenous genes**
  Lentiviral vectors for treating patients potentially may initiate interactions with endogenous retroviral sequences present in the transduced cells. Interactions may be recombination and transactivation, mobilisation of otherwise inactive endogenous retroviruses.
  The probability of integration at the wrong site, *i.e.* activating oncogenes for example, may be hard to determine.

- **Persistence**
  Replication-competent virus vector or plasmid may persist in the patient for extended periods and can increase in amount. A replication competent GM virus may also integrate in the patient's genome and stay latent. An infection by a related virus may induce replication and shedding at a later point in time. Therefore, the potential for shedding can be higher and could result in a greater likelihood of transmission.

  Persistence of naked DNA in the environment is estimated to be very unlikely, because of quick degradation. Also, in the stomach and intestine contact with bacteria will be minimal because of the degrading conditions. Uptake by bacteria is therefore estimated to be rather unlikely.

- **Shedding**
  Shedding as such is not a hazard. It is a mechanism through which dissemination, transmission and effects on the environment and public health may occur. It depends on the type of material and the environment what the consequences will be and whether shedding will have an adverse effect.

  Hazard characterisation will determine whether shedding needs to be assessed, *i.e*. if no hazard is identified shedding is not important. However, the evaluation of vector shedding is always a requirement for a phase I study (EMA, 2008a).

  Shedding at the wound is always expected when the therapeutic is injected in muscles. Also material may be accidentally spilled at the moment that samples are taken. Animals after intranasal administration of a vaccine will certainly shed the vaccine into the environment. Also, the GM vaccine may be present in the manure that will be spread on arable land.

  Shedding should not be confused with bio-distribution, *i.e*. the spread of a therapeutic within the patient's body from the site of administration. Data on bio-distribution are important in case of infective and transmissible organisms. Bio-distribution may help to understand whether and via which ways shedding may occur. If a vector may be spread through the blood vessels, shedding via urine and sperm might be possible. Data on the subject is particularly informative with conditionally replicating virus, where systemic distribution is very likely and the duration of the infectious state may be increased by replication.

  Pre-clinical bio-distribution and shedding data can be used to determine the sampling protocol (what, where and when). Also, assessment of shedding in lab animals will aid in the clinical monitoring plan. Shedding information can be used to guide monitoring for long-term adverse effects in both non-clinical and clinical studies (EMA, 2009b). The probability of release of the

GMO via shedding is, besides on initial dose, further depending on the half-life in the patient and his immune response to the GMO (clearing of the GMO).

- **Colonisation**
  Bacteria may be very restricted in their ability to survive in certain environments. Some are restricted to the human gut while there is no other animal reservoir. The possibility of environmental colonisation after shedding and spread of such a strain is therefore minimal (SACGM, 2007).

- **Immune reaction**
  Treatments based on the induction of an immune response may affect other people than the patient following contact through shedding for example. However, the likelihood of the effect occurring depends on the type of stimulation: the insert may stimulate an already existing response or induce a new response.

  Vectors based on adenovirus, to which the population in general has developed an effective immune response, will be quickly eliminated by the immune system. However, the immunological status of family members might be compromised (elderly or very young people) and clearance mechanisms might be inefficient.

3. Waste disposal
   Sensitivity of the GMO to inactivation methods will determine the risk management measures to be taken to prevent undesired effects on humans, animals and the environment. Mapping the different waste flows is a pre-requisite. Waste may consist of wound dressings, disposable gloves, disposable wipes, injection needles, empty containers of the investigational medicine, sampling vessels, etc. Waste may include urine and faeces. Also, bed-clothing, patient cloths, nurse gowns may be a source for exposure to the GMO.

   As pointed out before, there may be more diverse waste streams and exposure routes in the case of veterinary products. *E.g.* when housing cattle the manure will be collected and may be applied on farmland. If the veterinary medicine were excreted via faeces, the fate and effect of the product on the soil via manure application would need attention. With grazing animals the veterinary investigational product may enter the soil directly when spilled or lost at administration or indirectly when excreted. In case the herd stays close to water bodies exposure to aquatic organisms has to be assessed.

3.2.4 Step four: Risk characterisation

Finally the risk is assessed for each of the identified hazards. Risk is determined by combining the severity of the potential adverse effect and the likelihood for its occurrence. Each hazard that was confirmed in the second step of the ERA should be assessed.

Some examples:

- Toxic effects from the gene product may arise only when high concentrations are administered. Evaluation of the potential concentrations to which individuals might be exposed will determine the actual risk.
- Toxin-producing genes driven by a eukaryotic promoter are very likely not synthesised in bacteria. Consequently, an adverse effect happening in bacteria, if any, is therefore negligible and so is the risk.
- Strongly biologically contained GMOs, that are not able to survive in the environment and will be degraded very quickly do not pose a risk when shed.

- Recombination of a virus with its wild-type progenitor only takes place when both are present in the same cell. If bio-distribution studies show that this likelihood is very low, the probability of recombination will be very low. Therefore the risk of generating new viruses is very small.
- Shedding via sneezing or nose contact is certainly going to happen when veterinary therapeutics are applied intra-nasally in animals. If there is no adverse effect identified for other animals, the risk is negligible.
- Assessing the ability of a GMO to establish infection is in fact an evaluation of the 'fitness' of a GMO. Fitness is not coupled with pathogenicity per se. Theoretically, making the GMO more pathogenic may also render the GMO less fit, *i.e.* less able to survive. For example, a modified adenovirus with a non-functional E3 locus makes the virus more susceptible to immune surveillance and therefore reduces the risk of environmental spread (EMA, 2008a).

A conclusion may be that there is uncertainty on the likelihood or type of effect or precise working mechanism. Therefore more studies might be needed, but if the costs of gathering the information (by experimentation or review) for a more precise assessment are disproportionate, it is of course permitted to assume the worst (European Commission, 2006). When a worst-case scenario is found to result in a negligible risk, more realistic scenarios will also do. Indeed, quantification is often hard to accomplish, and not always necessary to make a decision.

3.2.5 Step five: Risk management

Risk management may be required to control an identified risk or to cover remaining uncertainties. It may include isolation measures where the likelihood of release is high. Another option is to identify a different release site at which contact with certain susceptible organisms can be avoided.

