2.- SUMMARY NOTIFICATION INFORMATION (SNIF)

ACCORDING TO FORMAT ESTABLISHED BY DECISION 2002/813/EC FROM COUNCIL of the EUROPEAN UNION (3 OCTOBER 2002).

SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAT HIGHER PLANTS
in accordance with article 11 of the Directive 2001/18/EC

A. GENERAL INFORMATION

1. Details of notification

(a) Member State of notification: Spain
(b) Notification number: B/ES/09/64
(c) Date of acknowledgement of notification: 31/08/09
(d) Title of the Project: Phase I clinical trial of intravenous administration of a conditionally replicating adenovirus ICOVIR-5 in patients with locally advanced or metastasic malignant melanoma.
e) Proposed period of release: First patient enrolment: Sep 2010. Alternative, 1rst December 2010. Last patient enrolment: Sep 2011. Alternative, 1rst December 2011.

2. Notifier

Name of institution or company: Institut Català d'Oncologia - IDIBELL

3. GMO characterisation

a) Indicate whether the GMO is a:

- **viroid** ☐
- **RNA virus** ☐
- **DNA virus** ☒
- **bacterium** ☐
- **fungus** ☐
- **animal** ☐
  - **mammals** ☐
  - **insects** ☐
  - **fish** ☐
  - **other animal** ☐ **specify phylum, class**

- **other, specify (kingdom, phylum and class)**

b) Identity of the GMO (genus and species)

ICOVIR-5 (HAd5-DM-E2F-K-Δ24-RGD) is an oncolytic adenovirus derived from HAd5. It has been designed for systemic treatment of disseminated tumors. ICOVIR-5 genome contains several modifications that confer selective replication in tumoral cells with deregulated retinoblastoma/E2F pathway. ICOVIR-5 includes the following modifications:

1. Endogenous E1A promoter substitution by E2F-1 promoter, to control viral E1A protein expression.
2. DM-1 genomic insulator insertion preceding E2F-1 promoter.
3. Kozak sequence insertion at E1A start codon (K)
4. 8-amino acid deletion at the pRb-binding domain in E1A protein (Δ24 deletion)
5. RGD-tripeptide insertion in the viral fiber protein.

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

Parental adenoviruses are very stable in nature. Although it has been described that recombination between different serotypes could have a role in the evolution of new strains with different immunogenic properties in immunosuppressed patients, such recombinants very rarely contain HAd5 sequences if never. Moreover, different scientific studies have demonstrated that genetically modified adenoviruses are stable as long as its genome size does not exceed 105% of the parental genome size. Longer genomes could result in delayed viral growth and spontaneous rearrangements that lead to loss of non-essential DNA sequences, usually the exogenous inserts.

ICOVIR-5 is a HAd5 based adenovirus and its genetic modifications modify genome size to a final length of 36928 base pairs (bp), which represents 102,76% with respect

to genome size of parental. Consequently the probability that ICOVIR-5 becomes genetically unstable is very low.

4. Is the same GMO release planned elsewhere in the Community (in conformity with Article 6(1)), by the same notifier?

Yes ☐	**No ☐**
If yes, insert the country code(s):	

5. Has the same GMO been notified for release elsewhere in the Community by the same notifier?

Yes ☐	**No ☐**
If yes: **- Member State of notification:** **- Notification number:**	

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 ☐**
If yes: **- Member State of notification:** **- Notification number:**	

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

There are not previous data concerning ICOVIR-5 environment release, since currently this virus has never been administered to human beings.

Analyzing data from clinical trials with systemically administered oncolytic adenoviruses similar to ICOVIR-5, adenovirus presence has been confirmed in urine, saliva and probably also stool until day 14 post-administration, and especially at higher doses (> 2.10^{12} viral particles/patient). That's probably associated to the relatively immunosuppressed status of cancer patients, that display a slower adaptative response.

Humans are the natural adenovirus hosts. Adenoviral infections are endemic and most population is sero-positive for anti-adenovirus neutralizing antibodies. Adenoviral infections are mostly asymptomatic, auto-limiting and restricted to few permissive tissues. Moreover, ICOVIR-5 genetic modifications restrict viral replication to human cancer cells, which reduces its potential environmental impact.

B. INFORMATION RELATING TO THE RECIPIENT OR PARENTAL ORGANISM FROM WHICH THE GMO IS DERIVED

In order to fill up requested information, the following premises have been taken into account:

- GMO: ICOVIR-5
- Recipient: HAd5
- Donor: human genome or synthetic fragments that have been added to HAd5 to generate the conditionally replicating adenovirus ICOVIR-5

1. Recipient or parental organism characterisation:

Indicate whether the recipient or parental organism is a:

(select one only)

- viroid ☐
- RNA virus ☐
- DNA virus ☐
- bacterium ☐
- fungus ☐
- animal ☐

- mammals ☐
- insects ☐
- fish ☐
- other animal ☐ **specify phylum, class**

- other, specify (kingdom, phylum and class)

2. Complete name

i)	**order and/or higher taxon (for animals):** Adenoviridae
ii)	**genus:** Mastadenovirus
iii)	**species:** wildtype human adenovirus (HAd) - C
iv)	**subspecies:** wildtype human adenovirus (HAd) serotype 5
v)	**strain:**
vi)	**pathovar:**
vii)	**common name:** HAd5

3. Geographical distribution of the organism

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

Yes ☐ **No** ☐ **Unknown** ☐

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

i) Yes ☐

If yes, indicate in which ecosystem is found:

Atlantic ☐

Mediterranean ☐

Arctic ☐

Alpine ☐

Continental ☐

ii) No ☐

iii) Unknown ☐

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

Yes ☐ **No** ☐

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

Yes ☐ **No** ☐

4. Natural habitat of the organism

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):

HAd5 is not present in natural ecosystems, except in human beings, since its activation is restricted to human cells. Vey recently it has been described that cotton rats (*Sigmodon hispidus*) and some hamster strains (as *Mesocricetus auratus*) are semi-permissive to human adenovirus replication.

