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AG013 (mouth rinse (MR) formulation of living GM *Lactococcus lactis* (*L. lactis*) bacteria, containing the human trefoil factor (*htff1*) gene)

Environmental Risk Assessment Dossier in accordance with Directive 2001/18/EC and Decision 2002/623/EC

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LIST OF ABBREVIATIONS

AG013	Mouth Rinse formulation of *Lactococcus lactis* strain sAGX0085, deficient in the gene coding for thymidylate synthase and producing human TFF1
CA	Competent Authority
CFU	Colony forming units
CT	Chemotherapy
DSMB	Data and safety monitoring board
DNA	Deoxyribonucleic acid
DS	Drug substance
EC	Ethics Committee
ERA	Environmental Risk Assessment
EFSA	European Food and Safety Authority
GCP	Good Clinical Practice
GI	Gastrointestinal
GLP	Good laboratory practices
GM	Genetically modified
GMO	Genetically Modified Organism
GMP	Manufacturing Practices
hIL-10	Human Interleukin-10
HNC	Head and neck cancer
hTFF1	Human Trefoil Factor 1
hTTF2	Human Trefoil Factor 2
hTTF3	Human Trefoil Factor 3
IMP	Investigational Medicinal Product
Laff	Lactococcal fertility factor
L. lactis	*Lactococcus lactis*
MA	Marketing Authorisation
MG1363	*L. lactis* subspecies *cremoris*, pLP712 cured (encodes genes for lactose and casein utilisation)
MR	Mouth rinse
OM	Oral Mucositis
QP	Qualified person
QPS	Qualified Presumption of Safety
pAGX0076	Plasmid carrying the hTFF1 expression cassette to modify *L. lactis*
PCR	Polymerase chain reaction
PK	Pharmacokinetics
PD	Pharmacodynamics
PthyA	Promoter of the thymidylate synthase A gene
Q-PCR	Quantitative polymerase chain reaction
RT	Radiation therapy

sAGX0085	*L. lactis* subsp. *cremoris* strain MG1363 genetically engineered to express hTFF1, deficient in the gene coding for thymidylate synthase A
Thy12	*Lactococcus lactis* strain producing humanised Interleukin 10, deficient in the gene coding for thymidylate synthase
thyA	Gene encoding thymidylate synthase A
ThyA- / ThyA+	ThyA-negative / positive strain
TFF	Trefoil Factor
usp45	Gene encoding a protein belonging to the nuclear receptor super family
UV	Ultraviolet light

INTRODUCTION

AG013 is a mouth rinse (MR) formulation of living Genetically Modified (GM) *Lactococcus lactis* (*L. lactis*) bacteria, containing the human trefoil factor (*htff1*) gene being developed as a therapeutic option and gain marketing approval for reduction in signs and the symptoms of radiation therapy (RT) and /or chemotherapy (CT) induced oral mucositis (OM).

Oral mucositis (OM) is a common, devastating toxicity of both drug and RT used for the treatment of cancer. The mucosal ulcerations associated with OM are excessively painful and debilitating and have profound clinical and economic implications. Clinically, the severity of OM ranges from focal areas of mild erythema and/or soreness reminiscent of a food burn, to diffuse erythema and full-thickness mucosal ulceration that are only marginally palliated with opioid-based analgesics (Villa and Sonis, 2015,Elting 2008). Severe OM is associated with significant morbidities including reduced oral intake and consequent weight loss, increases in use of narcotic pain medications and antibiotics, risk of local and systemic infections and febrile days, frequency of hospitalization and length of hospital stay, and unplanned and emergency room visits (Vera-Llonch *et al.*, 2007; Vera-Llonch *et al.*, 2006, Epstein, 2007). Significantly, the development of OM has a markedly negative impact on patients' quality of life (Mirabile et al., 2016; Trotti, 2003; Sonis, 2010; Elting 2008).

AG013 is composed of genetically modified *L. lactis* bacterium that stably expresses the human trefoil factor (*htff1*) gene. *L. lactis* are non-pathogenic, non-invasive, non-colonizing Gram-positive bacteria, critical in manufacturing dairy products such as buttermilk and cheese. In spite of the widespread use and massive discharge in the environment, *Lactococci* have not been identified as invasive or disruptive. Prior to the industrial use in the manufacture of dairy products, *L. lactis* may have been a commensal to specific plants. No *de novo* colonization of any other ecological niche has ever been reported.

The laboratory strain, MG1363 is designated as the recipient/parental organism for AG013 and is further restricted in its capacity for normal growth than naturally occurring *L.lactis* due to the removal of five plasmids by protoplast-induced curing. One of the plasmids plays a critical role in the bacterium's ability to process nutrients present in milk that are essential for *L. lactis* growth: lactose and caseins, providing a source for sugars (glycolysis) and amino acids respectively. MG1363 can therefore no longer survive in the natural niche of *L. lactis* and is confined to artificially supplemented culture conditions (Gasson, 1983).

AG013, a genetically modified version of MG1363, has been further restricted in its growth capacity due to the replacement of the *thyA* gene with the target therapeutic gene, *htff1* resulting in strict thymine/thymidine dependency, not only for growth but also for survival (thymine-less death).

The Trefoil Factor (TFF) family, which comprises TFF1, TFF2 and TFF3, is involved in protection of the gastrointestinal (GI) tract against mucosal damage and plays an important role in its subsequent repair. All three TFF peptides have shown a therapeutic effect in experimental models and are rapidly up regulated and secreted in an autocrine fashion in response to GI injury. Oral TFFs bind to salivary mucins and form a mucus layer over the epithelia of the mouth, acting as a physical barrier against bacteria and noxious environmental agents. Moreover, TFF peptides have wound-healing properties and are

important in protecting and healing mucosal tissues. The available non-clinical and clinical data suggest that hTFF1 provides a novel pharmacological tool for the prevention and treatment of human GI diseases. In addition, AG013 was well tolerated in rat, dog and hamsters (Appel *et al*., 2008a, 2008b, Prinsen *et al*., 2012, Caluwearts *et al*., 2012, Sonis *et al*., 2012) as well as human's studies. Two Phase 1 studies have been conducted to date, one in the USA and one in Belgium (Coulie 2012 and 2013). They both demonstrated the safety, tolerability, pharmacokinetics and efficacy of AG013 applied topically by repeat dosing. AG013 is expected to be present in the oral cavity in saliva, up to 24 hours after dosing and was not detected in fecal samples.

In summary, AG013 is a genetically modified *L. lactis* bacterium being developed to stably express the human refoil factor (hTFF1) protein for the treatment of OM in patients treated with RT and/or CT. Phase 1 clinical studies to date have demonstrated that AG013 is non-pathogenic, has restricted dispersal potential and survival and no replicating capacity when administered to humans. The risk to personnel and the environment coming into contact with the Genetically Modified Organism (GMO) is expected to be very low when AG013 is administered for use in proposed clinical studies prior to product marketing.

OBJECTIVE

The objective of this Environmental Risk Assessment (ERA) is to identify and evaluate potential adverse effects of AG013 on human health and the environment which the conduct of clinical studies to support the placing on the market of the GMO may exert, in accordance with Annex IIA of Directive 2001/18/EC.

METHODOLOGY

This ERA has been performed according to the precautionary principle using the methodology set down in Commission Decision 2002/623/EC. These general principles are:

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

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

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

- An analysis of the 'cumulative long-term effects' relevant to the release and the placing on the market is to be carried out.

1. IDENTIFICATION OF CHARACTERISTICS WHICH MAY CAUSE ADVERSE EFFECTS

1.1. Methodology

Characteristics which may cause adverse effects are considered based on the pathogenicity of the parent organism (wild-type *L. lactis* and laboratory strain MG1363), the nature of the genetic modifications made to generate AG013 and the receiving environment.

1.1.1. Parental organism

L. lactis is one of the most important micro-organisms in the dairy industry. It is critical for manufacturing dairy products like fermented milk products and cheese. When *L. lactis* subspecies *lactis* is added to milk, the bacterium uses lactose as an energy source and produces lactic acid as by-product. The lactic acid coagulates the milk, forming curds that are subsequently used to produce cheese and whey. *L. lactis* is also used to prepare pickled vegetables, beer, wine, some types of bread and sausages and other fermented foods. More recently *L. lactis*, has also been explored as a drug delivery vehicle.

L. lactis was originally isolated from raw milk and this niche remains one of the few that can sustain *Lactococcus* cultures (Hirsch, 1952). They can be found in man (Elliott *et al.*, 1991) and in animals (Klijn *et al.*, 1995a). *Lactococci* can be isolated from various environmental sources such as soil, effluent water and the skin of cattle, but none of these represent ecological niches. *Lactococcus* bacteria are thought to have plants as their natural habitat (Stark and Sherman, 1935) and have occasionally been identified in invertebrates and fish, although they are certainly not widely present there *(*Bahrndorff *et al.*, 2017*;* de Lacerda *et al.*, 2016; Opazo et al., 2016; Rungrassamee *et al.*, 2014). Environmental strains of *L. lactis* differ in a number of properties from those found in industrial starter cultures, indicating that the majority of the industrially produced *Lactococci* do not survive outside of the dairy environment (Stark and Sherman, 1935).

L. lactis lacks the ability to multiply in vivo in mammals, except in gnotobiotic mice (Gruzza et al., 1994). When live *L. lactis* were fed to animals and human volunteers, they passed rapidly through the GI tract without colonisation. Drouault and colleagues investigated the survival, physiology and lysis of *L. lactis* in the digestive tract of rats, in order to understand the fate of ingested lactic acid bacteria after oral administration (Drouault *et al.*, 1999). *Lactococci* that transited with the diet proved surprisingly resistant to gastric acidity (90-98% survival). In contrast, only 10-30% of bacteria survived in the duodenum. Viable cells were metabolically active in each compartment of the digestive tract, whereas most dead cells appeared to be subject to rapid lysis.