Risk management is about selection and assignment of appropriate control measures to reduce the risk to a negligible level. In contrast to CU, no standard list of measures corresponding to pre-defined containment levels has been described, leaving the specification to a case-by-case approach based on the ERA. The control measures proposed in the trial application may already be adequate, but extra measures may be included in the COGEM advice. Efficacy of the proposed risk mitigation should be demonstrated.

Examples from past applications include:

- Recombination of a virus with its wild-type progenitor during the production process can be avoided by testing for the absence of wild-type virus with *e.g.* quantitative PCR. In a CT patients may be tested for a wild-type virus infection before administration of the investigational product. The same is true for hospital and health care personnel; individuals with a related active virus infection should not be involved in the experiments.
- An exclusion criterion for CT might be that immunocompromised persons should not take part for instance to ascertain quick elimination of the GM virus.
- Wounds may leak the investigational product for some time until closure. Waterproof wound dressings may be advised with additional inactivation before disposal.
- An isolation room may be proposed with protective clothing for healthcare workers and visitors to prevent transmission via aerosols (shedding via coughing or sneezing). Also, disinfecting of all bedclothes and patient's cloths before cleaning as long as shedding is detected will reduce spread.

To reduce risks found to be originating from waste, the material is inactivated prior to disposal. Usually it is collected as medical hazardous waste in containers that, once closed, cannot be reopened. The sponsor of the trial may require collecting unused doses of the gene therapeutics for return. In veterinary trials animals may be euthanized and the carcasses destructed.

3.2.6 Step six: Overall risk evaluation and conclusion

The identified potential risks to the environment, including humans should finally be assessed on the basis that the control measures, proposed by the applicant and the COGEM, will be in place.

The COGEM then advises the competent authority, *i.e*. the Minister of IenM, on the outcome of the ERA, which will inform the decision-making process. Based on this general conclusion, the Minister may issue a permit with or without extra conditions or a denial.

3.2.7 Monitoring

As for CU, the COGEM may advise for monitoring in relation to different objectives:

- As a way to confirm or adjust assumptions made in the ERA (note that new information may require a change in the ERA with possibly an adjustment of the COGEM advice and permit situation),
- as confirmation that the imposed measures and conditions are adequately addressing the identified risks,
- as management tool to decide on reduction of containment measures (*e.g.* to determine whether protection measures can be removed on the basis that shedding no longer occurs),
- as alert system to enable quick response in case an adverse effect would occur, or
- as source of information that may be useful for future experiments (*e.g*. collecting shedding or bio-distribution data).

While monitoring for scientific purposes only, *e.g*. to test for efficacy of the therapeutic, may be of experimental interest, it would not fit within the context of the ERA and would therefore not be eligible to be included as a COGEM recommendation.

4 Discussion and recommendations

Applications of GMOs in medical and veterinary biotechnology require a risk assessment preceding decision-making. The COGEM handles ERAs for both contained use and deliberate release applications. In this analysis, the general approach to formulating an advice to the competent authority based on an ERA as well as the examples presented by COGEM advices were reviewed in order to describe and evaluate the deployed methodology. In the previous chapters general principles for performing a stepwise ERA have been described and documented, as well as the legal framework on which they are based. These principles can be applied to a range of activities, both within and outside the field of biotechnology.

4.1 Established ERA experience

From the development of legal indications as well as of different international guidance documents, it can be concluded that the past decades have been marked by a dynamic evolution of the principles of conducting an ERA. The approach that is used in the report is based on the most recent indications and documents, and is deemed very comprehensive. Nevertheless, it cannot be excluded that adjustments may be proposed in the future.

The COGEM has been involved in establishing classification lists of pathogens and assessing individual cases for many years already. In that period, a certain approach was developed, identifying points of concern, accumulating information and improving argumentations.

When analysing specific advices, it was not always obvious to discern each step of the described ERA process. This does not affect the validity of the performed ERA, as the COGEM in the past, in the absence of the stepwise methodology, focussed on key elements that were relevant for the overall ERA. For example, it can be easily argued that, if a risk were considered negligible, assessing and quantifying exposure routes would not reveal any additional relevant information.

In this report we used the stepwise methodology to relate the COGEM advices to the state-of-the-art practice, demonstrating that the same logic is already embedded in the COGEM advices. The stepwise methodology can be used further in the future as an additional verification to ensure that all relevant aspects of the ERA are taken into account.

4.2 Need for an ERA

In developing medicinal products more data will gradually become available on several aspects of the product as research and trials proceed. It is obvious that at the early stages many unknowns make it hard to obtain quantitative data. These investigations are therefore carried out under CU requirements and environmental studies are not yet required. But, as the product is nearing the stage for a market approval application, it will be necessary for example to study the fate of the product in the soil or to study its effects on aquatic organisms.

While strict containment measures of CU provide an excellent framework for R&D and production, it may leave developers with a false focus on measures addressing likelihood and exposure rather than fundamentally addressing hazards associated with release in the environment.

4.3 Similarity/difference of the ERA for CU and DR

During the study and interactions with the advisory committee, it became apparent that although an ERA for CU is treated differently from one for DR, the underlying methodology is basically the same (Table3).

Table 3 Relative importance of ERA steps in the evaluation of CU and DR cases.

	Contained Use		Deliberate Release
	Generic case Annex 5 of the '*Regeling*'	**Specific Case**	**Specific case**
COGEM involvement	Up-front general advice on generic classification	Individual advice	Individual advice
ERA emphasis			
• **Hazard identification**	X	X	X
• **Hazard characterisation**		X	X
• **Exposure assessment**		X	X
• **Risk characterisation**		X	X
• **Risk management**	X	X	X
• **Risk evaluation**	X	X	X
• **Monitoring**		X	X

In a CU assessment emphasis is put on containment, *i.e.* the whole of procedures and facilities intended to prevent exposure of the public and the environment in general to the GMOs. In DR exposure is to some extent assumed and therefore the consequences for the environment and human and animal health are assessed more broadly and in more detail.

Because the ERA of many activities that are conducted under the CU regime is relatively straightforward, the COGEM has established a systematic approach guiding users through a set of criteria to be checked for their planned activity in order to assign the activity to a containment level (Annex 5 of the '*Regeling*'). Complying with the corresponding containment requirements adequately mitigates risks (Annex 4 of the '*Regeling*'). The principle of the case-by-case assessment is preserved, while allowing for quick evaluation. Only in cases where not enough information is available or some aspects need specific consideration COGEM gives advice. In DR submissions the COGEM is involved in every case evaluation, as due to the wide range of experiments and applications standardisation is hardly possible.