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

N.A.

5. a) Paretal organism detection techniques

HAd's detection is performed by direct Real-time quantitative PCR from DNA extracted from desired tissue by specific oligonucleotides in HAd5 genome. Technique sensitivity = 10-20 genomes/20 ng de tissue DNA.

5. b) Paretal organism identity techniques

HAd's identity is performed at genomic level by PCR and restriction map of purified viral DNA.

6. ¿Is parental organim classified in terms of human health and environment protection according to current EC regulations?

Yes ☒	**No ☐**
If yes, specify: HAd5 is an human adenovirus of pathogenicity class 2.	

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

Yes ☒	No ☐	Not known ☐

If yes:

a) to which of the following organisms:

humans ☒
animals ☐
plants ☐
others ☐

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

HAd5 is a human virus with a pathogenicity class 2. HAd5 infections are mostly asymptomatic but eventually can produce respiratory tract diseases, together with ocular or gastrointestinal manifestations, especially in children. Incubation period is between 1 and 10 days. Some authors have described that lymphoid organs could generate persistent shedding, but such extreme is considered very unusual and only anecdotically demonstrated with children after bone marrow transplantation. Most population is seropositive for adenovirus, usually group C adenoviruses that are the most widespread distributed. Consequently, most population is able to easily neutralize any adenoviral infection.
Adenoviruses enter their host by respiratory tracts or eyes, through aerosols generated by infected individuals (by coughing or expectorations). Adenovirus transmission can be accomplished by saliva or by oral-faecal via. According to Canadian Pubic Health Agency, lower limit for inhalation-mediated infection is set at 150 plaque-forming units.
Most adenoviral infections are auto-limiting. Studies conducted with adenovirus live vaccines have demonstrated that transmission is possible after enteric administration, probably by oral-faecal via, but requires intimate contact. Infection after spontaneous contact is highly unprobable even with wild-type strains. Moreover horizontal transmission of adenovirus is only accomplished between humans, and it does not affect any other specie, excepting chimpanzee.

In contrast to retrovirus and lentivirus, adenovirus is not an integrative virus. Although some cases of long-term persistence in lymphoid tissues have been reported, such phenomenon seems to be episomal and no associated to low frequency integration events. Regardless of the mechanism, no adverse events related to long-term persistence of adenoviral genomes have been recorded. In addition efforts to generate transgenic mice by intra-testicular injection of adenovirus have failed.

8. Information concerning reproduction

a) Generation time in natural ecosystems:

Irrelevant, HAd5 is not found in natural ecosystems, just in human cells.

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

Irrelevant

c) Way of reproduction: **Sexual** ☐ **Asexual** ☐

Irrelevant

d) Factors affecting reproduction:

Irrelevant

9. Survivability

a) ability to form structures enhancing survival or dormancy:

i) endospores ☐
ii) cysts ☐
iii) sclerotia ☐
iv) asexual spores (fungi) ☐
v) asexual spores (fungi) ☐
vi) eggs ☐
vii) pupae ☐
viii) larvae ☐

ix) other (specify): viral amplification

b) relevant factors affecting survivability:

Adenoviruses quickly lose its bioactivity at room temperature. Bioactivity losses are logarithmic and consequently the virus retains a certain degree of stability at long time but remaining levels are below infectivity threshold. Adenoviruses are only partially resistant to chemical and physical agents. HAd5 is susceptible to 1% sodium hypochlorite, 2% glutaraldehyde and 0,25% sodium dodecylsulphate (SDS). It's also temperature-sensitive (>56ºC). HAd5 inactivation is accomplished by autoclaving at 121ºC for 15 minutes, with 100% efficiency. Higher temperatures or longer autoclaving periods can also be used.

10. a) Ways of dissemination

Adenoviruses affect different organs around human body. Most usual and known syndromes include:

- Respiratory tract infections: they are the most usual, especially those related to upper tract, such as pharyngeamygdalitis, that are active during all the year. Lower tract infections are also possible with adenoviruses, such as thraquea bronchitis, characterized by abundant coughing that can degenerate in pertuosic syndromes. Very rarely adenoviruses can be causative of pneumonia.
- Gastrointestinal infections. Most serotypes induce low-grade symptomatology, although infections with serotypes 40 and 41 can produce fever and gastroenteritis with an evolution period of 8 days or more.
- Ocular infections. Clinical manifestations include conjunctivitis, concomitant with pharyngytis or with a more pathogenic keratinoconjunctivitis, arising from a follicular conjunctivitis that invades the cornea.
- Genital-urinary infections: the most usual is hemorrhagic cystitis but eventually some cases of cervicitis or urethritis as sexual tramssion-derived pathologies have also been reported.
- Immuno-suppressed patients: occasionally, adenovirus can infect this kind of patients, provoking pneumonia or generalized infection, in which pathogens can be isolated from a range of organs, i.e. liver from a transplanted patient.

