PART 1 (COUNCIL DECISION 2002/813/EC)

SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS IN ACCORDANCE WITH ARTICLE 11 OF DIRECTIVE 2001/18/EC

In order to tick one or several possibilities, please use crosses (meaning x or X) into the space provided as (.)

A. General information

1. Details of notification

(a)	Member State of notification	Spain…
(b)	Notification number	B/ES/19/05
(c)	Date of acknowledgement of notification	16/JAN/2019
(d)	Title of the project	*A Phase I-IIa trial to assess the safety and antitumor activity of autologous CD44v6 CAR T-cells in acute myeloid leukemia and multiple myeloma expressing CD44v6*
(e)	Proposed period of release	*Duration of study: 48 months* *March 2019 to March 2023*

2. Notifier

Name of institution or company: *MolMed SpA , Via Olgettina 558 20132 Milano (Italy)*

3. GMO characterisation

The GMO (MLM-CAR44.1 T-cells) is defined as frozen patient T lymphocytes genetically modified to express a CD44v6-specific CAR (CAR CD44v6ΔNL) and the HSV-TK Mut2 suicide gene, which codes for a mutated form of the thymidine kinase of Herpes Simplex Virus I (HSV-TK).
It is conceived as treatment for patients suffering from AML (Acute Myeloid Leukaemia) or Multiple Myeloma (MM). Transduction of lymphocytes is carried out by a recombinant γ-retroviral vector (CAR-CD44v6ΔNL) derived from the Moloney Murine Leukaemia Virus (MoMuLV)-based LXSN vector and pseudotyped with the GaLV (Gibbon ape leukemia virus) envelope, able to transduce mammalian cells, including human cells, with the exception of mouse cells. The CAR-CD44v6ΔNL retroviral vector (RV) is replication-deficient by design and produced using the PG13 packaging cell line (ATCC # CRL-10686), characterized by the segregation of the viral sequences necessary for packaging into two different plasmids: one for gag and pol, the other for the GaLV env.

(a) Indicate whether the GMO is a:

viroid	(.)
RNA virus	(.)
DNA virus	(.)
bacterium	(.)
fungus	(.)
animal	

- mammals (x)
- insect (.)
- fish (.)
- other animal (.)

specify phylum, class *Chordata (vertebrates), Mammals*

(b) Identity of the GMO (genus and species)
Genus:Homo; Species:H. Sapiens (genetically modified human lymphocytes)

(c) Genetic stability – according to Annex IIIa, II, A(10)
Stable

4. Is the same GMO release planned elsewhere in the Community (in conformity with Article 6(1)), by the same notifier?
Yes (x) No (.)
If yes, insert the country code(s)
IT, CZ, DE…

5. Has the same GMO been notified for release elsewhere in the Community by the same notifier?
Yes No x
If yes:
- Member State of notification
- Notification number B/../../…

Please use the following country codes:
Austria AT; Belgium BE; Germany DE; Denmark DK; Spain ES; Finland FI; France FR; United Kingdom GB; Greece GR; Ireland IE; Iceland IS; Italy IT; Luxembourg LU; Netherlands NL; Norway NO; Portugal PT; Sweden SE

6. Has the same GMO been notified for release or placing on the market outside the Community by the same or other notifier?
Yes (.) No (x)
If yes:
- Member State of notification …
- Notification number B/../../…

7. Summary of the potential environmental impact of the release of the GMOs.

Environmental risks for medicinal products containing or consisting of GMOs are supposed to be addressed according to Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms.
MLM-CAR44.1 T-cells product is defined as frozen patient T lymphocytes genetically modified to express a CD44v6-specific CAR (CAR CD44v6ΔNL) and the HSV-TK Mut2 suicide gene, which codes for a mutated form of the thymidine kinase of Herpes Simplex Virus I (HSV-TK).
It is conceived as treatment for patients suffering from AML (Acute Myeloid Leukaemia) or Multiple Myeloma (MM). The MLM-CAR44.1 T-cells are mainly $CD3^+$ T lymphocytes. MLM-CAR44.1 T-cells are sensitive to ganciclovir.

Transduction of lymphocytes is carried out by a recombinant γ-retroviral vector (CAR-CD44v6ΔNL) derived from the Moloney Murine Leukaemia Virus (MoMuLV)-based LXSN vector and pseudotyped with the GaLV (Gibbon ape leukemia virus) envelope, able to transduce mammalian cells, including human cells, with the exception of mouse cells. It encodes a CD44v6-specific CAR (i.e. CAR CD44v6ΔNL) and a mutated form of HSV-TK (i.e. HSV-TK Mut2) under the control of the LTR and SV40 promoters, respectively. It does not encode for any antibiotic resistance markers or pathogenic, toxic, allergenic or oncogenic functions. It does not encode for any other functional sequences of HSV-TK than the TK Mut2.