This pragmatic approach allows the COGEM to focus on cases that deserve special attention. On the other hand, routine activities with GMOs that are expected to pose little or negligible risks can be quickly evaluated, provided that adequate containment measures are applied. As more experience is acquired, additional generic advices may be added.

4.4 Qualitative versus quantitative ERA

One of the difficulties encountered in conducting an ERA is the lack of data to quantify risks and their consequences. Although the GMO may be accurately described molecularly and hypothetical hazards may be formulated based on its characteristics, uncertainty often exists on the extent or severity of potential effects.

While Commission Decision 2002/623/EC has standardised a ranking system using the qualities “high”, “moderate”, “low”, and “negligible” for estimates of the consequences and their magnitudes, likelihoods, and risks of adverse effects occurring, the risk quotient (RQ) approach from veterinary medicine ERA may be used (EMA, 2007). The RQ is defined as the ratio between the predicted environmental concentration and the predicted no-effect concentration. The risk quotient indicates the likelihood of an adverse effect occurring. A value of one and a value less than one indicate no effect and no further testing are recommended. This can be translated into a RQ for infectious substances: predicted amount released compared to infectious dose, etc. However, one of the difficulties in applying this method is the fact that the no-effect concentration for the particular GMO is generally not known.

Literature searches or experiments specially designed to gain more knowledge on the frequency at which some events may happen, are often essential. For example, the rates of mutation and frequencies of recombination during viral replication are open to quantitative analysis and are known and published for many viruses. Nevertheless data are often not available and hence quantification of risks is often not possible. *E.g.* the infectious dose of an attenuated GMO may not be known and would require specific studies to provide a figure that eventually may be of limited value for the ERA. If costs would not be justifiable in relation to the relevance of the expected answer, other approaches may be used.

In the absence of data, estimates can provide an alternative, including an estimation of the level of uncertainty associated with each estimate of likelihood. If not quantifiable (*e.g.* the likelihood of an attenuated or disabled GMO reverting to wild-type status) the ERA can consider the number of discrete events that would need to take place, *i.e.* the more events needed, the less likely a reversion will occur.

Although a quantitative ERA may be perceived as more accurate, calculations should only be required when considered really necessary and reliable. In many cases it may be more applicable to work on the basis of worst-case scenarios. If a worst-case scenario identifies an important negative impact, then more detailed information and quantification of the components of the scenario may indicate if the negative impact may be realized. Again, in this case it may not be required to quantify each element, as the identification of very low likelihood of specific events in the worst-case scenario would render the entire scenario highly unlikely.

Sometimes it is not necessary to have quantitative data. If exposure is sure to happen, the focus should be on the consequences. Hence, experiments to exactly know what the likelihood of a certain event would be can be avoided. Also, if the consequences of an exposure are assessed to cause no harm, to know the exact likelihood of exposure is not relevant.

4.5 Tiered approach to data generation

Another alternative to exact quantification is based on a tiered approach in conducting experiments. A lab experiment with cell cultures may already show that it is very unlikely that an adverse effect would take place. In this way costly experiments with model lab animals may be avoided and animal lives may be saved. On the other hand, in a veterinary drug dossier for example, when the ERA is built on replication abortiveness of the microorganisms, this should be demonstrated in the target species. Experiments to gain more insight therefore need to be scientifically relevant and carefully designed.

Also, when no effect is observed the detection limits should be indicated. In general, experiments that are conducted to provide quantitative data for risk characterisation and likelihood should be statistically sound and fully described and analysed. Only then it will enable the assessors to make a correct judgement.

4.6 Cautious approach as basis

The systematic performance of a stepwise ERA for (GM) organisms is still relatively recent. Many ERAs take into account hypothetical cases ‘because it cannot be concluded that the risk is negligible’. This is in line with a cautious approach that imposes that in case the level of harm may be high, action is taken to prevent or minimise such harm, even when the absence of scientific certainty makes it difficult to predict the likelihood of harm occurring, or the level of harm should it occur. The need for control measures therefore increases with both the level of possible harm and the degree of uncertainty. The COGEM advices clearly reflect this principle.

4.7 Transparency

The COGEM takes great care to make guidance documents, general considerations and individual advices publically available. The advices provide an insight in the arguments that were deemed essential in reaching the conclusion. As such these also provide directions for applicants, who can evaluate to what extent a case can be seen as a relevant precedent.

This report may be another element in communicating on the methodology that has been developed by the COGEM. It may serve as reference for new GOGEM members, as direction for future applicants and as further clarification to stakeholders. The large experience that the COGEM has accumulated over years is a good basis for the safe use of GMOs in contained use and deliberate release, both at the level of R&D as well as at commercial scale.

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6 Annex COGEM Case Studies

In order to illustrate the ERA methodology, 4 cases were selected in consultation with the advisory committee for this project. The case studies were chosen to represent very different applications, relating viruses as well as bacteria, to contained use as well as deliberate release. Their selection does not mean that these cases entail particular problematic ERAs. Their description is purely intended as examples of the ERA.

Only the final COGEM advice was available and this advice may not systematically have covered all the elements of the ERA as indicated in the report and may lack other considerations that may have been part of the deliberation, but that were eventually not included in the advice. Consequently, only those elements that were available are listed. In case the authors made assumptions that could not be verified in the advice, this is specifically indicated.

1. Contained use case: Classification of activities with human parechoviruses.[5]

The research proposal covers the investigation of the function of several parechovirus genes. For this purpose, different research materials are proposed, including:

- human parechoviruses types 1-5 (HPeV1-5),
- human parechoviruses types 1-5 (HPeV1-5) with targeted point mutations and deletions,
- genetically modified human parechoviruses, also indicated as chimeras, resulting from combinations of coding and non-coding sequences of different parechovirus types or exchange of sequences between parechovirus and enterovirus sequences (enteroviruses and parechoviruses both belong to the family of *Picornaviridae)*, and
- chimeras with targeted point mutations and deletions

The proposal also covers the experimental infection of eukaryotic primary and established cell lines using these viruses.