10. b) Factors affecting dissemination

Irrelevant

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)

Previous notifications for ICOVIR-5 in confined-use regime have been notified for research purposes.

Corresponding authorizations have been issued by the Departament d'Agricultura Ramaderia i Pesca (DARP) de la Generalitat de Catalunya (local competent authority) .

Notification numbers for the facility and the operational procedure are, respectively:

A/ES/05I-14

A/ES/05/15

C. INFORMATION RELATING TO THE GENETIC MODIFICATION

In order to fill up requested information, the following premises have been taken into account:

- GMO: ICOVIR-5
- Recipient: HAd5
- Donor: human genome or synthetic fragments that have been added to HAd5 to generate the conditionally replicating adenovirus ICOVIR-5

1. Type of the genetic modification

i) insertion of genetic material ☐
ii) deletion of genetic material ☐
iii) base substitution ☐
iv) cell fusion ☐

v) Other (specify):

ICOVIR-5 (HAd5-DM-E2F-K-Δ24-RGD) is a HAd5-derived oncolytic adenovirus that has been designed for systemic treatment of disseminated tumors. Its genome contains different genetic modifications that confer selective replication in tumor cells with deregulated retinoblastoma/E2F pathway. ICOVIR-5 genetic modifications include:

1) partial substitution of endogenous E1A promoter by E2F-1 promoter to drive viral protein E1A expression.
2) insulator sequence (DM) insertion preceding E2F-1 promoter.
3) Kozak sequence (K) insertion at start codon of E1A protein (K)
4) deletion of 8 amino acids at pRB-binding domain of E1A (Δ24 deletion)
5) RGD tripeptide insertion at HI loop in adenoviral fiber.

2. Intended outcome of the genetic modification

Overall genetic alterations present in ICOVIR-5 genome lead to restricted adenovirus replication to tumor cells (with high free E2F-1 levels). Moreover these elements also preclude viral protein expression in cells different from tumor-forming cells, which allows systemic administration of the virus without significant hepatic toxicity. The presence of RGD-4C accounts for increased adenovirus infectivity in tumor cells, that has been demonstrated that over-express integrin in cell membrane. The intented use of ICOVIR-5 is the antitumoral treatment of patients with metastatic melanoma.

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

Yes ☒	**No ☐**
If no, go straight to question 5.	

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

Yes ☒	**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 ☐
cosmid ☐
transposable element ☐

other (specify):

b) Identity of the vector:

To generate conditionally replicating ICOVIR-5, plCOVIR-5 plasmid was digested with *Pac*I restriction enzyme and resulting material was purified and tranfected to HEK293 cells (from ATCC, *American Type Cell Collection*).

plCOVIR-5 is the recombination product between pShuttle-DM-E2F-K-Δ24 (plasmid that contains E1A promoter modifications, such as DM-1 genetic insulator, human E2F-1 promoter, and Kozak sequence, preceding the modified E1A-Δ24 coding region) and pVK503 plasmid (that contains HAd5 complete genome modified at aa 480 of fiber gene to include 27 base pairs coding for the RGD-4C peptide: Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys). pICOVIR-5 vector identity is confirmed by *Kpn*I digestion that results in a defined restriction pattern.

c) Host range of the vector

pICOVIR-5 is only able to replicate in bacteria.

Transfection of *Pac*I-digested pICOVIR-5 generates a complete copy of the ICOVIR-5 viral genome that is able to initiate its replication in human cells with high E2F-1 levels.

d) Presence in the vector of sequences giving a selectable or identifiable phenotype:

d1. In pICOVIR-5 plasmid

Yes ☒ No ☐

antibiotic resistance Yes☒
Other (specify):
Indication of which antibiotic resistance gene is inserted: ampicillin

d1. In ICOVIR-5 virus

Yes ☐ No ☒

antibiotic resistance No☒
Other (specify):
Indication of which antibiotic resistance gene is inserted:

e) Constituent fragments of the vector

plCOVIR-5 is the recombination product between pShuttle-DM-E2F-K-Δ24 (plasmid that contains region 34931-35935 from HAd5 genome, followed by 1-5790 region, including E1A promoter modifications, such as DM-1 genetic insulator, human E2F-1 promoter, and Kozak sequence, preceding the modified E1A-Δ24 coding region) and pVK503 plasmid (that contains HAd5 complete genome modified at aa 480 of fiber gene to include 27 base pairs coding for the RGD-4C peptide: Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys). Consequenly after recombination pICOVIR-5 contains ICOVIR-5 complete genome. Additionally pICOVIR-5 includes an ampicillin resistance gene and a bacterial origin of replication, both flanked by *Pac*I sites (in a way that after *Pac*I-digestion, elements related to bacterial relplication are removed)

f) Method for introducing the vector into the recipient organism

i) transformación ☐
ii) electroporación ☐
iii) macroinyección ☐
iv) microinyección ☐
v) infección ☐

vi) other (specify): Transfection of *Pac*I-digested pICOVIR-5 generates a complete copy of the ICOVIR-5 viral genome that is able to initiate its replication in human cells with high E2F-1 levels.