Klijn and co-workers reported a human feeding study using genetically marked *L. lactis* (Klijn *et al.*, 1995b). Cells could only be recovered from the faeces of the volunteers if they had passed the GI tract within3 days of ingestion, accounting for approximately 1% of the total number of cells consumed. The presence of related deoxyribonucleic acid (DNA), extracted from faeces, could be detected up to 4 days, when viable cells were no longer present.

Broad *L. lactis* multiplication can only be sustained in a select number of nutritionally favourable areas such as milk, specifically prepared meats, vegetable fermentations and laboratory culture broths. *L. lactis* cannot successfully propagate outside these very specific ecological niches, which is underscored by the fact that, despite ample opportunity –globally, live *Lactococci* are consumed and released in soil and sewage waters in tremendous amounts– no de-novo colonisation of any such niches has ever been reported.

The parental form of AG013, *L. lactis* strain MG1363 can no longer grow in milk or other natural environments. The carbon source for *L. lactis* in milk is lactose. In order to utilise this carbon source, wild type *Lactococci* are equipped with lactose utilisation genes as present on the lac operon. Furthermore, casein is used as amino acid source and PrtP protease is required to break the protein down to short oligopeptides for uptake (de Vos *et al*., 1989, Kunji *et al.*, 1995). In wild type *L. lactis*, the genes for both essential functions are carried by the pLP712 plasmid. As all plasmids were removed during the isolation of MG1363, the strain has essentially lost the capacity to access its main energy and amino acid sources. In consequence, the habitat of MG1363 is confined to artificially supplemented culture conditions.

L. lactis can grow at temperatures between 10°C and 45°C, with an optimum at 30°C. It is susceptible to infection by bacteriophages, the most important being 936, c2 and P335.

1.1.2. Genetic modifications resulting in AG013

The genetic modification introduced into the MG1363 *L. lactis* strain to produce the GMO sAGX0085 strain or AG013 are summarised below. These modifications reduce further the capacity of the GMO strain to growth or reproduce outside the artificially supplemented culture conditions provided in the laboratory and does not have any effect on the host range, stability or genetic capability of the wild-type bacteria.

The modification was based on targeted gene replacement by double homologous recombination and used a carrier plasmid, pAGX0076. Replacing the *L. lactis* MG1363 *thyA* gene and promoter by the new hTFF1 expression cassette was performed by double homologous recombination at 1 kb target areas 5' and 3' of *thyA* (5' and 3' target areas) (essentially as described, Steidler *et al*., 2003). The resulting *L. lactis* strain, sAGX0085 (AG013), carries the [Pxxx>>SSusp45>>hTFF1] DNA construct at the place of the *thyA* gene.

A full genome sequencing program was conducted and confirmed the predicted DNA sequence of AG013 strain sAGX0085 (Steidler *et al.*, 2003). Unexpected foreign DNA was not detected in AG013 and the sequence was as designed. Neither residual sequences of the *thyA* gene, nor the integration vector pAGX0076 other than the DNA construct and the *thyA*-flanking regions could be detected.

The removal of *thyA* gene in the generation of AG013 confers an additional safety feature than the ones already present in MG1363. The auxotrophic nature of the sAGX0085 strain (AG013) was confirmed by the decreased in viability of the bacterium in the absence of

thymidine which is identical to that of other *thyA*-deficient *L. lactis* strains (Steidler *et al.*, 2003; Vandenbroucke, 2008).

1.1.3. Receiving Environment

Following the approval of the proposed clinical studies by the appropriate national Competent Authorities (CA) and Ethics Committees (EC) in the selected countries, AG013 will be administered to humans as part of a clinical study to assess its safety, tolerability and therapeutic effect in the treatment of OM in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy.

The long-term objective is to develop AG013 to Marketing Authorisation (MA) where AG013 will be available across the European Community as a prescription medicine for the treatment of OM in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy.

AG013 is currently being developed as a MR formulation for use three times per day beginning from the start of RT until 2 weeks following its completion. Following sufficient rinsing in the mouth, AG013 is discarded into the sink/toilet. The active treatment phase lasts for 7 to 9 weeks, depending on the duration of chemoradiation therapy. The first treatment will take place in the clinical setting and subsequent dosing will occur outside of a clinical centre. It is expected that AG013 is released into the sewage system for a short time after administration.

1.2. Characteristics which may cause adverse effects

1.2.1. Effects on the environment

1.2.1.1. Effects on environmental processes

L. lactis is a non-pathogenic, non-invasive, non-colonizing Gram-positive bacterium critical in manufacturing dairy products. In spite of the widespread use and massive discharge in the environment, *Lactococci* have not been identified as invasive or disruptive. Although they can be found in very diverse sources (soil, manure, waste water), the bacteria depend on particular nutritional components for growth. MG1363 (AG013 parental strain) is restricted even more and as such confined to artificially supplemented culture conditions. In addition, the GMO (AG013) is dependent on thymine/thymidine supplementation.

There is no indication that AG013 itself is toxic, allergenic or pathogenic. The changes that were induced in the recipient strain MG1363 to result in the AG013, do not affect the basic toxic or allergenic features. In the unlikely event of infection, the GMO can quickly and easily be inactivated with standard antibiotics.

In a Phase 1 healthy volunteer study of AG013, consistent levels of AG013 (bacterial count and hTFF1 protein) could be recovered from the different sample sites in the oral cavity up to 24 hours after dosing. These data demonstrate that live AG013 bacteria adhere to the oral mucosa and actively secrete protein at the mucosal surface. There was no evidence for

systemic exposure neither to live AG013 bacteria (blood) nor to hTFF1 secreted (serum) and there was no recovery of live AG013 bacteria in faeces.

1.2.1.2. Transmission to non-human organisms in the environment

No specific interactions with non-target organisms have been identified. AG013 will be present in compartments which are natural for *L. lactis*, essentially the human GI and sewage system. With the exception of *htff1*, no other change has occurred and therefore it is expected that the impact will be similar or less than that of *L. lactis*. The possible interactions will be more limited given the specific growth disadvantage of AG013 and reduced life expectation as a result of being *thyA*-deficient strain.

The therapeutic protein, hTFF1 binds to salivary mucins and forms a mucus layer over the epithelia of the mouth, acting as a physical barrier against bacteria and noxious environmental agents. Moreover, TFF peptides have wound-healing properties and are important in protecting and healing mucosal tissues. In the pre-clinical trials healthy and diseased hamsters, rats and dogs have been administered AG013 without adverse effects. No local or systemic pathogenic effects were documented after chronic administration of AG013.

1.2.1.3. Transfer of genetic material into the environment

Exchange of genetic material between bacteria mostly occurs through plasmids. While different *L. lactis* strains, including those used in the dairy industry, can harbour several plasmids, MG1363 and AG013 have lost the 5 original plasmids present in the natural isolate. In addition, *L. lactis* MG1363 and AG013 do not contain conjugative transposons and AG013 is also *thyA* deficient, preventing phage replication (Pedersen *et al.*, 2002). Therefore, transduction of modified genetic material via phages is very unlikely.

Conjugative transposons, such as *Tn916*, are elements that transpose during conjugation from a donor cell harbouring the element to a recipient cell. Conjugative transposons have a broad host range: they are not only able to conjugatively transpose with frequencies of 10^{-4} to 10^{-9} among almost all species of gram-positive bacteria that have been investigated but can also transpose among gram-negative bacteria.

The first example of a limitation on the promiscuity of conjugative transposons is presented by *L. lactis* MG1363. In this strain, *Tn916* and *Tn919* do not excise (Bringel *et al.*, 1992). Although the MG1363 strain can act as a recipient for conjugative transposition from another genus, it has not been found to donate conjugative transposons in plate matings with *Bacillus subtilis*, *Enterococcus faecalis* or *Streptococcus pyogenes*. In intrageneric matings between *L. lactis* MG1363 derivatives, transconjugants can be established but the transposons will be present in the same location in the transconjugant chromosome as in the donor genome, indicating that no transposition has occurred. It is thought that *L. lactis* MG1363 lacks a factor required for excision of conjugative transposons (Bringel *et al.*, 1991).

Conjugative transfer of selectable, chromosomal markers from MG1363 to MG1363 derivatives has been reported for lactococcal fertility factor (*Laff*) (Bringel *et al.*, 1991). *Laff* is speculated to be identical to Clu/sex-factor (Gasson *et al.*, 1983; Stentz *et al.*, 2004)

however summarize the general knowledge that a) over a wide range of bacterial genera, cell aggregation provides the first cell-to-cell contact that is necessary for conjugal transfer; and b) cell aggregation has only been observed following sex factor and lactose plasmid cointegration. The lactose plasmid in MG1363 is absent (Gasson *et al.*, 1983, Wegmann *et al.*, 2007) as confirmed by full genome sequencing of MG1363. It is highly unlikely that MG1363 or its derivatives can serve as a conjugative donor, and, especially non-selectable, chromosomal traits could propagate into potential recipient (i.e. MG1363 related) populations.

It is possible that genetic elements could be released in the environment upon lysis of *L. lactis* and might be taken up by other bacteria. In the case of the GMO, the likelihood of release of intact naked DNA is reduced as thymine-less death triggers the degradation of DNA before the actual cell lysis.

None of the genetic modifications made to wild type *L. lactis* during construction of AG013 would be expected to enable the transfer or maintenance of genetic material into the environment (outside its obligate host species).