In the GOGEM advice there is no information on how the mutations and the chimeric GM viruses are made. Also the vectors used for modification are not mentioned. Likewise the type and scale of activities with the eukaryotic cell lines are not addressed and are consequently not covered in this case study. Nevertheless, the scope of the proposal clearly related to research activities in contained environment.

Step one: Hazard identification

Pathogenicity	- Viruses are classified based on their virulence and capacity for transmission within the host population. - HPeV1 and HPeV2 had already been classified as belonging to class 2 pathogens. - HPeV3, HPeV4 and HPeV5 had not been classified at the time of the request for advice. COGEM judged that, based on homology on the amino acid level (85%) and known pathogenicity of HPeV1 and HPeV2, classification of HPeV3, HPeV4 and HPeV5 as class 2 pathogens was justified. - Class 2 pathogens can cause diseases in humans (and/or animals); they are unlikely to spread within the population and there are effective means for prophylaxis and treatment.
Altered pathogenicity	- Mutagenesis may change the virus' virulence. - Production of chimeras may result in new or altered phenotypes. - Other picornaviruses possibly present in primary cell lines may recombine with the viruses under investigation in this study.
Altered tropism	- Production of chimeras may results in altered tropism. - Changes in the RGD motive may result in altered tropism (the RGD motive is involved in host cell receptor binding: HPeV3 differs from other parechoviruses as it lacks the RGD motive).

[5] COGEM-advies CGM/070115-01 Inschaling van handelingen met humane Parechovirussen.

Step two: Hazard characterisation

Human health impact	- Infections with HPeV occur mostly in children under the age of 3. - Symptoms caused by parechoviruses are very similar to those caused by an infection with enteroviruses. - HPeV1 causes disease predominantly in children. Symptoms range from mild gastro-intestinal and bronchial disorders, and in worst-cases encephalitis and muscular paralysis. - HPeV2 causes the same symptoms as HPeV1 but only rarely. - HPeV3 induces more severe symptoms like temporal paralysis and sepsis. One of the reasons for this may be that the symptoms are observed in very young children that may be more susceptible to infection.
Changed virulence	- Mutagenesis occurs frequently in nature. Mutagenesis (point mutations) may lead to higher, but most often to lower fitness. Inducing (targeted) mutations is not expected to result in new forms that would not already be present in nature. - Genomic deletions usually lead to attenuated viruses. - Recombination between enteroviruses can result in more virulent viruses. In nature such recombination occurs frequently and is an important cause of increased virulence. COGEM recognised that in most cases the chimeric viruses will be attenuated or will show no changes in virulence. However, as (very) little was known about HPeV3-5, it could not be excluded that a newly generated chimeric virus would be more virulent than the original virus. In the advice, reference is made to an earlier COGEM advice[6], which also covered mutations and recombinations. In that advice it was concluded that due to the lack of specificity of the composition of the recombinant viruses, COGEM was not able to evaluate their fitness and consequently had to assign activities to a higher biosafety level. - A risk exists for recombination with picornaviruses that would inadvertently be present in the primary cells that are to be used in infection experiments. As these cells are normally not tested for the presence of picornaviruses, the presence of relevant viruses and possible recombination has to be considered. Yet, COGEM estimated that the chance for this to occur was very small, as the presence of picornaviruses in the primary cells would most likely result in cell-death. Therefore COGEM indicated that testing for the presence of picornaviruses was not deemed necessary.
Changed tropism	- Recombination can lead to changed cell tropism (*e.g*. the tropism of HPeV3 may change when the sequence of the VP1 protein with the missing RGD motive is inserted).

Step three: Exposure assessment

Natural exposure/ protection	- HPeV1 is considered to be a widespread pathogen in the human population. More than 95% of the adults have antibodies against HPeV1. - Also antibodies against HPeV3 are common: 85% of Japanese children between 4 and 6 have antibodies, 87% of people older than 40 show antibodies against this serotype.
Route of transmission	- Wild-type native parecho- and enteroviruses are transmitted via the faecal-oral route, possibly also oral-oral, while no airborne transmission is known. - Recombination between enteroviruses can result in changed transmission routes. In nature such recombination occurs frequently and is an important cause of changed transmission routes. Although COGEM expected that transmission of chimeric viruses would remain limited to the faecal–oral route, the possibility that the transmission route would change could not be excluded, as the tropism of all recombinant viruses was not known.

[6] COGEM-advies CGM/020823-06 Molecular and cellular aspects of picornavirus replication

Exposed population	-	Only the trained staff performing the research project is potentially exposed to the viruses. Exposure under experimental conditions is already different from the normal transmission route. Furthermore, as the tropism of all recombinant viruses was not known, it was not possible to predict the chance that recombination results in changed transmission modes.

Step four: Risk characterisation

- The 5 native types of human parechoviruses were judged to have similar characteristics in relation to pathogenicity.
- Manipulations with mutated human parechoviruses (point mutations and deletions) were judged to present no higher risk than working with the parental viruses. The pathogenicity class (severity of disease, transmission mode) was expected to be the same (or lower) as the parental viruses.
- Working with chimeric viruses, however, was evaluated to present a higher risk compared to the parental viruses (uncertainty about HPeV3, HPeV4 and HPeV5 characteristics and subsequent uncertainty about possible changes of tropism and transmission mode).
- The risk for recombination with picornaviruses potentially present in primary cells was estimated to be low as the probability of their presence (exposure) was very low.

In the advice there is also a reference to another case[7] relating to polio and non-polio enteroviruses. Wild-type poliovirus was presented as a class 2 pathogen, considering that the virus had not yet been globally eradicated and vaccinations were still continued. In this case chimeric polioviruses were not deemed to belong to a higher risk class based on the following arguments:

- The entire sequence of the poliovirus is known.
- Nearly the entire world population has been vaccinated against polio.
- Polio is primarily transmitted via the faecal–oral route.
- Human enteroviruses are widely distributed among the human population. Therefore the likelihood that different enteroviruses occurred at the same time in a host and have spontaneously recombined is very high. If this had led to an important medical challenge then this would have been reported. Consequently, it is very unlikely that the experimental recombination will result in a chimera that has not yet occurred in nature and that would create specific medical challenges.