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:

ICOVIR-5 genome includes 4 insertions with respect to wild-type HAd5 genome:
- genetic insulator from human myotonic dystrophic gene (DM-1): Genbank L08835
- human E2F-1 gene promoter: Genbank S74230
- human Kozak sequence: CCACC sequence
- artificial α_v integrin-binding sequence: TGTGACTGCCGCGGAGACTGTTTCTGC sequence

b) Source of each constituent part of the insert:

- DM-1 insulator: human DM1 locus. Non-coding sequence from human origin that was obtained from DNA from normal human peripheral blood mononuclear cells by polymerase chain reaction (PCR) amplification, using oligonucleotides that amplify from nt 13006 to 13474 in human DM-1 genetic loci (GenBank accession number L08835). It functions by isolating E2F-1 promoter E2F1 in the viral genome context.

- E2F1 promoter: Non-coding sequence from human origin that was obtained from DNA from normal human peripheral blood mononuclear cells by polymerase chain reaction (PCR) amplification, using oligonucleotides that amplify from -218 to + 51 on E2F-1 sequence. It controls gene expression in an E2F-1-dependent manner.

- Kozak's sequence. Non-coding sequence from human origin that was synthesized as designed oligonucleotides that include the CCACC sequence and further PCR reaction. It facilitates mRNA recognition by translation cell machinery.

- synthetic integrin-binding: artificial sequence that was synthesized as designed oligonucleotides that include the TGTGACTGCCGCGGAGACTGTTTCTGC sequence and further PCR reaction. It allows viral binding to membrane integrins that are over-expressed in some tumor cells.

c) Intended function of each constituent part of the insert in the GMO:

See previous section.

d) Location of the insert in the host organism:

- on a free plasmid	☐
- integrated in the chromosome	☐

- other (specify): integrated in HAd5 genome.

e) (e) Does the insert contain parts whose product or function are not known?

Yes ☐ No ☐

If yes, specify:

D. INFORMATION ON THE ORGANISM(S) FROM WHICH THE INSERT IS DERIVED

In order to fill up requested information, the following premises have been taken into account:

- GMO: ICOVIR-5
- Recipient: HAd5
- Donor: human genome or synthetic fragments that have been added to HAd5 to generate the conditionally replicating adenovirus ICOVIR-5

1. Indicate whether it is a:

viroid	☐
RNA virus	☐
DNA virus	☐
bacterium	☐
fungus	☐
animal	☐
- mammals	☐
- insect	☐
- fish	☐
- other animal	☐ **(specify phylum, class)**

other (specify): Humans (*Homo sapiens sapiens*) and synthetic

2. Complete name

i) Order and/or higher taxon (for animals)

Primates.

ii) Family name for plants

Hominidae

iii) Genus:

Homo.

iv) Species:

Homo Sapiens.

v) Subspecies: Homo Sapiens Sapiens.
vi) Strain: N. A.
vii) Cultivar/breeding line N. A.
viii) Pathovar: N. A.
ix) Common name: Human being.

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

Yes ☐	**No ☒**	**Not known ☐**
If yes, specify the following: **a) 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 ☒**	**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☐	**No ☐**
If yes, specify:	

5. Do the donor and recipient organism exchange genetic material naturally?

Yes ☐	**No ☐**	**Not known ☐**

E. INFORMATION RELATING TO THE GENETICALLY MODIFIED ORGANISM

In order to fill up requested information, the following premises have been taken into account:

- GMO: ICOVIR-5
- Recipient: HAd5
- Donor: human genome or synthetic fragments that have been added to HAd5 to generate the conditionally replicating adenovirus ICOVIR-5

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 ☒** **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 ☐** **Not known ☐**

Specify: HAd5 is able to replicate in most human cells, whereas ICOVIR-5 just can replicate in human tumor cells (with high E2F-1 levels).

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

Yes ☐ **No ☒** **Not known ☐**

Specify:

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

Yes ☒ **No ☐** **Not known ☐**

Specify: ICOVIR-5 is defective in normal human cells and only lyses activated human cells. ICOVIR-5 does not reproduce respiratory tract pathogenicity associated to HAd5.

2. Genetic stability of the genetically modified organism

Stable.

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

Yes ☒	**No ☐**	**Not known ☐**	
If yes:			
a) to which of the following organisms?	**humans**	☒	(only tumor cells)
	animals	☐	
	plants	☐	
	other	☐	

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

Overall genetic modifications included in ICOVIR-5 impair both viral protein expression and viral replication in normal cells (without alterations in retinoblastoma pathway). Preclinical data obtained with ICOVIR-5 indicate that:

- In normal primary human astrocytes: ICOVIR-5 is unable to express detectable levels of E1A or late proteins (fiber), its replication is inactivated (viral titer 5 logs below wild-type HAd5 levels) and does not display significant cytotoxicity.
- In normal liver (from both human and murine origin) ICOVIR-5 is unable to express detectable levels of E1A. The virus is unable to replicate in human primary liver biopsies.
- In human normal fibroblasts, ICOVIR-5 does not express E1A protein.
- In human tumor cells (with alterations in retinoblastoma pathway) where the functionality of pRb pathway is restored by over-expression of pRb or the negative regulator p21), ICOVIR-5 is unable to express detectable levels of E1A or late proteins (fiber), and its replication is inactivated (viral titer 7 logs below the same cell line before pRb pathway restoration).