1.2.2. Effects on human health

1.2.2.1. Direct effects on human health

Transmission of AG013 to an unintended human recipient

L. lactis was originally isolated from raw milk and this niche remains one of the few that can sustain *Lactococcus* cultures (Hirsch, 1952). They can be found in man (Elliott *et al.*, 1991) and in animals (Klijn *et al.*, 1995a). *Lactococci* can be isolated from various environmental sources such as soil, effluent water and the skin of cattle, but none of these represent ecological niches. Environmental strains of *L. lactis* differ in a number of properties from those found in industrial starter cultures, indicating that the majority of the industrially produced *Lactococci* do not survive outside of the dairy environment (Stark and Sherman, 1935).

AG013 is being administered to patients as part of a clinical study to investigate its safety and efficacy in patients with OM as a result of concomitant chemoradiation therapy for the treatment of head and neck cancers (HNC). The first treatment will occur in a clinical setting where instructions will be provided to patients on the safe and controlled preparation and administration of subsequent doses in their home setting. Participants will rinse their mouths with the suspension for 30 seconds three times each day using AG013 or placebo, depending on their assignment. The suspension will then be expectorated into a sink or toilet. It is not intended to be swallowed. Unintended humans that could inadvertently come into to contact with AG013 could be medical staff or close family contacts that could be exposed to AG013 via aerosol or by contact of a surface or material such as AG013 bottle or packaging that contains the GMO.

AG013 is non-replicative, non-infectious and non-pathogenic. In addition, in the event of transmission to an unintended human, the number of viable bacteria will be considerably lower than the dose used by the patients. Phase 1 clinical data demonstrated that consistent levels of AG013 (bacterial count and hTFF1 protein) could be recovered from the different

sample sites in the oral cavity of trial participants up to 24 hours after dosing. It is expected that live AG013 bacteria adhere to the oral mucosa and actively secrete protein at the mucosal surface.

No specific interactions with non-target organisms have been identified. AG013 will be present in compartments which are natural for *L. lactis*, essentially the human GI and sewage system after administration by trial participants and not expected to be a risk to unintended human recipients. With the exception of *htff1* gene, no other change has occurred and therefore it is expected that the impact will be similar or less than that of *L. lactis*. Again, the possible interactions will be more limited given the restricted replicative capacity and reduced life expectation of AG013 comparted to the laboratory strain (MG1363) and wildtype *L. lactis*.

Capacity for genetic transfer between humans and AG013

AG013 does not infect human cells and tissues. It is being developed to express the hTFF1 protein which binds to salivary mucins and forms part of the mucus layer over the epithelia of the mouth, acting as a physical barrier against bacteria and noxious environmental agents. Moreover, TFF peptides have wound-healing properties and are important in protecting and healing mucosal tissues. In addition, in the two phase 1 clinical studies of AG013 there was no evidence for systemic exposure neither to live AG013 bacteria (blood) nor to hTFF1 secreted (serum). There was no recovery of live AG013 bacteria in fecal samples in the Phase 1 healthy volunteer study.

AG013 has been used in two clinical studies to date and was well tolerated with no significant adverse events. AG013 contains no plasmids or conjugative transposons and is *thyA* deficient, preventing phage replication (Pedersen *et al*., 2002). Therefore, transduction of modified genetic material via phages is very unlikely. Theoretically, genetic elements could be released into the environment upon lysis of AG013 and might be taken up by other bacteria but not human cells. The likelihood of release of intact naked DNA is reduced further in AG013 compared with its parental strain as thymine-less death triggers the degradation of DNA before the actual cell lysis.

In summary, the capacity for genetic transfer between humans and AG013 is limited and this risk is expected to be very low or negligible.

1.2.2.2. Indirect effects on human health

Transmission of a genetic variant of AG013 to an unintended human recipient

AG013 is an Investigational Medicinal Product (IMP) and is manufactured to Good Manufacturing Practices (GMP). After manufacturing it is thoroughly tested to confirm identity by specific manufacturing release tests with defined specifications and acceptance criteria. A batch of AG013 will not be released and used in the proposed clinical studies unless the specifications are met. For Drug Substances (DS), the presence and genetic identity are verified for the htff1 gene, including the promotor/secretion leader locus (hTFF1 expression cassette [Pxxx>>SSusp45>>htff1]).

During manufacture of the DS, approximately 20 generations of growth take place starting from the master seed. Analysis of genetic stability of *L. lactis* strain sAGX0085 (AG013) was performed on cultures obtained after a minimum of 100 generations of growth. Genetic stability was absolute for all of the parameters, for the following reasons:

1. All of the obtained sAGX0085 clones were unable to grow in thymidine-deficient medium.
2. All of the obtained sAGX0085 clones retained their initially reported high secretion level of hTFF1.
3. All of the sAGX0085 siblings analyzed, showed the correct Polymerase chain reaction (PCR) profile of the modified thyA locus, indicating the absence of [PthyA>>*thyA*] and the presence of [Pxxx>>SSusp45>>*htff1*].
4. DNA sequencing revealed that the [Pxxx>>SSusp45>>*htff1*] expression cassette of *L. lactis* strain sAGX0085 was unchanged in all of the sAGX0085 siblings tested.

AG013 contains no plasmids or conjugative transposons and is thymine/thymidine dependent due to *thyA* deficiency, severely hindering its own replication in the absence of thymidine/thymine as well as preventing phage replication

For AG013 to revert to its parental forms it would need to gain the thymidine gene and the five plasmids that are absent. The risk of this is infinitely small. In addition, wild type *L. lactis* are non-pathogenic, are, being used commercially in the dairy industry and distributed and released globally. Environmental strains of *L. lactis* differ in a number of properties from those found in industrial starter cultures, indicating that the majority of the industrially produced *Lactococci* do not survive outside of the dairy environment (Stark and Sherman, 1935) indicating that the risks to unintended human recipients is negligible.

Capacity for genetic transfer between humans and a genetic variant of AG013

AG013 and its possible genetic variant do not have the potential to transfer DNA into human cells. It does not have the capacity to infect human cells but also the heterologous expression unit is not known to be active in human cells. The only relevant risk is transfer of an intact *thyA* gene into *L. lactis* (Steidler *et al.*, 2003)

Under artificial laboratory conditions, a vector plasmid carrying an intact *thyA* has been shown to compensate a *thyA* mutation following electroporation into different bacteria (Ross *et al.*, 1990). However, this experimental scenario is unlikely in *L. lactis* bacteria, as they are not known to be naturally competent and therefore will not take up foreign DNA from other bacteria (Wydau *et al.*, 2006). Further, to the best of our knowledge, in the *Bacteriae* and *Archaeae*, *thyA* genes do not reside on plasmids, so plasmid-borne mobility of *thyA* inwards seems impossible. Closely related *Lactococcus* species could mobilise DNA into the GMO but again, mobile elements are unlikely to be present. Moreover, successful establishment of donor DNA would require double homologous recombination, which would resolve the GM trait.

Steidler and colleagues evaluated the possible integration of an intact wild type *thyA* sequence in strain Thy12, requiring double homologous recombination over the transgene and essentially removing the transgene (Steidler *et al.*, 2003). Donor bacteria were *L. lactis* MG1363, *L. lactis* subsp. *lactis* and subsp. *cremoris*, *Lactobacillus casei*, *Escherichia coli* subsp. DH5alpha and O157, and *Salmonella choleraesuis*. In none of the cases, forced acquisition of *thyA* from other microorganisms could be demonstrated, most likely due to high sequence diversity at *thyA* loci.

Compensation of the *thyA* deletion would be insufficient to circumvent the other specific metabolic requirements of *L. lactis* strain sAGX0085 (AG013) and its parent organism (inability to degrade lactose and casein). Also, because *lactococci* are not documented to multiply in the GI tract, there is no selective pressure on the acquisition of *thyA*.

1.3. Conclusions

In conclusion, based on the nature of the GMO, the parental organism and the receiving environment, the potential adverse effects which AG013 may exert by use in clinical studies and subsequently being placed on the market as a medicinal product are limited for the following reasons:

- Similarly, to its parental strain it is non-pathogenic. *L. lactis* is distributed globally and is used in the dairy industry with no adverse effects being reported in relation to its contact with humans, animals and the environment.
- Similarly, to its parental strain, AG013 is a poor competitor and has a limited ecological niche.
- AG013 is devoid of the metabolic pathways that enable the use of milk carbohydrate and amino acid sources and can therefore no longer grow in its ancestral ecological niche.
- AG013 is dependent on external supplementation of thymine or thymidine for growth and survival. Indeed, the thymidylate synthase gene was removed. When deprived of thymine and thymidine, an inherent suicidal system, “thymineless death” is induced.
- The transfer of genetic material between humans and AG013 and any variants of AG013 are expected to be unlikely due to the points listed above.

2. EVALUATION OF POTENTIAL CONSEQUENCES / MAGNITUDE OF EFFECT

The potential consequences of the possible adverse effects on human health identified in Section 1 of the ERA are considered in this section.

In the case of transmission to an unintended human recipient, the consequences for the individual are expected to be minimal. However, it is appropriate to consider potential scenarios in immune-competent healthy individuals, immune-compromised individuals (the very young or elderly) and those who are immunosuppressed due to an underlying condition or therapy (e.g. HIV patients, transplant recipients, cancer patients undergoing certain

therapy). It is also important to consider possible effects on the unborn child in the case of transmission to a pregnant woman.

2.1. Direct effects of the transmission of AG013 to an unintended human recipient

2.1.1. Magnitude of effect

Cases of the exposure of AG013 to an unintended human recipient are likely to be isolated. The medicinal product will be administered to (and administered by) a limited number of individuals.