Step five: Risk management

- The COGEM advised that activities with wild-type human parechoviruses and their mutants had to be conducted at biosafety containment level 2. Standard safe laboratory practices at this level were deemed adequate to avoid that exposure would occur leading to an infection of the worker and further spread in the environment. Open manipulations needed to be done in a biosafety cabinet class II.
- For activities with chimeras a biosafety containment level 3 was required and gloves had to be worn during all manipulations. Open manipulations needed to be done in a biosafety cabinet class II.
- Containment requirements for working with primary cell lines depended on the type and biosafety classification of the GM virus that they are infected with.

Step six: Overall risk evaluation and conclusion

Considering the type of organisms (wild-type and genetically modified) and the proposed containment level including additional biosafety measures to be adopted, the project was assessed to present negligible risk for the employees.

Monitoring

COGEM did not indicate any need for specific monitoring for these activities. However, there was a call for providing more data on HPeV3, HPeV4 and HPeV5 as they became available (literature).

[7] COGEM-advies CGM/021017-01 Reverse genetics van polio- en non-polio enterovirus

2. Contained use case: Classification of *in vitro* activities with a *Mycobacterium tuberculosis phoP* mutant.[8]

Mycobacterium tuberculosis (*MTB*) is the main cause of tuberculosis in humans. The proposed research project involves the use of a "knock-out" mutant, obtained by inserting a kanamycin resistance gene in the *phoP* gene. This mutant no longer produces the PhoP protein that is assumed to be involved in transcription regulation of important virulence genes.

The interactions of the mutant strain with dendritic cells *in vitro* would then be studied.

Step one: Hazard identification

Pathogenicity	- MTB is classified as a class 3 pathogen. Typical symptoms include a maintained cough, night sweats, loss of weight, fever and fatigue. Symptoms may remain unnoticed until the disease is already quite advanced. - Class 3 pathogens can cause serious diseases in humans (and/or animals); they are highly likely to be transmitted within a susceptible host population and there are means for prophylaxis and treatment. - It is expected that the *M. tuberculosis phoP* mutant is less pathogenic than wild-type (attenuation).
Impaired treatment	- Insertion of the kanamycin resistance gene may impair treatment of the disease by kanamycin and closely related antibiotics.

Step two: Hazard characterisation

Pathogenicity	- The insertion disrupts the *phoP* gene making it impossible to produce the protein. *M. tuberculosis phoP* mutant shows a reduced growth in *in vitro* studies (mouse microphagous cells). In SCID mice (immunocompromised) the mutant was clearly less virulent compared to wild-type *MTB* and the vaccination strain *M. bovis* BCG. Administration by inhalation or intravenously was not lethal, while wild-type *MTB* and *M. bovis* BCG this administration did cause death. It could therefore be expected that the *M. tuberculosis phoP* mutant was less pathogenic than wild-type *MTB*. Animal studies demonstrating attenuation of the mutant were, however, limited in time in which experiments could be conducted: infection may *e.g.* stay unnoticed or cause disease only later in life. This leaves some level of uncertainty. - The complete *phoP* sequence is still present in the genome. Deletion of the kanamycin resistance gene would result in a functional *phoP* gene, reverting the mutant to wild-type. Infection by this revertant could result in tuberculosis.
Impaired treatment	- Tuberculosis is difficult to treat and requires a combination of different antibiotics. Kanamycin was used as a second line treatment. - In the event that *M. tuberculosis* phoP mutant would result in disease, treatment cannot include kanamycin. This would not affect treatments based on first line products like isoniazide and rifampicine and other types of second line products like capreomycine, moxifloxacin or ethionamide.

Step three: Exposure assessment

Communicability	- *MTB* is transmitted by air (aerosols). The modification is not expected to result in a change in transmission mode. It was pointed out that manipulations with the bacterium in the laboratory could produce aerosols that lab workers might inhale. - Only about 10% of people infected with *MTB* ever develop tuberculosis disease. The infection may remain present in a latent form.
Restoration of	- To restore the *phoP* gene the kanamycin resistance gene would need to be

[8] COGEM-advies CGM/090212-01 Inschaling van *in vitro* werkzaamheden met een *Mycobacterium tuberculosis phoP* mutant.

mutation		very precisely excised. The probability was estimated to be very low and such an event had never been reported. Excision via recombination was also expected to be very unlikely since no homologous regions were present in the expression cassette. Also, the bacterium is considered to be a very genetically stable microorganism.
	-	In accordance with general hygiene measures, it is standard practice that lab personnel involved in activities with *MTB* are controlled for tuberculosis infection once a year.
Impaired treatment	-	The likelihood of kanamycin being the only antibiotic left to treat an infection by the mutant was deemed very low, as other antibiotics are available.

Step four: Risk characterisation

COGEM confirmed that the mutant strain showed reduced pathogenicity compared to the wild-type *MTB*. As the experience was still limited, three potential risks were retained:

- The risk of infection via inhalation was deemed high (*e.g*. in case aerosols are produced), but the risk of developing disease after infection by the mutant bacterium was estimated to be low due to its attenuation.
- The risk of reversion to wild-type was estimated to be very low, but left the theoretical possibility for restoration of virulence.
- The risk of not being able to cure an infection was deemed negligible.

It was noted that in an earlier COGEM advice on activities with genetically modified *Mycobacterium bovis* BCG strains[9], the arguments about strong attenuation and limited pathogenicity had also been made. However, as BCG was not known to be transmitted via aerosols, air transmission had not been deemed important in that case.

Step five: Risk management

- Safe microbiological practices (preventing aerosol formation) as applicable for biosafety containment level 2 and a biosafety cabinet for open manipulations (avoiding aerosols to be inhaled) were expected to reduce the risk of infection substantially.
- Culturing the strain under continued kanamycin selection pressure was requested in order to eliminate in an early phase any rare case of reversion to wild-type *MTB*.
- Each *M. tuberculosis phoP* batch needed to be tested for susceptibility to at least one primary antibiotic.
- All lab workers needed to be screened for tuberculosis infection once every year.

Step six: Overall risk evaluation and conclusion

The COGEM concluded that implementation of all safety measures would result in a negligible overall risk for the activities with the mutant *MTB*.

Monitoring

The COGEM did not indicate the need for any monitoring.

[9] COGEM-advies CGM/070402-05 Inschaling van vaccinatie van makaken met de genetisch gemodificeerde M. bovis stam BCG.