Mouse and rat tissues do not support efficient replication of human adenoviruses. However, mice can be used as host animal to grow xenograft human tumors when using immunodefficient strains (*nude* mice). More recently, Golden Syrian hamsters (*Mesocricetus auratus),* cotton rats (*Sigmodon hispidus*) and swine have also been proposed as semi-permissive models that can be used in preclinical studies to evaluate efficacy, toxicity and biodistribution of genetically modified variants of HAd5.

Single intravenous administration of ICOVIR-5 in mice models (both nude and immunocompetent strains) is cleared from blood with a median half-life ($t_{1/2}$) of 3 minutes, demonstrating that ICOVIR-5 clearance is very close to HAd5's one. The $t_{1/2}$ value for AdTL-RGD (viral vector with the same RGD insertion in its fiber) is also very similar to those obtained with HAd5 and ICOVIR-5 (2,1 minutes). These results indicate that RGD incorporation in fiber HI loop does not modify ICOVIR-5 pharmacokinetics with respect to other HAd5 derivatives. Consequently, although not clinical data are available about ICOVIR-5 intravenous administration profile, it can be assumed that this will be very similar to previous oncolytic adenovirus already tested in different clinical trials. In this sense, genome number data obtained by PCR in reported clinical trials with Onyx-015 and CV7870 after single endovenous administration indicate that adenoviruses are rapidly cleared from blood after administration with a $t_{1/2}$ value of 15 minutes, and genome nadir occurs typically between 9 and 12 hours post-administration.

In such mice model, ICOVIR-5 is mainly accumulated in the liver 1 hour after administration; however virus is detectable only at genome level since non-tumoral cells do not express viral protein, not allow its replication. A small fraction of administered virus reaches the tumor mass (in the subcutaneous model) where ICOVIR-5 begins to replicate. Viral replication can be detected by the presence of capsid proteins (late expression) from day 8 post-systemic

administration.

Several preclinical and clinical data demonstrate that main systemic toxicity of other oncolytic adenoviruses similar to ICOVIR-5 is hepatic. Administration of high adenovirus doses (up to a 10^{13} viral particles, vp) to more than 300 cancer patients led to an objectivity toxicity comprising a fever-related status, transitory and mild transaminase elevation and occasional vomiting. As in preclinical studies, liver was reported to be the main affected tissue due to hepatic transduction. In parallel, tolerance to high vector dosage has been reported in several phase I and II clinical trials with oncolytic adenovirus in cancer patients (for a revision, see Aghi & Martuza).

Acute toxicity studies after systemic administration of increasing doses ICOVIR-5 (from 1.10^{10} vp/mouse to 1.10^{11} vp/mouse) in immunocompetent BalbC mice have also been performed. As controls, wild type strain (HAd5) and an oncolytic virus without protein expression regulation (AdΔ24RGD) were included up to its maximal tolerated dose (from 1.10^{10} vp/mouse to 5.10^{10} vp/mouse). Obtained results demonstrated that:

- *In vivo* E1A liver expression is abrogated: Immunofluorescence against E1A in samples obtained at day 5 and 12 post-intravenous administration of increasing doses of ICOVIR-5 indicate that protein is not expressed even at higher assayed dose (1.10^{11} viral particles/mouse). Equivalent doses of HAd5 or AdΔ24RGD induced high levels of E1A expression.
- STD_{10} value for ICOVIR-5 (severe toxicity dose for 10% of animals) is set at 1.10^{11} vp/mouse. Analysis of the % body weight variation and associated lethality concluded that ICOVIR-5 is not lethal at doses below 1.10^{11} vp/mouse. This value is much lower that obtained for HAd5 or AdΔ24RGD.
- Anatomic/pathological study of tissues was performed at day 3 and 5 after intravenous administration of ICOVIR-5 in different tissues (liver, spleen, kidney, lung, etc.) by a pathologist blinded to sample identification. Results revealed that doses below 5.10^{10} vp/mouse do not produce any difference in liver biopsies. At 5.10^{10} vp/mouse some lymphoid aggregates were localized in a mainly healthy parenchyma, and no evidences of necrotic degeneration or steatosis were recorded. Higher dosage level led to the appearance of limited steatotic regions and isolated Councilman bodies. At such dose, it was also evident the presence of chronic infiltrating inflammation and significant proliferative activity. Such profile could be clearly distinguished from the massive hepatitis associated to the administration of 5.10^{10} vp/mouse of HAd5 ó AdΔ24RGD, where the presence of large necrotic areas and macrosteatosis was evident. Analysis of lung biopsies obtained at day 5 post administration revealed no significant morphological differences at any dosage level (up to 1.10^{11} vp/mouse of ICOVIR-5). Pathological study of kidney samples was also unable to differentiate control groups (vehicle, PBS) from animals injected with ICOVIR-5 up to 5.10^{10} vp/mouse, excepting the presence of interstitial lymphoid inflammation at low degree. At 1.10^{11} vp/mouse ICOVIR-5-injected kidneys did not displayed significant differences, although lymphoid inflammation was increased, and single apoptotic cells were occasionally present.
- Biochemical analysis of hepatic function in mice plasma after ICOVIR-5 administration: At day 5 post-administration ICOVIR-5 injection did not altered creatinin levels at any assayed dose. By contrast, transaminitis was detected at highest doses of ICOVIR-5 (5.10^{10} and 1.10^{11} vp/mouse) although AST/ALT values were statistically reduced to equivalent doses of wild-type strain (HAd5) or AdΔ24RGD. Results at day 12 p.a. demonstrated that transaminase levels tended to normalize at 5.10^{10} vp de ICOVIR-5, but higher doses (1.10^{11} vp) resulted in slower decreasing rates.
- Hematological analysis in total peripheral blood after ICOVIR-5 administration: At day 5 post-administration, ICOVIR-5 virus induced a marginal decrease in platelets only at the highest dose (1.10^{11} vp/mouse), whereas HAd5 or AdΔ24RGD were able to induce much dramatic reduction at lower doses. By contrast, lymphocyte counts were