The CAR-CD44v6ΔNL retroviral vector is replication-deficient by design and produced using the PG13 packaging cell line (ATCC # CRL-10686) characterized by the segregation of the viral sequences necessary for packaging into two different plasmids: one for gag and pol, the other for env. After transduction, the CAR-CD44v6ΔNL provirus is stably integrated into the host cell genome.

Quality control of the final batch of MLM-CAR44.1 T-cells was tested as being free of adventitious viruses and potential microbial contaminants. Furthermore, batches were tested negative for replication competent retrovirus, RCR, with a RT-PCR assay detecting GaLV and gag-pol genes as a recombination indication.

MLM-CAR44.1 T-cells is formulated as frozen cell suspension at the concentration of 1 to $10x10^6$ cells/ml. It will be administered to patients to achieve a dose from 0.5 to $2x10^6$ cells/kg body weight.

Treatment of patients will be performed under strict aseptic conditions in a hospital setting under safe administration conditions analogous to contained use. Hand washing before and after administration either soap and running water or appropriate antiseptic hand cleaner. Overcoat, gloves, mask and lab safety glasses shall be worn for administration. MLM-CAR44.1 T-cells are administered to the patient through the use of special valves connected to a venous catheter, so that as use of needles for infusion is not required. It will be performed by trained hospital personnel, only, minimizing the risk of unintended transmission of either MLM-CAR44.1 T-cells or possibly associated free CAR-CD44v6ΔNL retroviral vector particles to non-target human subjects or the environment.

Under these safe administration conditions exposure of hospital personnel can solely occur by accident. Release into the environment is hardly possible at all as not only regular disinfection, decontamination and waste inactivation takes place but also because potential spills are very small and emergency clean up procedures are in place for spills to reach the environment outside the administration site. In the previous experience with similar GMO, used by MolMed in two clinical trials, the Phase I/II trial (TK007) and a currently on-going Phase III trial (TK008), no unexpected events or accidents have been recorded during administration.

Concerning the generation of replication competent retrovirus, the PG13 packaging cell line use for the production of the CAR-CD44v6ΔNL retroviral vector is widely used and recognized as a safe tool for retroviral vector production (Miller, et al. J Virol 1991), because complementation of a replication-deficient retroviral vector provirus with cell line sequences has not been observed to occur. It is characterized by the segregation of the viral sequences necessary for packaging into two different helper plasmids: one for gag and pol (pLGPS), the other for the GaLV envelope (pMOV-GaLV), both containing a deletion of the Ψ+ packaging sequence. As a consequence of the packaging cell line engineering, helper plasmid sequences are neither inserted into the CAR-CD44v6ΔNL retroviral vector genome, nor are they packaged into the CAR-CD44v6ΔNL retroviral vector particles. This so-called 3rd generation packaging system minimizes the risk of RCR generation. Indeed, a multi-institutional study including samples from 10 research groups involved with 14 clinical trials, all but one using the PG13 cell line, showed no evidence of RCR in 282 transduced cell products as well as in the 241 research subjects screened for RCR post-treatment (Cornetta et al. Mol Ther:Meth & Clin Dev, 2018). Furthermore, RCR assays based on FDA RCR-testing recommendations, were performed which provided no evidence for any RCR, not in the

packaging cell line nor in the CAR-CD44v6ΔNL retroviral vector to be used to transduce T-cells. These recommendations from the FDA can be found in the Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors. Testing has been done with the Mus dunni cell assay, known not to contain endogenous retroviral sequences, with the aim to verify the absence of retrovirus competent for the replication (Allan et al. Leukemia & Lymphoma 1996).

Concerning the post-administration generation of RCR, the results reported by Cornetta et al (Mol Ther:Meth & Clin Dev, 2018) are in agreement with the post-administration monitoring performed by Molmed for the TK007 and TK008 studies, which did not indicate any detectable generation of RCR in 150 samples from 30 patients over 12 months. The assay was performed with a highly sensitive quantitative PCR with a LoQ of 1 positive cells / 75,000 cells with 95% confidence limit.

All together these results support the conclusion that the risk of RCR generation post-administration is negligible as: a) at least three recombination events with CAR-CD44v6ΔNL would be necessary for gain of replication competency, b) human endogenous retroviruses (HERVs) sequences did not show homology with CAR-CD44v6ΔNL according to a BLAST alignment test, c) co-infection of MLM-CAR44.1 T-cells with animal retrovirus, with which recombination could take place, do not infect human cells, and recombination of lentivirus with retrovirus has not been detected, so far, despite of a large number of in vivo gene therapy clinical trials with retroviral and lentiviral vectors. In order for RCR to have an adverse effect on people and the environment, significant numbers would have to be generated and shed from patients. Furthermore, survival in the environment would have to be ascertained for the RCR to reach potential non-target hosts. Retroviruses and retroviral vectors are sensitive to any standard detergent, non-physiological pH conditions or temperatures to which they are exposed. In the remote event that RCR happens, a subject must be exposed to repeated infections with high number of RCR to show the negative effect of infection, as pathogenicity is extremely low. (Anderson et al. Hum Gene Ther 1993).