3. Deliberate release case: Clinical study with a conditionally replicating adenoviral vector.[10]

In this phase I/II clinical trial a conditional-replicating adenoviral vector (Ad5-Delta24-RGD) is proposed to be administered to patients with a brain tumour (glioblastoma multiforme). The wild-type virus from which the vector is derived is a group C serotype 5 adenovirus. Twenty-four base pairs from the viral *E1A* gene have been removed from the genome, limiting the replication of the vector to tumour cells. Furthermore the viral coat protein is modified by addition of the sequence coding for the arginine-glycine-aspargine-4C peptide (RGD motive). The RGD motive enables the vector to bind to and integrate in cells that express specific integrines on their cell membrane.

The concept underlying the clinical study is that infected tumour cells die due to the absence of a functional adenoviral E1 that normally would delay host cell apoptosis. The tumour cells that die, release progeny viral particles that will infect other neighbouring tumour cells. When all tumour cells are destroyed, the replication-selective viral vector will not be able to further replicate in or release progeny viral vectors from normal non-tumour cells.

In the trial a dose varying between $1x10^7$ and $1x10^{11}$ viral particles is proposed for administration by infusion via catheters for 50-68 hours in or around the tumour. The patients are expected to stay in an isolation room with negative pressure and antechamber until 24 hours after the treatment. After the removal of the catheters the wound is sutured and covered with an adhesive. Dexamethasone is administered to prevent oedema formation.

Patients with an active adenoviral infection are excluded from the trial. Tissue samples are taken to study persistence and shedding. Measures aiming at preventing dissemination during transport of samples are in place.

Step one: Hazard identification

Pathogenicity

- The wild-type adenovirus had been classified as a class 2 pathogen[11].
- Class 2 pathogens can cause diseases in humans (and/or animals); they are unlikely to be spread within the population and there are effective means for prophylaxis and treatment.
- Disease symptoms are rather mild, resembling a cold. Medical treatment usually is not necessary. Adenoviral infections are self-limiting. Immuno-incompetent persons may develop a kidney or lung infection.
- The administration of dexamethasone before, during and after treatment may have an effect on the receptor and integrines on cell membranes resulting in changed transduction efficiency.
- The addition of the RGB-motive enables the viral vector to bind effectively to certain integrines, thereby enlarging the range of cells that can be infected. This could result in enhanced infection risk.
- The adenoviral vector is only replication competent in dividing tumour cells due to a deletion in the E1A gene and a defective Rb-pathway in tumour cells. This change in replication competence results in reduced pathogenicity.
- Replication to some extent remains possible in diving cells (*e.g.* intestinal cells), but the replication is limited in comparison to tumour cells.
- The administration of dexamethasone before, during and after treatment may reduce the working of the immune system. A wild-type virus infection may stay unnoticed (masking of symptoms).

[10] COGEM advies CGM/090429-04 Klinische studie met een conditioneel-replicerende adenovirale vector.
COGEM advies CGM/091021-02 Aanvullende informatie over een klinische studie met conditioneel-replicerende adenovirussen.
COGEM-advies COGEM advies CGM/110112-01 Verzoek tot wijziging vergunning fase I/II klinische studie met conditioneel-replicerende adenovirussen

[11] Ministerie van Infrastructuur en Milieu: Lijst van pathogene micro-organismen en agentia - December 2010

Altered tropism	-	Introduction of the RGD-motive in the virus coat allows the virus to recognise certain integrines on cell membranes, apart from normal receptors, therefore enlarging the number of cell types the virus can transduce. The modified virus can now infect, amongst others, endothelium cells, fibroblasts and epithelium cells.
Pathogenicity of new recombinant viruses	-	Recombination between the GMO and wild-type hAd5 virus, may lead to two new variant forms: wild-type adenovirus with a deletion in the E1 gene (Ad5-Delta24) and wild-type adenovirus with an insertion of the RGD-motive (Ad5-RGD).
Dissemination	-	Healthcare workers and family members may come in contact with the genetically modified virus and become infected.
	-	The adenoviral vector may be disseminated in the environment and could lead to exposure of people not related to the trial.

Step two: Hazard characterisation

Pathogenicity	-	It is assumed that the modification does not result in an immune response shift, *i.e.* the GM virus triggers the same immune response as the wild-type adenovirus (author's assumption). In such case, the GM virus is presumed to be less pathogenic (attenuated). The rationale is that, although the RGD-motive in the GM virus allows infection of more cell types, this would not be followed by replication in resting cells and therefore not result in disease. In healthy dividing cells replication efficiency is much lower compared to wild-type and the virus only replicates for a few hours.
	-	The effect of dexamethasone on receptor and integrines may increase or decrease transduction efficiency to tumour and healthy cells. As only the patient would receive dexamethasone and no effect is envisioned on healthcare personnel, family members or the broader environment, this hazard is considered to be beyond the scope of the ERA.
Recombination/ pathogenicity	-	The first potential new variant recombinant Ad5-Delta24 is a deletion mutant of the wild-type hAd5, without new characteristics. This deletion mutant is no longer capable of replication in cells with a functional Rb-pathway, as present in 'healthy' cells. COGEM concluded that the potential risks of this Ad5-Delta24 are negligible low, as this recombinant is highly attenuated compared with hAd5.
	-	The second potential new variant recombinant Ad5-RGD consists of the wild-type adenovirus with an extended host range. Lacking the *E1A* deletion, this recombinant would be able to infect and replicate in healthy cells, possibly inducing disease. No predictions could be made about its pathogenicity as no information was available.
Recombination/ altered tropism	-	The recombinant Ad5-RGD would show an enlarged host range in comparison with the wild-type, possibly leading to more severe disease symptoms. The implications of severity of the putative consequences are, however, unknown.
Dissemination	-	Exposure to the recombinant adenovirus may result from contact at the time of administration of the GM virus (handling preparations, disposal of packaging), shedding via wound leakage, sample taking, care taking (urine, faeces), inhalation of airborne particles, and when handling waste.

Step three: Exposure assessment

Natural exposure/ protection	-	Almost the entire human population has been exposed to an adenoviral infection and has acquired (some level of) immunity against human adenoviruses (humoral and cellular). Normally, the immune system of healthy people will be able to clear the virus very quickly. This is, however, not the case for people with a reduced immune response. It is assumed that the patients that will participate would elicit an immune reaction to the vector, resulting in its inactivation.
	-	Furthermore Kupffer cells in the liver efficiently eliminate adenoviral vectors that are present in blood.
	-	These above-mentioned natural defence systems will limit the period of

potential shedding.