not affected at any dose of ICOVIR-5. ICOVIR-5 also induced monocyte and neutrophyl count increases, but not eosinophylia, at doses higher than $2{,}5.10^{10}$ vp/mouse. All these alteration were reverted by day 12 post-administration.

Preliminary acute toxicity data in Golden Syrian hamster (were adenovirus replication is semi-permissive) is also available. In this case ICOVIR-5 was systemically administered up to 4.10^{11} vp/hamster. Animals did not experienced significant body weight alteration with respect to control group up to day 29 post-administration. Results from biochemical analysis at day 5 p.a. pointed up that ICOVIR-5 did not affect hepatic function, and creatinine and transaminase levels (AST/ALT) were not increased at any dose.

With respect to environmental release, ICOVIR-5 has never been intentionally released or administered to humans and consequently, no data is available. Data from clinical trials performed with other oncolytic adenoviruses similar to ICOVIR-5 indicate that shedding of life adenovirus following systemic exposure may take place via the urine, stool and saliva for up to 14 days post-treatment, especially at higher viral doses (> 2.10^{12} vp/patient). That is probably associated to the relatively immuno-compromised status of cancer patients that present a delayed adaptative response (see 2i section of the Environmental Risk Assessment document). However inadvertent transmission of virus to personnel or spread to thirds has never been reported in any of the studies.

Intended use for ICOVIR-5 includes its endovenous administration. After systemic exposure, rapid blood clearance of the virus is expected during initial hours after administration (according to previous clinical experience). When ICOVIR-5 reaches the tumor, replication of the virus in the distant tumor loci should occur. From preclinical biodistribution studies in mice, it is expected that ICOVIR-5 will display similar tissue distribution with respect to HAd5. According to published data on HAd5 biodistribution after systemic administration in human, swine and chimpanzees, tissular biodistribution of adenovirus is wide.

Also in terms of persistence it is expected that ICOVIR-5 behaves similar to HAd5 or Onyx-015. Viral genomes of these viruses have been detected in the patients several days after last systemic administration, but not viable viruses were recovered in any case.

4. Description of identification and detection methods

a) Techniques used to detect the GMO in the environment

Quantitative polymerase chain reaction (Real-time-PCR).

b) Techniques used to identify the GMO:

ICOVIR-5 identity will be determined at viral genomic DNA level by PCR and further restriction analysis of purified viral DNA.

a) PCR amplification from 321 bp to 2013 on ICOVIR-5 genome. Such 1692 bp transcript includes DM-1 insulator, E2F-1 promoter and Δ24 mutation in E1A gene.
b) Restriction analysis of genomic viral DNA with *Kpn* I restriction enzyme. Such restriction generates a pattern that comprises 2 characteristic fragments for ICOVIR-5: one which sizes 1284bp and a second one sizing 1738bp (complete restriction pattern for ICOVIR-5 + *Kpn* I is: 6.4 / 5.7 / 5.1 / 4.8 / 3.6 / 2.9 / 2.3 / 1.7 / 1.6 / 1.2 / 1.0 Kb). In spite of such 2 characteristic bands, HAd5 generates a single band of 2052 bp.

Also viral DNA sequencing can be used to identify ICOVIR-5. ICOVIR-5 genome sequencing is performed in differential regions with respect to HAd5. The following oligonucleotides are used:

DM-1 region: DM-1-Up (5'-GGGCAGATGGAGGGCCTTTTATTC-3')
E2F promoter region: E2F-Up (5'-GTGTTACTCATAGCGCGTAA-3')
Δ24 deletion region : Δ24-down (5'-CTCCGGTGATAATGACAAG-3')
Tropism-modified fiber (RGD sequence): FiberUp (5'-AAACGCTGTTGGATTTATG-3').

F. INFORMATION RELATING TO THE RELEASE

1. Purpose of the release (including any significant potential environmental benefits that may be expected)

Current clinical trial objective is the determination of the maximal tolerated dose (MTD) corresponding to the infusion of the conditionally replicating adenovirus ICOVIR-5 in patients with advanced malignant melanoma. Moreover this study also envisions establishing the safety and toxicological profile for ICOVIR-5, together with its preliminary efficacy data. These estimations will allow determining a safe dose for subsequent efficacy clinical trials (recommended dose). Concomitantly, a biodistribution study of ICOVIR-5 in sera and tumor will be obtained from malignant melanoma patients after treatment with ICOVIR-5.