Besides the potential of RCR formation, the following characteristics of MLM-CAR44.1 T-cells were taken into account for the environmental risk assessment:

a) MLM-CAR44.1 T-cells consists of terminally differentiated transduced T lymphocytes not able to survive outside the patient they are dedicated to, unless injected into immune-compromised individuals. MLM-CAR44.1 T-cells are sensitive to ganciclovir. Treated patients are excluded from donating blood.

b) CAR-CD44v6ΔNL is replication-defective and does not express traits, which could lead to adverse effects in non-target hosts. According to the manufacturing protocol free CAR-CD44v6ΔNL is not expected in batches of MLM-CAR44.1 T-cells, because MLM-CAR44.1 T-cells are immunoselected in order to obtain a highly purified population of transduced cells and this step, along with the several washing performed throughout the process, allow the MLM-CAR44.1 T-cells to be free of remaining CAR-CD44v6ΔNL retroviral vector particles. Integration into MLM-CAR44.1 T-cells cells is stable and no gene rearrangements have been detected so far. CAR-CD44v6ΔNL does not harbour any sequence that would give it a competitive advantage in itself or when recombined with retrovirus and does not affect any potential medical treatment. Even though being pseudotyped with the GaLV envelope and free CAR-CD44v6ΔNL viral particles could infect a number of animal hosts including humans and a number of different cell types, only actively replicating cells can be infected when exposed to a significant number of viral particles. Infection may take place only in the presence of specific agents (e.g. protamine) which facilitate viral adhesion to the cell membrane and entry. Free viral particles would be eliminated by the complement system of immune competent, healthy individuals.

From these considerations, no adverse effects from MLM-CAR44.1 T-cells or free CAR-CD44v6ΔNL and thus no negative consequences to people and the environment are expected. Furthermore,

generation of RCR is highly unlikely. Thus, it we can concluded that the risk of using MLM-CAR44.1 T-cells for people and the environment is negligible.
In the event of accidental exposure of hospital personnel to MLM-CAR44.1 T-cells, the only event where exposure cannot be completely excluded, no adverse effects would be expected as no immune compromised individuals are allowed to participate in the administration of MLM-CAR44.1 T-cells and potential exposure would be a fraction of what is injected into patients. Furthermore, exposed individuals could be monitored in the context of the pharmacovigilance plan for patients.
Post-administration monitoring of treated patients for RCR will be performed at the following times 3 months, 6 months, 1 year, then annually (ref: Guidance for Industry - Supplemental Guidance Retroviral Testing for Retroviral Retroviral Vector Based Gene Therapy Products and Follow-up of Patients in Clinical Trials Using Retroviral Vectors - FDA November 2006). RCR in patient blood would be the first indication of RCR generation and anti-retroviral treatment would be considered before RCR would be shed into the environment.

B. Information relating to the recipient or parental organism from which the GMO is derived

1. Recipient or parental organism characterisation:

(a) Indicate whether the recipient or parental organism is a:

(select one only)

viroid		(.)
RNA virus		(.)
DNA virus		(.)
bacterium		(.)
fungus		(.)
animal		
-	mammals	(x)
-	insect	(.)
-	fish	(.)
-	other animal	(.)

(specify phylum, class) *Chordata (vertebrates), Mammals, (lymphocytes)*

other, specify …

2. Name

(i)	order and/or higher taxon (for animals)	*Primates*…
(ii)	genus	*Homo*
(iii)	species	*Homo sapiens…*
(iv)	subspecies	-…
(v)	strain	-…
(vi)	pathovar (biotype, ecotype, race, etc.)	-…
(vii)	common name	*lymphocytes*

3. Geographical distribution of the organism: *Not Relevant (NR)*

(a) Indigenous to, or otherwise established in, the country where the notification is made:

Yes (.) No (.) Not known (.)

(b) Indigenous to, or otherwise established in, other EC countries:

(i) Yes (.)

If yes, indicate the type of ecosystem in which it is found:

Atlantic	x
Mediteranean	x
Boreal	x
Alpine	x
Continental	x
Macaronesian	x

(ii) No (.)

(iii) Not known (.)

(c) Is it frequently used in the country where the notification is made?

Yes (x) No (.)

(d) Is it frequently kept in the country where the notification is made?

Yes (x) No (.)