The most likely individuals who may be affected would be:

- Health workers involved in the initial administration of AG013
- Health workers or laboratory staff involved in analysis of biological samples.
- Close contacts of the treated individual (partners and family members)

The capacity for widespread dissemination of AG013 is expected to be severely limited due to:

- *L. lactis* strains are already distributed in the environment, globally and are non-infectious and non-pathogenic.
- AG013 is being used in clinical studies where its use is controlled, and all containers/packaging used and destroyed are monitored as per Good Clinical Practise (GCP) guidelines related to IMP accountability.
- Only designated, trained healthcare professionals will distribute AG013 to patients who will be given instruction on its preparation, administration and disposal at the home setting as well as risks for transmissions and procedures in case of spillage.
- AG013 is expected to be released into the sewage system however it has limited survival or replicative capacity outside the laboratory setting.

2.1.2. Consequences of transmission of AG013 to an unintended individual

2.1.2.1. Potential effects of the transgene

The only gene inserted into AG013 is *htff1* gene. The therapeutic protein, hTFF1 binds to salivary mucins and forms a mucus layer over the epithelia of the mouth, acting as a physical barrier against bacteria and noxious environmental agents. Moreover, TFF peptides have wound-healing properties and are important in protecting and healing mucosal tissues.

2.1.2.1.1. Potential toxicity of *htff1* gene

The *htff1 gene* is expressed in mucosal cells and human TFF1 protein is secreted by human salivary glands and acts as a barrier against bacteria and noxious environmental agents (Devine *et al*., 2000). In non-clinical studies, healthy and diseased hamsters, rats and dogs

have been administered AG013 encoding the *htff1* gene without adverse effects. No local or systemic pathogenic effects were documented after chronic administration of AG013.
Two Phase 1 studies have been conducted to date with AG013 encoding *htff1* gene, one in the USA and one in Belgium. They both demonstrated the safety, tolerability, pharmacokinetics and efficacy of AG013 applied topically by repeat dosing over several weeks.

2.1.2.1.2. Potential oncogenicity of *htff1 gene.*

In conducting a PUBMED search using the search terms oncogenicity AND *htff1* gene (March 2018) there were no publications matching this search criterion.

2.1.2.1.3. Potential for alteration of cell cycle

In conducting a PUBMED search using the search terms cell cycle AND *htff1* gene [Title/Abstract] (March 2018) there were no publications matching this search criterion.

2.1.2.2. Potential effects of the bacteria

2.1.2.2.1. Virulence and Pathogenicity

L. lactis is non-pathogenic, non-invasive, non-colonizing Gram-positive bacterium, critical in manufacturing dairy products. In spite of the widespread use and massive discharge in the environment, *Lactococci* have not been identified as invasive or disruptive. Although they can be found in very diverse sources (soil, manure, waste water), the bacteria depend on particular nutritional components for growth. MG1363 (AG013 parental strain) is restricted even more and as such confined to artificially supplemented culture conditions. In addition, the GMO (AG013) is critically dependent on thymine/thymidine supplementation for growth and survival.

There is no indication that the AG013 itself is toxic, allergenic or pathogenic. The changes that were induced in the recipient strain MG1363 as well as in the AG013, do not affect the basic toxic or allergenic features. In the unlikely event of infection, the GMO can quickly and easily be inactivated with standard antibiotics.

Animal models:
In the pre-clinical trials healthy and diseased hamsters, rats and dogs have been administered AG013 without adverse effects. No local or systemic pathogenic effects were documented after chronic administration of AG013.

Safety pharmacology, Pharmacodynamics (PD) and Pharmacokinetics (PK) studies using AG013 established that:

- That limited systemic absorption of AG013 is likely as confirmed by in vivo study to determine the risk for systemic absorption of live *L. lactis* bacteria in hamsters in which concomitant OM and severe neutropenia (induced by myelosuppressive

agents) were present. There were no indication of clinical infections, i.e. bacteria could survive neither the systemic circulation, nor in the peripheral tissues.

- Systemic absorption of the bacteria did not cause systemic infection, even under neutropenic circumstances in an *in vivo* study in severely neutropenic rats (induced by myelosuppressive agents). IV inoculation was used which represents the worst-case scenario where AG013 would enter the blood stream in neutropenic human subject.
- The engineered bacteria are metabolically active in the oral cavity (hamsters) and GI tract (rats and dogs), and that neither the bacteria, nor the secreted hTFF1, enter into the systemic circulation.
- that the hTFF1 expression cassette in healthy hamsters is cleared from the oral cavity and oropharyngeal-GI with similar kinetics as the bacteria and confirmed that the expression cassette does not accumulate in tissues and organs that are predisposed by the route of administration.

The repeated dose toxicology studies (3 Good Laboratory Practice [GLP] studies in 2 healthy animal species) up to 3-month duration did not indicated any treatment-related effects. All safety pharmacology and toxicology studies were found to be negative for side effects of administration of *L. lactis* and therefore support safety administration of AG013 to OM patients at risk of developing RT and/or CT induced neutropenia, whose non-intact oral mucosa might represent additional risks for bacteremia.

Clinical trials:

To date, AG013 has been studied in humans in a Phase 1b study (AG013-ODOM-101) in the US and a Phase 1 pharmacokinetic (PK) study in healthy volunteers (AG013-CSM-MU-004) in Belgium.

The Phase 1b study was a multicenter, single-blinded, placebo-controlled, sequential dose-escalation study that evaluated the safety, tolerability, and PK profile of AG013 in subjects experiencing OM during induction chemotherapy for the treatment of Head and Neck Cancer (HNC) [ClinicalTrials.gov Identifier: NCT00938080].

A total of at least 21 subjects were planned to be enrolled in 3 successive groups of at least 7 subjects each (at least 5 subjects were assigned to AG013 and at least 2 subjects were assigned to placebo). An independent data and safety monitoring board (DSMB) reviewed the safety results from each group prior to dose escalation. The study achieved its primary objective by demonstrating that AG013 was generally safe and well tolerated. The incidence of sepsis due to AG013 was followed as an event of special interest in this clinical study and no subjects experienced an adverse event of this type.

The second objective of the study was to evaluate the PK of AG013. In general, live bacterial levels of AG013-sAGX0085 were high immediately following dosing and decreased by 90 minutes post-dose. No differences were noted among the active treatment groups; no dose relationship was seen. In all treatment groups, AG013-sAGX0085 levels were 0 by the End Of Study visit. AG013-sAGX0085 could not be detected in blood. No dose frequency-related differences in hTFF1 levels could be detected in saliva or oral mucosa amongst the

active treatment groups. Levels of hTFF1 in serum were not significantly different between treatments groups at all time points measured.

The Phase 1 PK study in healthy volunteers was a single-center, open-label Phase 1 study to assess the effect of food/beverage and to characterize the PK of single and multiple oral doses of AG013 in healthy subjects. Ten subjects were enrolled in the study.

AG013 was generally safe when applied by mouth rinse once or three times on one day. Overall, consistent levels of AG013 (bacterial count and protein) could be recovered from the different sample sites in the oral cavity, up to 24 hours after dosing. Furthermore, live AG013 bacteria levels coincided with protein levels. These data demonstrate that live AG013 bacteria adhere to the oral mucosa and actively secrete protein at the mucosal surface. This results in homogeneous exposure to the entire mucosal surface. There was no evidence for systemic exposure neither to live AG013 bacteria (blood) nor to hTFF1 secreted (serum) and there was no recovery of live AG013 bacteria in feces.

Overall, the *in vivo* safety pharmacology studies, and the 2 completed phase I studies support safe administration of AG013 for attenuation of OM in patients with cancer of head and neck receiving concomitant chemoradiation therapy.

2.1.2.2.2. Potential to cause encephalitis

L. lactis is distributed globally and is used in the dairy industry with no adverse effects being reported in relation to its contact with humans, animals and the environment.

2.1.2.2.3. Potential for reversion to wildtype

For AG013 to revert to its parental forms it would need to gain the thymidine gene and the five plasmids that are absence. The risk of this is negligible. In addition, *L. Lactis* is distributed globally, being used commercial in the dairy industry and is non-pathogenic. Environmental strains of *L. lactis* differ in a number of properties from those found in industrial starter cultures, indicating that the majority of the industrially produced *Lactococci* do not survive outside of the dairy environment (Stark and Sherman, 1935) indicating that the risks to unintended human recipients is negligible.

2.1.2.2.4. Susceptibility to antibiotics agents

L. lactis bacteria are sensitive to a wide array of antibiotics including ampicillin and other beta-lactams (oxacillin, penicillin, pipericillin), cephalosporin, chloramphenicol, erythromycin, amikacin, gentamicin, tetracycline, sulphonamide, trimethoprim/sulfamethoxazole and vancomycin. Somewhat lowered susceptibility was reported towards carbenicillin, ciprofloxacin, dicloxacillin and norfloxacin, while intrinsic resistance was observed towards colistin, fosfomycin, pipedimic acid and rifamycin (De Fabrizio *et al*., 1994).

The following antibiotics susceptibility/resistance profile of MG1363 was determined as part of a nonclinical program:

- MG1363 are resistant to metronidazole (metronidazole), nalidixic acid (first generation quinolone), trimethoprim, sulfamethoxazole and a combination of both in a ratio of 1/19 (sulfonamides).

- MG1363 are sensitive to all other tested antibiotics: gentamicin (aminoglycoside), imipenem (carbapenem), vancomycin (glycopeptide), clindamycin (lincosamide), erythromycin (macrolide), nitrofurantoin (nitrofuranes), linezolid (ozazolidinones), ampicillin, amoxicillin, penicillin G (penicillins), chloramphenicol (phenicole), bacitracin (polypeptide), ciprofloxacin and levofloxacin (second and third generation quinolones), tetracycline (tetracycline) and cefepime (third generation cephalosporin).

MG1363 is the parental strain of AG013 therefore it is expected to have similar susceptibilities to antibiotics.