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 ☐
If yes, specify:	

3. Information concerning the release and the surrounding area

a) Geographical location (administrative region and where appropriate grid reference):

Hospital Duran y Reynals. Av. Gran Via de l'Hospitalet 199, Hospitalet de Llobregat. (Barcelona).

b) Size of the site (m²):

i) actual release site (m²): 7

ii) wider release site (m²): Hospital Duran y Reynals

Patients will be treated in isolated rooms, and they will not be allowed to exit the room until blood, urine and stool samples become negative for adenovirus presence (by real-time PCR technique analysis).

c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected:

N.A.

d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO:

N.A.

4. Method and amount of release

a) Quantities of GMOs to be released:

Initial dose is set at 1.10^{11} vp of ICOVIR-5 per patient. Initially a single patient will be injected for each dose level. If no grade 2-toxicity or higher level is observed, dose will be scaled until next dose level. On the contrary, a cohort of 3 to 6 patients will be included in each dose level.

b) Duration of the operation:

Viral infusion is performed by systemic administration of 50 ml of viral solution in 10 minutes (aprox.).

A treatment cycle is defined as the infusion of a single dose + 4 weeks of monitoring

c) Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of the release:

Protocols to be adopted by personnel and third persons (visitors, etc.) to be in contact with patients administered with ICOVIR-5, are defined in an additional SOP (standard operation procedure) that includes all instruction to be followed. A summary of such SOP is appended below:

- Personnel that harm themself with needles or sharp instruments that have been in contact with ICOVIR-5 must follow standard current procedures associated to such kind of accidents. The accident must be notified to study responsible person and to Risk Prevention Department.
- Any member of the study-associated personnel that feels sick must inform to study responsible person and to Risk Prevention Department.
- Visits of immuno-compromised, transplanted, chemotherapy or corticoid-treated persons, children or pregnant women are not allowed.
- A maximum of two persons will be simultaneously accepted.
- Visitors must wear disposable coats, security glasses and masks when accessing to patient room.

All clinical specimens from ICOVIR-5-treated patients must be introduced in a double sealed-bag and carried into tightly closed rigid containers.

Containers and bags will be labeled as biosafety material.

Samples to be analyzed will be manipulated by study personnel, which warrant proper transfer to laboratory.

5. Short description of average environmental conditions (weather, temperature, etc.)

Mediterranean climate and controlled conditions in Hospital Area.

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.**

ICOVIR-5 has never been intentionally released or administered to humans and consequently, no data is available. Data from clinical trials performed with other oncolytic adenoviruses similar to ICOVIR-5 indicate that shedding of life adenovirus following systemic exposure may take place via the urine, stool and saliva for up to 14 days post-treatment, especially at higher viral doses (> 2.10^{12} vp/patient). That is probably associated to the relatively immuno-compromised status of cancer patients that present a delayed adaptative response

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):** Primates.
ii) **Family (plants):** Hominidae.
iii) Genus: Homo.
iv) Species: Homo Sapiens.
v) Subspecies: Homo sapiens sapiens.
vi) Strain: N.A.
vii) Cultivar/breeding line: N.A.

viii) Pathovar:

N.A.

ix) Common name:

Human being.

2. Anticipated mechanism and result of interaction between the released GMOs and the target organism (if applicable)

Main aim of ICOVIR-5 administration is antitumoral treatment of human melanoma tumors with oncolytic adenoviruses. Based in the characteristics of tumor cells (with high E2F levels) and overall genetic modifications included in ICOVIR-5 genome, ICOVIR-5 replicates selectively in tumor cells leading to selective eradication of malignant cells and amplification of ICOVIR-5 input dose in tumor mass. When virus reaches tumor margin, ICOVIR-5 will be internalized by non-tumoral cells, where it is unable to express viral proteins and initiate its replication, and consequently its propagation is stopped. This mechanism allows selective destruction of melanoma tumors in patients. Modifications included in ICOVIR-5 also allows for systemic administration to blood stream, since:

- In normal primary human astrocytes: ICOVIR-5 is unable to express detectable levels of E1A or late proteins (fiber), its replication is inactivated (viral titer 5 logs below wild-type HAd5 levels) and does not display significant cytotoxicity.
- In normal liver (from both human and murine origin) ICOVIR-5 is unable to express detectable levels of E1A. The virus is unable to replicate in human primary liver biopsies.
- In human normal fibroblasts, ICOVIR-5 does not express E1A protein.
- In human tumor cells (with alterations in retinoblastoma pathway) where the functionality of pRb pathway is restored by over-expression of pRb or the negative regulator p21), ICOVIR-5 is unable to express detectable levels of E1A or late proteins (fiber), and its replication is inactivated (viral titer 7 logs below the same cell line before pRb pathway restoration).

3. Any other potentially significant interactions with other organisms in the environment

Host-range of HAd5 is very restricted to human cells. In spite that non-human cells can be efficiently infected by human adenovirus, such infections are abortive. Genetic modifications included in ICOVIR-5 do not modify its human host-specificity with respect to HAd5, and consequently the possibility that ICOVIR-5 expand its host-range is negligible.

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?

Yes ☐	No ☐	Not known ☐

Give details:

ICOVIR-5 is defective in normal human cells, and non-human cells. It can only invade human tumor cells.