4. Natural habitat of the organism: *Not Relevant. Human lymphocytes are able to survive only in very restricted in vitro conditions in the presence of cytokines or inside the human body.*

(a) If the organism is a microorganism

water	(.)
soil, free-living	(.)
soil in association with plant-root systems	(.)
in association with plant leaf/stem systems	(.)
other, specify …	

(b) If the organism is an animal: natural habitat or usual agroecosystem:

…

5. (a) Detection techniques *Not Relevant.*

(b) Identification techniques

Human lymphocytes can be identified for the expression of lineage specific markers such as the CD3, by immunofluorescence with specific antibody or by molecular techniques such as the PCR

6. Is the recipient organism classified under existing Community rules relating to the protection of human health and/or the environment?

Yes (.x) No (

If yes, specify

… *Biosafety Level 1*

7. Is the recipient organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?

Yes (.) No (x) Not known (.)

If yes:

(a) to which of the following organisms:

humans	(.)
animals	(.)
plants	(.)
other	(.)

(b) give the relevant information specified under Annex III A, point II. (A)(11)(d) of Directive 2001/18/EC

…

8. Information concerning reproduction *Not Relevant*

(a) Generation time in natural ecosystems:

(b) Generation time in the ecosystem where the release will take place:

…

(c) Way of reproduction: Sexual .. Asexual ..

(d) Factors affecting reproduction:

…

9. Survivability : *Not relevant for this product since human lymphocytes are able to survive only inside the human body or in very restricted in vitro conditions in the presence of cytokines.*

(a) ability to form structures enhancing survival or dormancy:

(i)	endospores	(.)
(ii)	cysts	(.)
(iii)	sclerotia	(.)
(iv)	asexual spores (fungi)	(.)
(v)	sexual spores (funghi)	(.)
(vi)	eggs	(.)
(vii)	pupae	(.)
(viii)	larvae	(.)
(ix)	other, specify …	

(b) relevant factors affecting survivability:

10. (a) Ways of dissemination

Not relevant for this product since human lymphocytes are able to survive only inside the human body or in very restricted in vitro conditions in the presence of cytokines.

(b) Factors affecting dissemination

Not relevant for this product since human lymphocytes able to survive only inside the human body or in very restricted in vitro conditions in the presence of cytokines.

11. Previous genetic modifications of the recipient or parental organism already notified for release in the country where the notification is made (give notification numbers)
…, B/../../…None

C. **Information relating to the genetic modification**

1. Type of the genetic modification

(i)	insertion of genetic material	(x)
(ii)	deletion of genetic material	(.)
(iii)	base substitution	(.)
(iv)	cell fusion	(.)
(v)	others, specify …	

2. Intended outcome of the genetic modification

To obtain the expression of the two transgenes by the MLM-CAR44.1 T-cells: 1) the Chimeric Antigen Receptor (CAR) CD44v6ΔNL specific for the antigen CD44v6 expressed by tumor cells; 2) the suicide gene HSV-TK Mut2 that allows killing of the transduced cells by GCV administration if adverse events occur. CAR-expressing MLM-CAR44.1 T-cells exert their effector functions such as cytokine production and cytotoxicity, which ultimately result in the elimination of tumor cells.

3. (a) Has a vector been used in the process of modification? ,

Yes (x) No (.)

If no, go straight to question 5.

(b) If yes, is the vector wholly or partially present in the modified organism?

Yes (x) No (.)

If no, go straight to question 5.

4. If the answer to 3(b) is yes, supply the following information

(a) Type of vector

plasmid	(.)
bacteriophage	(.)
virus	(x)
cosmid	(.)
transposable element	(.)
other, specify …	

(b) Identity of the vector

The CAR-CD44v6ΔNL is replication defective γ-retroviral vector derived from the Moloney Murine Leukaemia Virus (MoMuLV)-based LXSN vector and pseudotyped

with the GaLV (Gibbon ape leukemia virus) envelope, able to transduce mammalian cells, including human cells, with the exception of mouse cells.

(c) Host range of the vector
The host range of recombinant MoMuLV vectors is dependent on the specificity of the viral envelope. The GaLV (Gibbon ape leukemia virus) allows infection of a wide range of mammalian cells including human cells with the exception of mouse cells.