2.1.2.3. Observed effects of AG013 in patients

To date, AG013 has been studied in humans in a Phase 1b clinical study (AG013-ODOM-101) and a Phase 1 PK study in healthy volunteers (AG013-CSD-MU-004). A total of 17 patients undergoing chemotherapy for HNC and 10 healthy volunteers have been administered AG013.

AG013 was generally safe when applied by mouth rinse once or three times on one day.

In the Phase 1 healthy volunteer study, consistent levels of AG013 (bacterial count and protein) could be recovered from the different sample sites in the oral cavity, up to 24 hours after dosing. Furthermore, live AG013 bacteria levels coincided with protein levels. These data demonstrate that live AG013 bacteria adhere to the oral mucosa and actively secrete protein at the mucosal surface. This results in homogeneous exposure to the entire mucosal surface. Exposure to AG013 was reduced by food intake while the intake of a beverage did not impact on exposure to AG013. There was no evidence for systemic exposure neither to live AG013 bacteria (blood) nor to hTFF1 secreted (serum) and there was no recovery of live AG013 bacteria in faeces.

For the Phase 1b study, in subjects that received active treatment, levels of live AG013 bacteria were detected immediately and at 90 minutes after rinsing in oral mucosal and saliva samples on days 1, 7, and 14. No bacteria could be detected at the end of study visit. The number of live AG013 bacteria recovered decreased by 90 minutes after dosing. Overall, oral bacterial levels were equivalent on days 1, 7, and 14 in all active treatment groups. The levels of hTFF1 at the oral mucosa, in saliva, and in serum were not significantly different between the treatment groups at all time points measured.

Overall, the two-completed phase I studies support safe administration of AG013 for attenuation of OM in patients with cancer of head and neck receiving concomitant chemoradiation therapy.

2.1.2.4. Likely effects of AG013 in unintended individuals

2.1.2.4.1. Effects in immune-competent individuals

Exposure of immune-competent individuals to AG013 is unlikely to produce any adverse effects, for the following reasons:

- Its non-pathogenic similarly to its parental strain. *L. lactis* is distributed globally and is used in the dairy industry with no adverse effects being reported in relation to its contact with humans, animals and the environment.
- It is highly susceptible to treatment with a broad spectrum of antibiotics

2.1.2.4.2. Effects in immune-compromised individuals

Exposure of immune-compromised individuals to AG013 is unlikely to produce any adverse effects for the reasons listed in Section 2.1.2.4.1. However, data to support this is limited and not available for AG013. On conducting a PUBMED search (05Apr2018) using the search terms (*L. lactis* [Title/Abstract] AND immunocompromised [Title/Abstract]) and (*L. lactis* [Title/Abstract] AND vulnerable groups [Title/Abstract]) there were no hits.

In the proposed phase 2 study special attention is given to clinically significant bacteraemia and clinical sepsis, which should be recorded as AE or SAE. In the event that a subject develops symptoms suggesting clinically significant bacteraemia or sepsis, the subject should be treated per the site's standard of care, including commonly used antibiotics.
A disposition plan for the management of clinical sepsis is provided in the clinical study protocol.

2.1.2.4.3. Potential effects on the unborn child

Potential effects on an unborn child are unlikely to produce any adverse effects for the reasons listed in Section 2.1.2.4.1. In addition, systemic absorption from treatment withAG013 is not expected to occur.

2.2. Indirect effects of the transmission of a genetic variant of AG013 to an unintended human recipient

2.2.1. Magnitude of effect

As with *L. lactis* and the parental strain of AG013, cases of the transmission of a genetic variant of AG013 to an unintended human recipient are likely to be **very low** (see Section 2.1.1).

2.2.2. Consequences of transmission of a genetic variant of AG013 to an unintended individual

The possibility for the generation of genetic variants of AG013 is very low for the reasons discussed in section 2.1.1. The consequences of transmission of a genetic variant of AG013 to an unintended individual are negligible.

2.3. Conclusions

The potential magnitude of unintended spread within the human population is considered very low, given the attenuated nature of the GMO. For those unintended individuals that may be exposed to AG013 or any possible genetic variants, the adverse effects are expected to be very low as they will have already been exposed to *L. lactis* strains, AG013 is further attenuated than wildtype *L. lactis* and the parental stain resulting in its in ability to replicate and survival outside of a selective growth environment. In the very unlikely event that AG013 comes into contact with a vulnerable group e.g. immunocompromised individual resulting in sepsis, AG013 is susceptible to a wide range of antibiotics. In the proposed phase 2 clinical study special attention is given to clinically significant bacteraemia and clinical sepsis, which will be recorded as AE or SAE.

In conclusion, the potential consequences in the case of transmission of AG013 or its possible genetic variants are expected to be very low.

3. EVALUATION OF LIKELIHOOD OF OCCURRENCE OF IDENTIFIED ADVERSE EFFECT

The likelihood of the occurrence of the possible adverse effects on human health identified in Section 1 of the ERA is considered in this section.

3.1. Likelihood of Direct effects of the transmission of AG013 to an unintended human recipient

The potential direct adverse effects, magnitude and consequence of any potential effects described in the preceding sections of the ERA are dependent on the likelihood of exposure of unintended recipients to AG013

This in turn is influenced by the manner, scale and environment of release, the potential mechanisms of exposure and the specific risk management measures in place to minimise exposure.

3.1.1. Manner, scale and environment of release

The proposed clinical studies will be initiated after approval by the appropriate national CA and Ethics Committees in the selected countries. AG013 will be administered to humans as part of a clinical study to assess its safety, tolerability and therapeutic effect in the treatment of OM in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy.

The long-term objective is to develop AG013 to Marketing Authorisation (MA) where AG013 will be available across the European Community as a prescription medicine for the treatment of OM in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy. Thereafter, it is expected to be used routinely in this indication throughout the European Community in accordance with its approved product labelling.

AG013 is lyophilised powder of bacterial strain sAGX0085 (2 x 10^{11} Colony forming Units [CFU]**)** mixed with cryoprotectants, formulated for oral administration as a MR. This MR suspension is prepared by adding a solution containing water, an aroma and a sweetener to the AG013 powder mixed with cryoprotectants. The AG013 powder is packed in clear glass vials with tamper-evident, child-resistant screw caps. Three of these vials are (for one day of dosing) in turn packed into a sealed aluminium bag. The solution for re-suspension (reconstitution) is delivered in a dark glass bottle with tamper-evident, child-resistant screw caps.

AG013 will be delivered to the clinical trial centre pharmacy as single dose packages that do not require further manipulation. Direct contact with the lyophilised powder is excluded until opening of the single doses. Only at the moment of administration, exposure to the suspended powder is possible. The MR is applied for 30 seconds and then the suspension is expectorated into a sink or toilet. The MR is not expected to be swallowed reducing the dose of bacteria that could be shed via faecal matter.

A total of 200 participants will be involved in the study worldwide: 140 in the US and 60 in Europe divided equally between Belgium and Germany. Therefore, in Belgium, and Germany, approximately 15 participants will be recruited per country in the AG013 treatment group. Based on the treatment design and the number of subjects planned to be enrolled, it can be estimated that a maximum quantity of 5.6x10^{14} CFU will be released per country (supposing that every AG013 bacteria in the mouth rinse is released viable, which is impossible and a worst-case scenario).

3.1.2. Potential mechanisms of exposure and risk management measures

3.1.2.1. Mechanism of transmission and survivability

The first dose of AG013 is administered in the clinical trial centre supported by trained healthcare professionals to ensure the patient understands how to store, prepare, administer and dispose of AG013, bottles and packaging contaminated with the GMO. Subsequent dosing is conducted on an ambulatory/outpatient basis, i.e. participants do not reside in the clinical study centre during administration of further doses. Detailed instructions will be provided to patients including information on its controlled storage, dose preparation, dispensing and discarding material contaminated with the GMO.

AG013 will be primarily released into the environment at the clinical setting for the first dose and then at the participant's home into the sink or toilet for subsequent doses. In addition, it maybe be shed at a very low dose in saliva shortly after administration. During the active treatment phase the participants will come for observations twice weekly. This frequency is reduced to once weekly in the short-term follow-up phase.
The risk of shedding via the faecal route is expected to be insignificant as patients are not required to swallow AG013. It is expected to be held for 30 seconds in the mouth and then discarded into the sink or toilet. During the Phase 1 studies, live bacteria were not detected in faecal matter.

Klijn and co-workers reported a human feeding study using genetically marked *L. lactis* (Klijn *et al.*, 1995b). Cells could only be recovered from the faeces of the volunteers if they

had passed the GI tract within 3 days of ingestion, accounting for approximately 1% of the total number of cells consumed. The presence of related DNA, extracted from faeces, could be detected up to 4 days, when viable cells were no longer present.

In the Phase 1 healthy volunteer study, consistent levels of AG013 (bacterial count and protein) could be recovered from the different sample sites in the oral cavity, up to 24 hours after dosing. There was no evidence for systemic exposure neither to live AG013 bacteria (blood) and there was no recovery of live AG013 bacteria in faeces.

In addition, AG013 PK studies were carried out in healthy hamsters, rats and dogs, as well as in hamsters with radiation-induced OM. In healthy hamsters and hamsters with radiation-induced OM it was demonstrated that the *L. lactis* sAGX0085 bacteria were metabolically active in the oral cavity following topical application of AG013, with or without subsequent rinsing, and that neither the bacteria, nor the hTFF1 secreted, distributed into systemic circulation. Living bacteria, administered topically to the cheek pouch of healthy and diseased hamsters, adhere to the oral mucosa following rinsing for a maximum of 24 hours. Live bacteria could no longer be detected 48 hours after the last dosing indicating that there was no colonisation. The PK study in healthy rats and dogs also showed that, during transit through the GI tract, the bacteria were metabolically active and moved along with the faecal stream, again without systemic exposure.