Recombination

- COGEM indicated that recombinations are considered as "worst-case" scenarios, as there is no scientific literature about the chance of occurrence and about the potential risks.
- Recombination may happen in patients as well as in other people that may become infected with both the GM and wild-type virus at the same time. However, both the GM and wild-type virus need to be present in the same cell. Adenovirus usually infects a small number of lung cells and is usually quickly cleared. The likelihood of co-infection of a cell is therefore very low.
- The simultaneous presence of the GM and wild-type virus in patients is low due to patient pre-screening for the presence of an active adenoviral infection.
- It is unclear whether dexamethasone would increase infection frequency in general and therefore mask a wild-type adenovirus infection. However, if the modified adenovirus would remain longer in the patient's body due to administration of dexamethasone and the patient would at the same time undergo a wild-type adenovirus infection unnoticed, the probability of recombination increases. The effects would be the same in patients having a weakened immune response regardless of dexamethasone treatment.
- Whereas *in vitro* studies have confirmed that recombination between different adenoviruses is possible, it has never been confirmed in *in vivo* studies that such recombination leads to replication competent adenoviruses.
- COGEM considered the chance of recombination that may have occurred during production of the vector batch. Based on information provided on the production method and additional quality data, the chance for such an occurrence was deemed negligible.

Dissemination

- Earlier studies showed that the virus is not able to pass the brain–blood vessel barrier. However, brain tumours may disrupt this barrier directly (invasiveness) or indirectly (due to surgery) as indicated in bio-distribution studies.
- It had been shown that shedding of adenoviral vectors (replication competent as well as replication deficient) could occur. Shedding is possible in 2 waves: before the immune system is able to respond and after several replication rounds in the tumour cells. It is unclear to what extent the different transmission routes will actually lead to virus exposure. While shedding via urine, body liquids and faeces is possible, COGEM estimated that aerosol formation (*e.g.* when sneezing) is the most likely source for secondary infection. Yet while the likelihood of such transmission could not be evaluated, COGEM expected it to be low, as replication in lung cells was deemed unlikely due to the presence of a functional Rb-pathway.
- During the administration and 24 hours after the completion, the patients reside in an isolation room with airlock. After this period the wounds should normally have healed sufficiently to prevent shedding. If not, then the patient is expected to remain until sufficient healing.
- Due to dexamethasone treatment, the GM virus may not be cleared efficiently, making it residing for a longer period in the patient's body. This could also result in a prolonged shedding period.
- Patients may leave the hospital during the trial and thereby expose other people and the environment in case of shedding. The likelihood of this scenario is deemed very small.

Step four: Risk characterisation

- By assuming safe hospital practices (author's assumption) and taking into account the appropriate measures the applicant has proposed a protocol to protect people at critical points. Due to the absence or mild disease symptoms following an infection, the risk of becoming ill because of the GM virus is negligible. Even for immunocompromised persons the risk was estimated to be low as a combination of low likelihood and a moderate potential adverse effect.
- Although recombination with a wild-type virus would possibly result in the restoration of the *E1A* gene or a wild-type adenovirus acquiring the RGD-motive, the occurrence of recombination was estimated to be very low and was considered in the context of "worst-case scenarios".

Considering such worst-case, COGEM identified that this recombinant would have an extended host range and could potentially be more pathogenic.
- Uncertainty exists about the consequences of the administration of dexamethasone in relation to the ability of the immune system to cope with the virus and as a result thereof about (the period of) shedding.
- Chance for dissemination and exposure of people different than the patients (*e.g*. hospital staff, relatives) is limited because of the biosafety management measures installed.

Step five: Risk management

- The possibility for simultaneous presence of wild-type adenovirus (and potentially leading to recombination in the patient) is reduced by
    - Excluding patients with fever and/or with reduced immune response,
    - Assessing the clinical situation of the participants,
    - Testing participants for adenoviral infections shortly before the start of the trial (proposed by applicant max 5 days and required to be reduced by COGEM to 1 day).
- The isolation room is equipped with an antechamber. The likelihood of exposure during treatment is reduced by implementing standard hospital hygiene and the following additional safety measures:
    - Contact with the patient should be as much as possible limited, in particular with other people that may be infected with wild-type virus.
    - Caretakers and visitors have to wear protective clothing (gloves, face mask, cap, watertight gown). The protective clothing needs to remain within the isolation room.
    - Bedclothes, protective gowns and disposables need to be separately removed and sterilised.
    - In case the patient needs to leave the isolation room for another treatment, then he has to wear a mouth mask and clean clothes. Caretakers would need to take the same precautions as in the isolation room. In a similar way, conditions were formulated in case of emergency evacuations.
- After removing the catheters, the wounds are to be sutured and a watertight wound dressing preventing leakage is to be applied.
- Twenty-four hours after removing catheters and after checking for wound closure, the patient is transferred to a normal hospital room without further containment. It should be mentioned that in a later advice the period in the isolation room after treatment was reduced. This was based on the fact that the applicant proposed to refresh the wound dressing every 2 hours. If no wound leakage as discovered at 3 consecutive inspections patients are allowed to leave the isolation chamber (min. 6 hours after removing the catheters).
- The measures to protect healthcare workers and visitors have to be maintained for as long as shedding was detected (see Monitoring).
- If a patient decides to leave before the end of the trial, the patient needs to take care that the chance for dissemination is limited. Recognizing that it is difficult to impose measures on a patient that has left the trial, COGEM nevertheless recommended to make sure that such patients are adequately informed to package material exposed to wound fluids in plastic and to remove it for destruction. Based on information provided by the applicant, COGEM accepted that it is very unlikely that patients would prematurely leave the trial.

Step six: Overall risk evaluation and conclusion

The GMO, the adenoviral vector Ad5-Delta24-RGD, is assessed to be less pathogenic than the wild-type adenovirus serotype 5 strain.

In the worst-case, recombination with wild-type adenovirus would result in a virus with unknown pathogenicity. This uncertainty triggers the implementation of further measures to make sure that no opportunities for recombination, either directly in the patient or after dissemination to other persons, are created.