(d) Presence in the vector of sequences giving a selectable or identifiable phenotype
Yes (x) No (.)

antibiotic resistance ()
other, specify *viral LTR identifiable by PCR;*

Indication of which antibiotic resistance gene is inserted
In the integrated provirus there are no antibiotic resistance gene

(e) Constituent fragments of the vector
…

Genetic Element	**Position**	**Function**
5' LTR (Long terminal Repeat)	1-589	Transcriptional promoter
Ψ+ gag	659-1468	Viral sequences containing the Ψ+ signal, responsible for the formation of complete virions, (Miller and Rosman 1989) and 700bp of the MoLV gag gene, which does not generate any functional transcript, since the ATG start codon was modified to a TAG stop codon
HSV-TK Mut2	1493-2623	Suicide gene
SV40 (Simian Virus 40)	2633-2987	Transcriptional promoter
CAR CD44v6ΔNL (ORF)	3020-4996	Therapeutic and selection gene
Viral sequence and 3' LTR	5317-5910	Transcriptional termination and polyadenylation

(f) Method for introducing the vector into the recipient organism

(i) transformation (.)
(ii) electroporation (.)
(iii) macroinjection (.)
(iv) microinjection (.)
(v) infection (x)
(vi) other, specify …

5. If the answer to question B.3 (a) and (b) is no, what was the method used in the process of modification?

(i) transformation (.)
(ii) microinjection (.)
(iii) microencapsulation (.)
(iv) macroinjection (.)
(v) other, specify …

6. Composition of the insert :
   (a) Composition of the insert
   (b) Source of each constituent part of the insert
   (c) Intended function of each constituent part of the insert in the GMO

Long Terminal Repeats (LTR):

The LTRs are of retroviral origin. The 5' LTR serves as promoter of transcription of the down-stream therapeutic gene (HSV-TK Mut2) while the 3' LTR serves as termination of transcription and polyadenylation signal

Structural genes

The structural genes are composed of a 420 bp fragment of the gag gene and the Ψ+ packaging signal and are both of retroviral origin.

The gag gene is deleted of 700 bp. Thus, the gag portion included into the vector corresponds to 420 bp. Noteworthy, the gag sequence is not expressed, because in the original vector the ATG start codon for the gag protein was mutated to generate a TAG stop codon to prevent synthesis of viral proteins (Miller et al. Biotechniques, 1989). Therefore, only a limited portion of the entire original genome of the parental virus is present in the parental vector since it lacks of both env and pol genes and only a limited portion of gag is present.

The function of the Ψ+ packaging signal is to allow for packaging of the vector genome into viral particles during the manufacturing process of the retroviral vector. There are no proteins expressed from this sequence.

HSV-TK Mut2

The HSV-TK Mut2 suicide gene encodes a mutated form of the thymidine kinase enzyme of herpes simplex virus I strain CL101 (Abelson et al. Cancer Res, 1970). It was generated by site-directed mutagenesis with the introduction of a silent T to C transition at a splicing donor site of the original HSV-TK (European Patent EP 1 781 789 in the name of MolMed SpA). The mutation has been proven to avoid the generation of GCV-resistant HSV-TK spliced forms.

HSV-TK Mut2 makes the transduced cell sensitive to GCV, thus allowing the negative selection of the transduced cells if adverse events occurred. Following its administration, GCV is first monophosphorylated by the HSV thymidine kinase enzyme expressed in the genetically modified cells and then transformed to the triphosphate form by other cellular kinases. This active, triphosphate form of GCV blocks the synthesis of new DNA strands during mitosis, and hence provokes the death of proliferating cells. There are no functional elements derived from HSV other than the sequence coding for the TK.

SV40

The SV40 region promotes the expression of the CAR CD44v6ΔNL gene. The SV40 promoter is derived from the original LXSN backbone. As the LXSN backbone is one of the most widely used plasmids for preparation of transfer vectors for gene therapy applications, the SV40 promoter has been used for a relevant number of years for vector preparations, using both murine and human cells as packaging substrate, without recording any specific safety concern (Miller et al. Biotechniques, 1989).

CAR CD44v6ΔNL (Casucci et al. Frontier Immunol 2018)

It is a recombinant fusion protein including:

i) The single-chain variable fragment (scFv, which represents the extracellular binding region specific for the CD44v6 antigen, derived from the humanized monoclonal antibody

(mAb) BIWA-8 (Verel et al. Int J Cancer 2002),
ii) A spacer formed by the extracellular domain of the human low-affinity nerve growth factor receptor (ΔLNGFR), which provides a flexible link between the scFv and the transmembrane domain. In general, it allows the antigen-binding domain to accommodate different orientations and facilitate antigen recognition. Moreover, this spacer allows the in vitro immunoselection and in vivo tracking of the transduced cells by the used of LNGFR-specific antibodies (Casucci et al. Frontier Immunol 2018).
iii) The transmembrane and intracellular domain of the human costimulatory molecule CD28, essential for the production of cytokines and the in vivo proliferation and long-term survival of CAR T cells. The transmembrane domain provides a physical link between the spacer and intracellular signaling domains and allows the selection of cells to be infused in patients and their monitoring using specific antibodies (Casucci et al. Frontier Immunol 2018).
iv) The intracellular signaling domain of the human CD3ζ chain that is essential for T cell activation.