In an *in vitro* study to assess viability of *L. lactis* strains MG1363 and sAGX0085 (AG013) in pooled, complement-preserved human serum, there was a sharp decrease in viability, and no viable MG1363 or sAGX0085 bacteria could be observed after 96 hours of incubation at 37°C. In contrast, *S. aureus* (positive control) initially showed growth to saturation and further remained at high viability. This confirmed that *L. lactis* strains MG1363 and sAGX0085 were incapable of surviving in pooled, complement-preserved human serum.

An *in vivo* study was performed to determine the risk for systemic absorption of live *L. lactis* bacteria in hamsters in which concomitant OM and neutropenia (induced by myelosuppressive agents) are present. Although limited systemic absorption might occur following topical administration of the bacteria to the ulcerated cheek pouch of severely neutropenic hamsters, there were no indications of clinical infection (Caluwaerts *et al.*, 2009; Vandenbroucke *et al.*, 2009). The bacteria could survive neither in systemic circulation, nor in the peripheral tissues. Furthermore, *in vivo* and *in vitro* safety pharmacology studies, in neutropenic rat and pooled, complement-preserved serum respectively, confirmed that the bacteria cannot survive in systemic circulation (Vandenbrouck *et al.*, 2009, Caluwaerts *et al.*, 2009) .

The impact to the environment is considered very low/negligible, as the GMO cannot survive outside the target ecosystem, cannot replicate, and is non-pathogenic to humans and other organisms in the environment. The gene of interest has not been shown to be toxic.

3.1.2.2. Potential for exposure during administration

The most likely mechanism of exposure during administration is by contact with contaminated packaging or surface, by aerosol when AG013 mouth rinse formulation is discarded into the sink or toilet or by sharps injury in the event of breakage of the glass bottle

containing the mouth rinse formulation. The subject, healthcare professional or close family contacts could be exposed to AG013 at this stage.

Subject will be supplied weekly only with AG013 or placebo kits. The full supply as per the clinical protocol will not be provided to participants at one time. Minimal manipulation of the AG013 as supplied is required prior to administration; the procedure is limited to resuspension of the lyophilised AG013, mixing and subject rinsing their mouths for 30 seconds before discarding into the sink/toilet. Participants are not required to swallow the mouth rinse. There is the likelihood of aerosol formation splashes (droplets) but the dose will be low compared to that in the initial mouth wash formulation and AG013 is non-infectious, non-pathogenic, non-replicating and has minimal survival capabilities outside of an artificial culture environment.

The subjects must return all used and unused bottles to the study site even in the case when a subject discontinues the study.

3.1.2.3. Potential for exposure from the environment at the site of administration

AG013 is to be administered initially at a hospital setting and subsequent doses are administered via the study subject in their home setting. Subjects are required to discard the mouth rinse into the sink or toilet and to not swallow the product. As a result, the GMO will be primarily released into the environment via the sewage system initially at the hospital setting and then at the participant's home environment. It may also be shed in saliva and, potentially in stools (risk of shedding in stools is very low as the MR is not swallowed and no live bacteria were detected in stool samples of patients in the Phase 1 healthy volunteers study).

Instructions for the disposal of waste and for the handling of accidental spills and breakages are provided to the subject on receiving their first dose. Appropriate waste disposal procedures are universally applied in a hospital setting and detailed instructions are provided to subjects to be applied in their home setting.

In addition, routine cleaning procedures and practices are universally applied in a hospital setting where the initial dose will be administered and where the potential for contamination from other agents is potentially much more hazardous than that presented by AG013. Instructions for good hygiene will be provided to subjects at the time of their first dose of AG013.

The impact to the environment is considered very low/negligible, as the GMO cannot survive outside the target ecosystem, cannot replicate, and is non-pathogenic to humans and other organisms in the environment. The gene of interest is non-toxic.

3.1.2.4. Potential for exposure following administration

3.1.2.4.1. Bacterial shedding

There is the potential for bacteria shedding in saliva of participants shortly after administering the mouth rinse. The bacteria have not been detected in blood after administration and in stool samples. The risk of shedding as a consequence of subjects inadvertently swallowing the mouth rinse is expected to be very low as discussed below.

In the Phase 1b study (AG013-ODOM-101) conducted in the USA, twenty-two subjects completed the study per protocol. Serum samples, mucosal smears, and saliva samples were collected and used to assess the PK of AG013 including the viability status of the bacteria. For subjects in all 3 active treatment groups, live AG013 bacteria were detected immediately (oral mucosa) and 90 minutes (oral mucosa and saliva) after dosing on Day 1, Day 7, and Day 14. The number AG013 bacteria recovered from oral mucosa decreased by 90 minutes post-dose, but viability was still significant. In general, viability of AG013 bacteria detected in oral mucosa remained above 10% and was higher than compared to the viability in saliva. Overall, bacterial levels were consistent on Day 1, Day 7, and Day 14 in all 3 active treatment groups. For all active treatment groups, AG013 bacteria could no longer be detected in saliva and oral mucosal by the end of study visit. For subjects in all 3 active treatment groups, AG013 bacteria could not be detected in blood.

In the other study which was a single-centre, open-label Phase 1 study to assess the effect of food/beverage and to characterise the PK of single and multiple oral doses of AG013 in Healthy Subjects (AG013-CSD-MU-004), exposure to AG013 and hTFF1 was significantly reduced by food intake however not by beverage intake. Therefore, these results indicate that AG013 must be dosed after meal intake to ensure optimal exposure. Three rinses of AG013 resulted in a 1-day exposure period of AG013, though there is no evidence of accumulation of bacteria, since the number of live bacteria did not increase after three rinses. For none of the subjects and for none of the periods were live nor dead bacteria recorded in blood. Thus, there was no evidence for systemic exposure to live AG013 bacteria. There was no recovery of live AG013 bacteria in faeces.

In the event that participants were to inadvertently swallow the mouth rinse there is the potential for the bacteria to be shed in faecal matter. In clinical studies where *L. lactis* expressing the *hIL-10* gene for the treatment of Crohns disease was swallowed to provide clinical efficacy in the GI tract, live bacteria were shed for 3 days after the last treatment (Braat *et al*., 2006). The same study reported that 10^4 viable genetically modified *L. lactis* expressing the *hIL-10* gene (strain Thy12) cells/g stool were present two days after the treatment period of patients with Crohn's disease who received a daily dose of 2.0 x 10^{10} CFU for seven days. In contrast, live bacteria were not detected in stool samples from subjects in the Phase 1 AG013 healthy volunteer study where patients, as part of the clinical study protocol were required to swallow and not discard the mouth rinse. In the AG013 PK study in healthy rats and dogs, AG013 bacteria were metabolically active during transit through the GI tract and moved along with the faecal stream, without systemic exposure.

AG013 is being developed for the treatment of the oral cavity and not the GI tract. Study subjects are not required to swallow the mouth rinse and therefore the risks of shedding in stools is very low. In addition, if this was to occur the dose would be considerable low.

Shedding in saliva is expected immediately after the use of the mouth rinse but the dose is low compared to that in the oral cavity and drops after 90 minutes. It is expected that any *L. lactis* AG013 entering the immediate environment and the sanitary sewer system will be inactivated and/or removed by the physical, biological, and/or chemical treatments in place in wastewater treatment plants.

3.1.3. Summary of clinical trial experience relating to likelihood of unintended transmission

An integrated summary tabulation of all previous clinical uses/ releases of AG013 is provided in Table **1**.

Table 1: Summary of previous clinical uses / releases of AG013

Protocol/ Study title	Countries	# Sites per country	# Patients treated with AG013 per country	Containment precautions
AG013-ODOM-101	USA	N/A	17	No additional precautions required
AG013-CSD-MU-004	Belgium	N/A	10	

AG013 has not been known to be transmitted to patient contacts, hospital or other attending personnel in the trials conducted to date, where 27 patients have been treated with AG013.

Sharps injuries to healthcare professionals and study participants or closed contacts have NOT been reported during clinical trials.

3.2. Likelihood of Indirect effects of the transmission of a genetic variant of AG013 to an unintended human recipient

The Drug Substance (DS) of AG013 is a homogeneous, lyophilized powder of *Lactococcus lactis* (*L. lactis*) strain sAGX0085 mixed with cryoprotectants. Each batch of drug substance manufactured will undergo release testing for its genetic identity using a PCR method for the *httf1* gene and in addition, gene sequencing. Prior to its release to manufacture the drug product these tests are required to meet defined specifications approved by regulatory authorities and signed off by a Qualified Person (QP) at the manufacturing facility.

Genetic variants of AG013 are not manufactured or released for use in the clinical study. If they were to occur this would be spontaneously in patients or in the sewage system. It is unlikely that a genetic variant could be generated. The risks of transmission of a genetic variant of AG013 is considered negligible as discussed in Section 1.2.2.2.

In addition, the number of incidences of the generation of such variants will be considerably less than the number of purposeful administrations of AG013. No genetic variants have been detected in clinical trials to date (see Section 3.1.3).

There is no risk of exposure to a genetic variant of AG013 related to its administration as part of a clinical study. The possibility of exposure after administration will be the same as that described above.

As such the risk of transmission of a genetic variant of AG013 to an unintended human recipient is considered far lower than the risk of transmission of AG013 itself.

3.3. Conclusions

Consideration of the manner, scale and environment of release, the mode of transmission and survivability of the parent organism (*L. lactis* and MG1363 strain), the available data relating to shedding of AG013 in clinical trials and the risk management measures in place, it is considered that the risk of exposure of an unintended recipient to AG013 or a genetic variant of AG013 is very low.