Initially COGEM pointed out that it could not be concluded that the risks associated with the proposed activities were negligibly low despite having reviewed and selected all practically possible biosafety measures, to reduce the remaining potential risk as much as possible. Nevertheless COGEM underlined the importance of the proposed clinical trial in view of the importance of developing a treatment of multiform glioblastoma and for obtaining information on shedding. Subsequently extra information was provided on the physical and mental health of selected patients, as well as on the

plans for professional care at the patient's home, in case patients would leave the hospital before the end of the trial. Based on this additional information, the COGEM concluded that the risks of the clinical study for humans and the environment were negligibly low.

Monitoring

To gain more insight in bio-distribution, persistence and shedding of the GMO, the COGEM advised to monitor shedding more closely than suggested by the applicant. Selected excreta and secreta had to be expanded by inclusion of faeces samples to investigate whether replication in dividing tissue of the intestines is possible.

During the first week samples were to be taken every day. Sampling was to continue until shedding has not been ben detected for at least 3 consecutive days.

4. Deliberate release case: Clinical study with a *Lactococcus lactis* strain expressing recombinant human interleukine-10 (hIL-10).[12]

The bacterium *Lactococcus lactis* strain AG011 has been genetically modified to produce the therapeutic protein human interleukin-10 (hIL-10). This protein is expected to reduce the symptoms (such as pain and bloody diarrhoea) of patients suffering from inflammatory bowel disease. Local expression of hIL-10 should limit and terminate inflammatory responses and regulate the growth of several immune cells.

Preceding studies had shown that this investigational medicinal product was safe and does not survive for a long time outside the human body. An initial clinical study with humans demonstrated safety and efficacy. In that study patients were administered 4 capsules with 10^{12} cfu a day for 1 week at the hospital. The present case study will focus on a second study in which patients administer themselves the freeze-dried powder orally and/or rectally at home. The trial aims at assessing safety, tolerability and efficacy of the drug. During 28 days, patients visit the hospital once a week for sample taking (blood, urine, faeces, bowel biopsies), returning packages of the investigational product from the previous period as well as receiving the necessaries quantities for the subsequent week.

Step one: Hazard identification

Pathogenicity

- The parent strain is not pathogenic and is not capable of colonizing the bowel.
- The wild-type organism *L. lactis* could be found in bowels, *e.g.* after consumption of dairy products that contain the organism. *L. lactis* is widely used in the dairy industry for the production of buttermilk, yoghurt and cheese.
- In the advice for the initial clinical study COGEM questioned whether the presence of the hIL-10 gene would change the status in relation to pathogenicity. That initial study confirmed the safety of the product.

Dissemination

- The GMO will be dispersed in the environment, either via unused product (remnants in packages, accidental release) or via shedding.
- For the production of AG011 a weakened strain of *L. lactis* was used. In this strain all natural plasmids have been removed, resulting in the weakened parental strain *L. lactis* MG1363 that was no longer capable to survive in the natural environment. In practice, the strain could only survive under specific laboratory conditions where essential elements are supplied in the growth media.
- During the genetic modification the gene for thymine production in *L. lactis* MG1363 was replaced by the gene coding for hIL-10. Thymine is essential for DNA replication and is not available freely in nature. In consequence *L. lactis* AG011 is dependent on an external source of thymine/thymidine in order to replicate. Lack of an external source of thymine/thymidine results not only in an arrest of growth, it also initiates an early decay of the organisms, described as thymine-less death.
- During the initial clinical study, the strong biological containment of AG011 had been proven to be effective.

Step two: Hazard characterisation

Dissemination

- After renewal of the bowel content, *L. lactis* was not expected to be present anymore. It was indicated that this could take on average 3 days. During this period, shedding could occur together with faeces, released into the sewage system. The stool would not be disinfected.
- Because of the combination of the thymine/thymidine dependence and the total lack of plasmids *L. lactis* AG011 was estimated to be highly biologically contained.

[12] COGEM-advies CGM/020823-02 Advies Klinische toepassing van *Lactococcus lactis* met daarin een recombinant humaan interleukine-10 (hIL-10) gen bij patiënten met de ziekte van Crohn.
COGEM-advies COGEM-advies CGM/080821-01 Fase 2a klinische studie met L. lactis stam AG011 tegen matige ulceratieve colitis.

Step three: Exposure assessment

Dissemination

- Health care workers and family members may be exposed via empty product packaging and shedding. The environment will be exposed to the GMO via shedding.
- The freeze-dried investigational product is sensitive to heat, moist, UV etc. and will degrade quickly. The duration of shedding is expected to be short as the bacterium is not able to colonise the intestines. This was confirmed by monitoring during the first trial.
- In case of spillage normal hygienic procedures (decontamination with standard detergent or bleach) were deemed sufficient to kill the bacteria.

Step four: Risk characterisation

- In the initial clinical trial (see COGEM-advies CGM/020823-02), there remained some uncertainty, as although exposure could be limited, the effect of the GMO on healthy people was not clear. In the second trial this was no longer considered an issue (see COGEM-advies CGM/080821-01).
- Due to the biological containment and the limited exposure time the GMO was not expected to spread into the environment.

Step five: Risk management

- In the initial clinical trial (see COGEM-advies CGM/020823-02), faeces had to be inactivated (chemical toilet). Following confirmation of the efficient biological containment, normal hygiene was deemed sufficient in the second trial (see COGEM-advies CGM/080821-01). In case of a spill cleaning with soap or bleach were indicated as adequate.
- No further requirements were deemed necessary.

Step six: Overall risk evaluation and conclusion

COGEM concluded that within the limits of the proposed trial and based on the characteristics of the organism, the chance that *L. lactis* could establish in the environment was negligible low.

The overall risk of the clinical trial with hIL-10-producing *L. lactis* for human health and the environment was deemed negligibly low.

Monitoring

In the initial clinical trial (see COGEM-advies CGM/020823-02), monitoring for presence of GM bacteria in the faeces was advised to confirm the assumption that shedding will be limited in time. In the second trial (see COGEM-advies CGM/080821-01), samples are taken from the patients at weekly intervals. In order to verify the occurrence of shedding of AG011 and to monitor the effectiveness of the biological containment the ratio living to dead *Lactococci* in faeces had to be determined.