(b) Location of the insert in the host organism

- on a free plasmid (.)
- integrated in the chromosome (x)
- other, specify …

(c) Does the insert contain parts whose product or function are not known?
Yes (.) No (x)
If yes, specify …

D. Information on the organism(s) from which the insert is derived

1. Indicate whether it is a:

viroid (.)
RNA virus (.)
DNA virus (x) *cDNA encoding HSV-TKMut2*
bacterium (.)
fungus (.)
animal
- mammals (x) *scFv of humanized murine mAb + all the other sequences are of human origin*
- insect (.)
- fish (.)
- other animal (.)

(specify phylum, class) …
other, specify …

2. Complete name *Not relevant*

(i) order and/or higher taxon (for animals) …
(ii) family name for plants …
(iii) genus …
(iv) species …

(v) subspecies …
(vi) strain …
(vii) cultivar/breeding line …
(viii) pathovar …
(ix) common name …

3. Is the organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?
Yes (.) No (x) Not known (.)
If yes, specify the following:

(b) to which of the following organisms:

humans (.)
animals (.)
plants (.)
other ..

(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism
Yes (.) No (x) Not known (.)

If yes, give the relevant information under Annex III A, point II(A)(11)(d):
…

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?
Yes x No
If yes, specify Level 2
…

5. Do the donor and recipient organism exchange genetic material naturally?
Yes (.) No (x) Not known (.)

E. Information relating to the genetically modified organism

1. Genetic traits and phenotypic characteristics of the recipient or parental organism, which have been changed as a result of the genetic modification

(a) is the GMO different from the recipient as far as survivability is concerned?
Yes (.) No (x) Not known (.)
Specify …

(b) is the GMO in any way different from the recipient as far as mode and/or rate of reproduction is concerned?
Yes (.) No (x) Unknown (.)
Specify …

(c) is the GMO in any way different from the recipient as far as dissemination is concerned?

Yes (.) No (x) Not known (.)

Specify …

(d) is the GMO in any way different from the recipient as far as pathogenicity is concerned?

Yes (.) No (x) Not known (.)

Specify …

2. Genetic stability of the genetically modified organism

Expression of the transgene has been tested after 6 month of storage at <130°C. Moreover, data from the literature have demonstrated that very similar GMO (i.e. human lymphocytes transduced with the same LXSN-based vector enconding the HSV-TK Mut2 and the cell surface marker ΔLNGFR instead of the CAR (i.e. Zalmoxis, a cell-based advanced therapy granted by EC for a conditional Marketing Authorization (EU/1/16/1121)), are genetically stable 14th years after in vivo administration to patients (Oliveira G, Sci Transl Med. 2015).

3. Is the GMO significantly pathogenic or harmful in any way (including its extracellular products), either living or dead?

Yes (.) No (x) Unknown (.)

(a) to which of the following organisms?

humans (.)
animals (.)
plants (.)
other …

(b) give the relevant information specified under Annex III A, point II(A)(11)(d) and II(C)(2)(i)

…

4. Description of identification and detection methods

(a) Techniques used to detect the GMO in the environment

Due to the nature of the product and its intended use for specific patients in hospital settings only, no release of the GMO in the environment is expected. However, in case of need, the presence of the GMO in the environment can be monitored through the analyses of the inserted genes. Cells samples can be analysed by:

• Expression of the CAR CD44v6 by FACS analysis for the LNGFR spacer;

• Detection of specific transgene sequences by PCR.

Swabs or fluids can be analysed by PCR to detect specific transgene sequences

(b) Techniques used to identify the GMO

The GMO can be identified PCR analysis with specific primers

F. Information relating to the release

1. Purpose of the release (including any significant potential environmental benefits that may be expected)
There is no deliberate release of MLM-CAR44.1 T-cells into the environment foreseen other than the administration to the respective patients.

2. Is the site of the release different from the natural habitat or from the ecosystem in which the recipient or parental organism is regularly used, kept or found?
Yes (.) No (x)
If yes, specify …

3. Information concerning the release and the surrounding area

(a) Geographical location (administrative region and where appropriate grid reference):
Limited to the administration site
Hospital Sanata Creu I Sant Pau
Servicio de Hematologia
Mas Casanovas 90
08041 Barcelona

(b) Size of the site (m^2): *Regular size hospital room*
(i) actual release site (m^2): *10 m^2*
(ii) wider release site (m^2): *18 m^2*

(c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected:
No physical proximity to humans and other biota is foreseen

(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO
Administration to patients, no intended deliberate release into the environment

4. Method and amount of release

(a) Quantities of GMOs to be released:
MLM-CAR44.1 T-cells administered to patients vary from a minimum of 0.5x10^6 cells to a maximum of 2x10^6 cells/kg.

(b) Duration of the operation:
The administration per patient itself takes approximately one hour.