Therefore, the likelihood of direct or indirect effects of the transmission of AG013 or a genetic variant of AG013 to an unintended human recipient is considered very low or negligible.

4. ESTIMATION OF RISK POSED BY EACH IDENTIFIED CHARACTERISTIC

The risk posed by AG013 or a genetic variant of AG013 on human health (specifically an unintended recipient) is considered by combining the estimated consequences of the effect with the estimated likelihood of effect (in accordance with 2001/18/EC and 2002/623/EC). This estimation is made with reference to the risk attributed to the parental organism (*L. lactis* and MG1363 strain) for context. The estimate is also influenced by the degree of scientific uncertainty in those estimates, in accordance with the Precautionary Principle.

4.1. Risk associated with the parental organism (*L. lactis* and MG1363 strain)

For context, it is useful to consider the risk associated with wild-type *L. lactis* and MG1363 strain, itself.

L. lactis is a non-pathogenic, non-invasive, non-colonizing Gram-positive bacterium, critical in manufacturing dairy products. In spite of the widespread use and massive discharge in the environment, *Lactococci* have not been identified as invasive or disruptive. Although they can be found in very diverse sources (soil, manure, waste water), the bacteria depend on particular nutritional components for growth. MG1363 (AG013 parental strain) is restricted even more and as such confined to artificially supplemented culture conditions. In addition, the GMO (AG013) is dependent on thymine/thymidine supplementation.

L. lactis is not considered a human pathogen given its history of safe use in the food industry. *L. lactis* infections generally occur in patients with co-morbidities and are often associated with the consumption of unpasteurized dairy products. Overall, reported cases are scarce and infectivity is not severe in patients with underlying conditions. There are no reported cases of allergic reaction linked to any strain of *L. lactis*.

L. lactis is not categorised in a risk group in the European Community according to Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work.

The species *L. lactis*, is considered by the European Food and Safety Authority (EFSA) to fulfil criteria for assessment by Qualified Presumption of Safety (QPS). This relates to a generic approach for safety assessment of micro-organisms used in food/feed and the production of food/feed additives. The list is reviewed annually by EFSA's Panel on Biological Hazards (BIOHAZ). *Lactococcus lactis* received QPS status recommendation in 2013 as a gram-positive non-sporulating bacteria. The latest scientific opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA, published in September 2017, concluded that there is no need to change the QPS recommendation of *L. lactis* (Ricci *et al*., 2018).

Similar classifications of hazard have been assigned to *L. lactis* by the World Health Organisation (WHO), and in the USA, Canada and Australia as summarised in Table **2**. Thus, the risk for healthy individuals working with wild-type *L. lactis* is widely considered to be negligible or limited risk to the immunocompromised individuals.

Table 2: Global Biosafety classifications for wild type *L. lactis*

Territory	Category	Definition	Reference
WHO	**Risk Group – not listed**	N/A	WHO Laboratory Biosafety Manual, 3rd Ed (2004)
US	**Biosafety Level - not listed**	N/A	NIH Recombinant DNA Guidelines (USA, 2011) Appendix B-II-D. CDC/NIH Guidelines (1999) "Biosafety in Microbiological and Biomedical Laboratories" 5th Edition, 2009. Section VIII-E. Not listed under 42CFR73.3 – Select Agents and Toxins
Canada	**Risk Group – not listed**	N/A	Canadian Laboratory Safety Guidelines (2004) Human Pathogens and Toxins Act. S.C. 2009, c. 24. Schedule II
Australia/NZ	**Group – not listed**	N/A	Standard AS/NZS 2243.3:2010. Safety in laboratories Part 3: Microbiological aspects and containment facilities. Standards Association of Australia, Sydney.

4.2. Risk associated with the transmission of AG013 to an unintended recipient

AG013 lacks all necessary components for survival and multiplication in the environment and transmission to unintended recipients. Furthermore, stability studies have shown that the lyophilised powder needs to be stored in tightly sealed aluminium bags (vapour barrier), protected from moisture and refrigerated (2-8°C) to preserve quality. In liquid conditions under room temperature, a log 6 reduction of the number of bacteria is observed within 8 hours. In addition, the liquid form of AG013 is extremely sensitive to temperatures above 40°C, low pH, air drying, direct sunlight, Ultraviolet light (UV) and high salt.

The AG013 lyophilised powder is packed in child-proof bottles. Subjects will be instructed how to prepare and administer the AG013 MR. Risks associated with the transmission of AG013 to an unintended recipient are very low due to the following reasons:

- Similarly, to its parental strain it is non-pathogenic. *L .lactis* is distributed globally and is used in the dairy industry with no adverse effects being reported in relation to its contact with humans, animals and the environment.
- Similarly, to its parental strain, AG013 is a poor competitor and has a limited ecological niche.
- AG013 is devoid of the metabolic pathways that enable the use of milk carbohydrate and amino acid sources and can therefore no longer grow in its ancestral ecological niche.
- AG013 is dependent on external supplementation of thymine or thymidine for growth and survival. Indeed, the thymidylate synthase gene was removed. When deprived of thymine and thymidine, an inherent suicidal system, "thymineless death" is induced.
- The protein encoded by the transgene (hTFF1), is of human origin and has been used in several clinical studies to date with no significant adverse events reported.

Exposure of immune-competent individuals to AG013 is therefore unlikely to produce any adverse effects, due to the severely attenuated nature of the bacteria. At worst, an adverse event profile similar to that observed in clinical trials where AG013 was used as a single agent would be expected. However, such reactions would be expected to be both less severe and less frequent in an unintended recipient.

Since *L. lactis* has a safe history of use and is considered as non-pathogenic to the general population and that the recombinant *htffl* gene and protein have shown to be safe for humans, the use of AG013 is not expected to cause adverse effects to the general population. Its potential hazard to human health is considered very low. The consequences of transmission of AG013 to an unintended recipient are considered very low (See Section 2.1).

The likelihood of transmission of AG013 is considered 'very low (see Section 3.1), and certainly no greater than that of wild-type *L. lactis*. Its mode of transmission and survivability are greatly reduced by the genetic modifications made to the wild type bacteria.

Data on the shedding of AG013 gathered in animal and human models provide a high level of scientific certainty in this regard.

Thus, through a combination of the very low-level consequences of transmission and the very low likelihood of this occurring, the overall risk posed by AG013 to the unintended recipient is considered **very low** (with reference to Table **2**: Guideline on Environmental Risk Assessments for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs).

4.3. Risk associated with the transmission of a genetic variant AG013 to an unintended recipient

The consequences of transmission of a genetic variant of AG013 to an unintended recipient are considered '**very low'** (See Section 2.2) and the following:

- The likeliness of genetic variant developing is very low as per Section 1.2.
- In the unlikely event that a genetic variant was generated initial exposure will be considerably less than that in study subjects and likely on a single occasion as opposed to multiple exposure received by patients enrolled in the clinical study.

Exposure of immune-competent individuals to genetic variants of AG013 is therefore unlikely to produce any adverse effects beyond those observed in clinical trials where AG013 is used as a single agent.

The consequences of exposure are therefore considered lower for genetic variants of AG013 than wild type *L. lactis* (see above). The risks of genetic variants being developed are very low since manufacturing. clinical and non-clinical data to date have not detected the development of genetic variants of AG013.

The likelihood of transmission of a genetic variant of AG013 is considered to be no greater than that of wild-type *L. lactis*. As a result of the reduced survival capacity of AG013 compared to wildtype *L. lactis* and the fact that the wildtype strain is non-pathogenic, non-infectious, distributed globally, any genetic variant of AG013 is not expected to be of any further risk to an unintended recipient.

Further, the transmission of a genetic variant of AG013 to an unintended recipient would be an indirect effect, relying on a chain of events, namely

- The development of a genetic variant in the environment
- Transmission of the genetic variant to the unintended recipient by contact with a surface containing the variant or via the sewerage system
- Genetic variant like AG013 being unable to survive outside of the laboratory in the absence of nutrients that sustain its growth.

Overall therefore it is concluded that the likelihood of transmission of a genetic variant of AG013 to an unintended recipient is very low or negligible (see Section 3.2).

Thus, through a combination of the very low-level consequences (applying the precautionary principle) of transmission and the very low likelihood of this occurring, the overall risk posed by a genetic variant of AG013 to the unintended recipient is considered **very low** (with reference to Table **2**: Guideline on ERA for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs).

5. APPLICATION OF MANAGEMENT STRATEGIES FOR RISKS

5.1. Design of GMO

In the development of AG013, there has been several steps taken to ensure its safety for use in humans for the treatment of OM and these are listed below:

- The choice to use a non-pathogenic, non-infectious and widely distributed bacterium used in the food industry like *L. lactis*
- The parental strain (MG1363) has reduced growth and replication capabilities than the wild type strain as a result of the removal during the isolation of all plasmids. This strain has lost the capacity to access its main energy and amino acid sources. In consequence, the habitat of MG1363 is confined to artificially supplemented culture conditions.
- *L. lactis* strain, sAGX0085 (AG013) incorporates hTFF1 expression cassette which replaced the *thyA* gene. The removal of the thymidylate synthase function generates a dependence of the strain on thymine or thymidine supplementation for growth and survival. Thymine starvation leads to rapid cell death.

5.2. Control of release

The proposed clinical studies will be initiated after approval by the appropriate national CA and EC in the selected countries. AG013 will be administered to humans as part of a clinical study to assess its safety, tolerability and therapeutic effect in the treatment of OM in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy.

The long-term objective is to develop AG013 to MA where AG013 will be available across the European Community as a prescription medicine for the treatment of OM in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy. Thereafter, it is expected to be used routinely in this indication throughout the European Community in accordance with its approved product labelling.

The manufacture, supply and traceability of AG013 is therefore tightly controlled and monitored in accordance with medicines regulation.

Since AG013 administration will be controlled by a healthcare professional in a hospital environment initially and then by the patients following training, the following precautions are expected to be implicit:

- Safe storage
- Established routine practices for dealing with GMO in the hospital setting (autoclaves, sharps bins, incinerators, disinfectants and appropriate cleanable surfaces).
- Patients are restricted to one-week supply at a time. In addition, are trained and given instructions on storage, preparation, administration and destruction of bottles/packaging that have come in to contact with AG013. As well as instruction in the event of spillage of AG013 as a result of breakage of the glass bottles.

5.3. Administration precautions

The first dose of AG013 is administered in the clinical trial centre supported by trained healthcare professionals to ensure the patient understands how to store, prepare, administer and dispose of AG013, bottles and packaging potentially contaminated with the GMO. Subsequent dosing is conducted on an ambulatory/outpatient basis, i.e. participants do not

reside in the clinical study centre during administration of further doses. In the clinical study, a fixed dose of AG013 will be administered at a dose frequency of three times daily.

Detailed instructions will be provided to patients including information on its controlled storage, dose preparation, dispensing and how to discard material contaminated with the GMO.

5.4. Cleaning and waste management

Wildtype *L .lactis* has not been classified as meeting the criteria for assignment to a biosafety risk group (Table 2). In the hospital setting, bottles/packaging and other materials that come into contact with AG013 will be managed as per institutional procedures and guidelines for biologicals products. Typically, standing procedures for disposal within hospitals (where the potential for contamination from other agents is potentially much more hazardous than that presented by AG013 will be consistent with the guidance given in the WHO Laboratory Biosafety Manual, 3rd Ed (2004) for BSL 1).

The study participant will return all used and unused bottles to the study site during their weekly visits. The bottles will be inactivated as hazardous medical waste according to the institutional standards. It should be noted that this is an ultimate precaution, as it can be expected that by the time of return, no more living AG013 bacteria will be present.

The genetic modifications made during the construction of AG013 from wild type *L. lactis* do not affect its sensitivity to physical and chemical inactivation.

Physical inactivation: The IMP is stored in tightly sealed aluminium bags (vapour barrier), protected from moisture and refrigerated (2-8°C) in order to preserve quality. The powder of AG013 is sensitive to temperatures above 40°C, low pH, air drying, direct sunlight, UV and high salt. The viability of the reconstituted mouth rinse solution drops by approx. 40% after 24 hours at 23°C.

Chemical inactivation: Materials should be disinfected according to standard medical procedures suitable for the equipment or handled according to standard hygienic procedures (*e.g.* washing of exposed textiles using standard household product).

In the event of an accidental spillage, the use of standard detergent (soap) or bleach immediately will completely eradicate the AG013 and decontaminate the affected area. AG013 is short-lived when dissolved in water at room temperature.

5.5. Containment precautions

The AG013 powder is packed in clear glass vials with tamper-evident, child-resistant screw caps. Three of these vials are in turn packed into a sealed aluminium bag. The solution for re-suspension (reconstitution) is delivered in a dark glass bottle with tamper-evident, child-resistant screw caps.

AG013 will be delivered to the clinical trial centre pharmacy as single dose packages that do not require further manipulation. Direct contact with the lyophilised powder is excluded

until opening of the single doses. Only at the moment of administration, exposure to the suspended powder is possible. The study participants will only be issued with one-week supply at one time and will be required to return all used and unused bottles to the hospital setting on a weekly basis for IMP accountability and disposal.

5.6. Product Labelling

The product label meets EU GMP Annex 13 labelling requirements and any country specific requirements for an IMP. The patient dosing instructions includes reference to the IMP being a GMO and provides essential information alongside the patient's Questions and Answers booklet to minimise the risk of transmission to an unintended individual.

5.7. Monitoring activities

To detect AG013, a specific quantitative Q-PCR method has been developed based on the detection of the synthetic *htff1* gene which uniquely identifies the GMO. The *htff1* gene allows for the detection and quantification of total numbers of GM bacteria (live and dead), while a viable count assay allows for the detection and quantification of the total number of live bacteria. These data provide a ratio between live and dead bacteria. The *htff1* gene in the GM *Lactococcus* is a unique, synthetic gene which can be distinguished from native *htff1*.

5.7.1. Monitoring during treatment of patients

Patients will be monitored throughout treatment by their treating physician and adverse effects will be managed accordingly and reported as per regulatory requirements for an IMP. The data gathered by the sponsor will be monitored and regularly reviewed for safety signals as for any medicinal product. Regular reporting requirements to the Competent Authority will be fulfilled according to pharmaceutical law.

5.7.2. Follow-up of patients after treatment

At the end of treatment, patients will be followed up by their clinician as per normal standard of care for HNC.

5.7.3. Monitoring of unintended recipients

Monitoring of unintended recipient is not required due to the non-infectious, non-pathogenic nature of wildtype *L. lactis* and that of AG013 which is significantly reduced in its capabilities compared to the wildtype strains in growth and survival outside of a laboratory environment.

5.8. Conclusions

Appropriate risk management strategies are in place to minimise any potential risks and consequences of exposure to unintended individuals. Wildtype *L. lactis* has not been classified as meeting the criteria for assignment to a biosafety risk group (Table

2) and is not considered to be a risk to humans. Appropriate monitoring strategies are however proposed to add to the scientific certainty of the environmental risk assessment related to shedding in saliva as part of the clinical study.

6. DETERMINATION OF OVERALL RISK OF THE GMO

L. lactis is a non-pathogenic, non-invasive, non-colonizing Gram-positive bacteria, critical for manufacturing dairy products. In spite of the widespread use and massive discharge in the environment, *Lactococci* have not been identified as invasive or disruptive to humans or the environment. The bacteria can be found in very diverse sources (soil, manure, waste water), depend on particular nutritional components for growth. MG1363 (AG013 parental strain) is restricted even more and as such confined to artificially supplemented culture conditions. In addition, the GMO (AG013) is also dependent on thymine/thymidine supplementation.

There is no indication that the AG013 itself is toxic, allergenic or pathogenic. The changes that were induced in the recipient strain MG1363 as well as in the AG013, do not affect the basic toxic or allergenic features. In the unlikely event of infection, the GMO can quickly and easily be inactivated with standard antibiotics.

Exposure to the secreted hTFF1 protein is limited in time as the bacteria are short lived. AG013 mouth rinse is now swallowed by clinical study participants. In the event it was accidentally swallowed, *L. lactis* does not colonize the GI and will not survive in the harsh conditions of the sewage system.

None of the genetic modifications made to wild type *L. lactis* or MG1363 strain during construction of AG013 or its theoretical variants, would be expected to alter its effect on environmental processes. As such, there is no expected impact to the environment as a whole following the release of AG013.

Therefore, based on the nature of the GMO, the parental organism and the receiving environment, **the potential adverse effects which AG013 may exert by its use in clinical studies are limited to:**

- **Direct effects of the transmission of AG013 to an unintended human recipient.**
- **Indirect effects of the transmission of a genetic variant of AG013 to an unintended human recipient.**

The potential magnitude of unintended spread within the human population is considered very low, given the attenuated nature of the GMO. For those unintended individuals that may be exposed to AG013 or its possible genetic variants, the adverse effects are expected to be of a considerably lower severity than those observed with wild type *L .lactis* which itself is universally unclassified as a risk to humans and the environment.

Therefore, the potential consequences in the case of transmission of AG013 or its possible genetic variants are expected to be very low level and isolated.

Consideration of the manner, scale and environment of release, the mode of transmission and survivability of the parent organism (wild-type *L. lactis*), the available data relating to shedding of similar vector systems and AG013 in clinical trials and the risk management measures in place, it is considered that the risk of exposure of an unintended recipient to AG013 or a genetic variant of AG013 is very low.

Therefore, the likelihood of direct or indirect effects of the transmission of AG013 or a genetic variant of AG013 to an unintended human recipient is considered very low.

The risk posed by AG013 or a genetic variant of AG013 on human health (specifically an unintended recipient) is considered by combining the estimated consequences of the effect with the estimated likelihood of effect (in accordance with 2001/18/EC and 2002/623/EC). This estimation is made with reference to the risk attributed to the parental organism (wildtype *L. lactis*) for context. The estimate is also influenced by the degree of scientific uncertainty in those estimates, in accordance with the Precautionary Principle.

Thus, through a combination of the very low-level consequences of transmission and the very low likelihood of this occurring, the overall risk posed by AG013 to the unintended recipient is considered **very low** (with reference to Table **2**: Guideline on Environmental Risk Assessments for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs). Similarly, through a combination of the very low-level consequences (applying the precautionary principle) of transmission and the low likelihood of this occurring, the overall risk posed by a genetic variant of AG013 to the unintended recipient is considered **very low** (with reference to Table **2**: Guideline on Environmental Risk Assessments for Medicinal Products consisting of, or containing, genetically modified organisms (GMOs).

Appropriate risk management strategies are in place to minimise the risks and consequences of exposure to unintended individuals particularly those that are fall in to the venerable group e.g. immunocompromised individuals. The manufacture, supply and traceability of AG013 is implicitly controlled and monitored in accordance with medicines regulation. In the event of an unexpected infection, the individual concerned may be treated with approved antibiotics if clinically indicated. **Appropriate monitoring strategies are proposed to add to the scientific certainty of the environmental risk assessment** through the collection of additional data in a larger number of individuals during the clinical studies, under the conditions of real life clinical use; it is likely that reduced monitoring can occur as certainty is increased through data collection prior to product marketing**.**

In conclusion, overall the environmental impact of the deliberate release of AG013 as an IMP for use in clinical studies, under the conditions of the release and the precautions and monitoring activities applied, is considered acceptable.

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