(c) Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of the release
The hospital personnel is properly instructed on best practices on handling the product before and during the infusion, and on waste management .
Hand washing before and after administration either soap and running water or appropriate antiseptic hand cleaner. Overcoat, gloves, mask and lab safety glasses shall be worn for administration. MLM-CAR44.1 T-cells are administered to the patient through the use of special valves connected to a venous catheter, so that as

use of needles for infusion is not required. The material used is then be disposed of according to the rules governing the disposal of biological material.

5. Short description of average environmental conditions (weather, temperature, etc.)
The environmental conditions usually present in hospital rooms

6. Relevant data regarding previous releases carried out with the same GMO, if any, specially related to the potential environmental and human health impacts from the release.
No data are available

G. Interactions of the GMO with the environment and potential impact on the environment, if significantly different from the recipient or parent organism

1. Name of target organism (if applicable)

(i)	order and/or higher taxon (for animals)	…
(ii)	family name for plants	…
(iii)	genus	…
(iv)	species	…
(v)	subspecies	…
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	…

2. Anticipated mechanism and result of interaction between the released GMOs and the target organism (if applicable)
The GMO CD44v6 CAR T cells are aimed at controlling tumor growth in treated patients. In case of adverse events, the cells can be eliminated by GCV administration…

3. Any other potentially significant interactions with other organisms in the environment
All individuals not subjected to the therapy with MLM-CAR44.1 T-cells but potentially being exposed to MLM-CAR44.1 T-cells can be considered as non-target organism. As MLM-CAR44.1 T-cells is not released into the environment, no other organisms might be potential non-target organisms.
Transfer of MLM-CAR44.1 T-cells to non-target individuals may occur through accidental contact. In this light, patient administration is the most critical phase and hospital personnel involved are the potentially exposed subjects. However, healthy individuals, when in contact with MLM-CAR44.1 T-cells by accidental blood contact or mucosal exposure, are expected to eliminate the cells as the immune system of the individual would recognize the donor cells as non-self. The chance that two individuals have an identical HLA is extremely low. Immune compromised persons are not allowed to participate in the administration of MLM-CAR44.1 T-cells. As MLM-CAR44.1 T-cells is not expected to contain free CAR-CD44v6ΔNL vector particles, MLM-CAR44.1 T-cells retroviral vector, exposure of contact persons is not expected during administration

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?
Yes (.) No (X) Not known (.)
Give details

In the proposed clinical protocol, the cells used for transduction are differentiated T lymphocytes. Although stimulated to cycling using IL15 and IL7, once infused they do not likely possess any selective growth advantage in vivo. Moreover, the therapeutic approach based on a suicide strategy should in principle limit the risk of any uncontrolled growth of transduced cells, since they can be eliminated by GCV administration.

5. Types of ecosystems to which the GMO could be disseminated from the site of release and in which it could become established

The GMO is not able to survive out of the body and therefore dissemination in the environment is not expected.

6. Complete name of non-target organisms which (taking into account the nature of the receiving environment) may be unintentionally significantly harmed by the release of the GMO

All individuals not subjected to the therapy with MLM-CAR44.1 T-cells but potentially being exposed to MLM-CAR44.1 T-cells can be considered as non-target organism. As MLM-CAR44.1 T-cells is not released into the environment, no other organisms might be potential non-target organisms.

(i)	order and/or higher taxon (for animals)	…
(ii)	family name for plants	…
(iii)	genus	…
(iv)	species	…
(v)	subspecies	…
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	…

7. Likelihood of genetic exchange in vivo

(a) from the GMO to other organisms in the release ecosystem: *none*
(b) from other organisms to the GMO: *none*
(c) likely consequences of gene transfer: *none*

Neither MLM-CAR44.1 T-cells nor the CAR-CD44v6ΔNL retroviral vector is expected to get into contact with other organisms. It is administered in a hospital setting to patients, only. There is no release into the environment during administration of MLM-CAR44.1 T-cells, which could lead to contact with sensitive people or populations of other organisms at large. For the administration procedure hospital personnel wears personal protective equipment and is trained for the specific procedure. In case of accidental exposure through needle stick injury or mucosal exposure, a minimal number of MLM-CAR44.1 T-cells could be injected (a needle stick injury would only be possible with a contaminated needle after administration). However, immune-compromised hospital personnel is not allowed to participate in the administration procedure or in patient treatment after administration, and thus, any minimal number of MLM-CAR44.1 T-cells would be rejected by the immune system of the accidentally exposed person.

As MLM-CAR44.1 T-cells is not expected to contain free CAR-CD44v6ΔNL vector particles, the number of free CAR-CD44v6ΔNL retroviral vector particles to which hospital personnel could be exposed to from a needle stick injury would be close to Zero and no effect from such an event can be envisioned.

8. Give references to relevant results (if available) from studies of the behavior and characteristics of the GMO and its ecological impact carried out in stimulated natural environments (e.g. microcosms, etc.):
Studies on this specific GMO expressing the CD44v6 CAR and the HSV-TKMut2 suicide gene are not available since the first in man treatment is planned for the next year. However, studies with human lymphocytes genetically modified with retroviral vectors encoding CARs or the HSV-TK genes have been performed. In particular, lymphocytes genetically modified with LXSN-based RV vector as this GMO, has been / are used for the clinical development (phase I/II and Phase III) of Zalmoxis, a cell-based advanced therapy granted by EC for a conditional Marketing Authorization (EU/1/16/1121). From these studies, no negative consequences to people and the environment are expected.

9. Possible environmentally significant interactions with biogeochemical processes (if different from the recipient or parental organism)
No known or predicted interactions in biogeochemical processes or any other potential interactions with the environment conceivable.

H. Information relating to monitoring

1. Methods for monitoring the GMOs
CD44v6 CAR T cells will be detected in patient blood samples by a quantitative Real Time PCR assay targeted to TK sequences and by cytofluorimetry
Moreover, detection of GaLV+ viral particles is also performed to assess any possible mobilization of viral vector sequences.
The test will be performed at baseline, on M3, M6 and M12 post-administration. Samples are also taken and stored yearly thereafter for 5 years. This design is in compliance with recommendation described in a FDA/CBER document (FDA-CBER, 2006).

2. Methods for monitoring ecosystem effects
Due to the nature of the product and its intended use for specific patients in hospital settings only, no particular measures have been taken to monitor the ecosystem. However, in case of need, the ecosystem can be monitored through the analyses of the inserted genes. Cells samples can be analysed by:
   - *Expression of LNGFR by FACS analysis;*
   - *Detection of specific transgene sequences by PCR.*

Swabs or fluids can be analysed by PCR to detect specific transgene sequences

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms
Transfer of donated genetic material from the GMO to other organisms is not conceivable. However, in case of need, constitutive expression of the inserted gene would be analysed by:
   - *Expression of LNGFR by FACS analysis;*
   - *Detection of specific transgene sequences by PCR;*
   - *GCV sensitivity;*

4. Size of the monitoring area (m^2) *Not relevant. The product intended use is for specific patients in hospital settings only,*
… m^2

5. Duration of the monitoring *not relevant*

…

6. Frequency of the monitoring *In case of need, the ecosystem can be monitored through the analyses of the inserted genes..*

…

I. Information on post-release and waste treatment

1. Post-release treatment of the site

The MLM-CAR44.1 T-cells is susceptible to inactivation under ambient environmental conditions and alcoholic disinfectants. Surface that could have been in contact with the GMO must be decontaminated with disinfectants, adhering to proposed contact time indicated for the specific brand of disinfectant used.

2. Post-release treatment of the GMOs

Solid waste: all material having been in contact with the GMO should be handled and disposed of as biohazardous waste in the appropriate container for biohazard waste
Liquid waste: liquid containing the GMO can be inactivated with chlorine bleach 1/50 (2% final concentration) for 2 minutes and then be dispose in container for biohazard waste

3. (a) Type and amount of waste generated

Solid and liquid wastes

3. (b) Treatment of waste

Wastes are inactivated with sodium ipochloride. Whenever possible they are gathered in specific closed containers and treated as potentially infective biologic material.

J. Information on emergency response plans

1. Methods and procedures for controlling the dissemination of the GMO(s) in case of unexpected spread

The GMO is to be manipulated in restricted area only and under specific procedures. In this light, dissemination of the GMO in the environment is a very unlikely event and emergency plans are not in place.

2. Methods for removal of the GMO(s) of the areas potentially affected

In case of accidental spillage on surfaces

- *Continue wearing personal protective equipment.*
- *Put on a second pair of gloves.*
- *Cover spill with absorptive paper towels.*
- *Spray/soak with disinfectant.*
- *Remove after immersion time of 30 minutes. Use forceps if sharps have been produced and dispose sharps into a sharps container. Dispose paper towels and gloves in waste bag.*
- *Dispose all waste, including gloves, as potentially infectious medical waste.*

- *Disinfect and wash hands with soap and water*

In case of accidental spillage on clothing:

- *Remove all contaminated clothing and put into laundry bin.*
- *Disinfectant skin surfaces that were in potential contact.*
- *Remove gloves and discard as potentially infectious medical waste.*
- *Re-dress with clean clothes.*
- *Close bin with contaminated clothing and have it laundered according to the hospital procedure for clothing potentially contaminated with infectious biological material*

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread
Not applicable.

4. Plans for protecting human health and the environment in the event of an undesirable effect
Not applicable