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

It's not expected that ICOVIR-5 interacts with organisms different from target human patients, due its narrow host-specificity and for its intended administration system. As previously stated, humans are exclusive hosts for HAd5, and ICOVIR-5 genetic modifications do not alter such host-range. Consequently the probability of productive infection of ICOVIR-5 in animals is very low. Moreover, intrinsic tumor-specificity of ICOVIR5 would also prevent further spreading to non-tumoral cells.

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

i) order and/or higher taxon (for animals):

N.A.

The probability of productive infection of ICOVIR-5 in animals is very low.

ii) Family name (for plants):

N.A.

The probability of productive infection of ICOVIR-5 in plants is very low.

iii) Genus: N.A.
iv) Species: N.A.
v) Subspecies: N.A.
vi) Strain: N.A.
vii) Cultivar/breeding line: N.A.
viii) Pathovar: N.A.
ix) Common name: N.A.

7. Likelihood of genetic exchange in vivo

a) from the GMO to other organisms in the release ecosystem: N.A.
b) from other organisms to the GMO: N.A.
c) likely consequences of gene transfer: N.A.

8. **Give references to relevant results (if available) from studies of the behaviour and characteristics of the GMO and its ecological impact carried out in stimulated natural environments (e.g. microcosms, etc.)**

N.A.

9. **Possible environmentally significant interactions with biogeochemical processes (if different from the recipient or parental organism)**

N.A.

H. INFORMATION RELATING TO MONITORING

1. Methods for monitoring the GMOs

Control of direct and indirect effects of ICOVIR-5 (antitumoral activity + toxicity) in patients will be performed through periodic clinical evaluations that include physical exploration, ECG, vital constants (cardiac frequency and arterial tension), adverse events notification, estimation of putative reactions at injection site, histology and immune populations evaluation. Ideally, patients will be monitored for 2 years after ICOVIR-5 administration.

In parallel, different samples will be collected for viral shedding evaluation. Such study will include blood samples to monitor ICOVIR-5 titers in plasma, viral replication and anti-adenovirus neutralizing antibodies (NAb's) (day 0, 1, 2, 5, 12, 19, 26 and at the ending of treatment). Biological samples from faeces, urine and stool will also be analyzed at day 0,5,12,19,26 and at the ending of treatment.

2. Methods for monitoring ecosystem effects

Based in the low probability of ICOVIR-5 transmission to non-treated individuals, together with the defective replication of the virus in non-tumoral tissues, it is assumed that the probability of ICOVIR-5 spreading by its transmission from third parties is negligible.

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms

N.A.

It is not expected ICOVIR-5 interacting with organisms different from target individuals, based in its narrow host-specificity, proposed diffusion and transitory nature of its genetic expression. If necessary, the technique to be used is Real-time PCR.

4. Size of the monitoring area (m²)

7 m^2 room at Hospital Duran i Reynals.

5. Duration of the monitoring

Monitoring period for ICOVIR-5 presence will last until patient biopsies demonstrate to be negative for viral presence, but in any case it will last at least 1 month post-administration.

6. Frequency of the monitoring

Frequency of monitoring will be variable:

- Samples will be obtained daily during first week.
- Samples will be obtained weekly during first month.

If all samples are negative during the monitoring period, monitoring of shedding will be stopped.

I. INFORMATION ON POST-RELEASE AND WASTE TREATMENT

1. Post-release treatment of the site

The place where ICOVIR-5 solution is prepared for administration will be decontaminated before and after manipulation, using standard disinfectants (1% sodium hypochlorite or 0,25% dodecyl sulfate).

Once treated patients are externalized, rooms and towels will be decontaminated using standard disinfectants for surfaces.

2. Post-release treatment of the GMOs

All wastes generated during direct manipulation of ICOVIR-5 will be disposed in biosecurity sealed containers (residue class 3; biological residues) that are appropriately labeled. Residue management will be subcontracted to a specialized company.
Patient specimens for further analysis must be introduced in a double sealed-bag and carried into tightly closed rigid containers that are appropriately labeled as biological risk residues, and will be manipulated by study personnel, which warrant proper transfer to laboratory.
Samples to be analyzed by external laboratories will also be carried into tightly closed rigid containers. Receptor laboratory will be informed of sample shipment before its execution.

3. a) Type and amount of waste generated

Different type of residues will be generated during the study:

- material coming from the preparation of ICOVIR-5 solution to be injected: residues will be manipulated as class 2 biological residues, and will be disposed in specific containers which management will be subcontracted to a specialized company.
- Clinical material: syringes, needles, vials, disposable bandages, coats, gloves, cleaning material, etc. : solid material will be decontaminated by steam sterilization and further incineration. Liquid residues and surfaces will be treated with a disinfectant (i.e. Virkon™). Remaining waste will be incinerated at Hospital dependences as usual clinical residues.

3. b) Treatment of waste

All wastes generated direct manipulation of ICOVIR-5 will be considered as infectious material. Consequently, all wastes must be maintained in appropriate containers for biodangerous substances and decontaminated before disposal. Decontamination and disposal form such materials (used or not) will be performed at central dependences indicated by promoter.

According to the material nature, protocol for disposal will be different:

- specialized company management
- steam inactivation by autoclaving at 121ºC for 15 minutes. Higher temperatures will be allowed.
- Surface cleaning with soap and disinfectants (i.e. Virkon™).

J. INFORMATION ON EMERGENCY RESPONSE PLANS

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

A detailed description of the preparation procedures will be facilitated to all personnel involved in ICOVIR-5 preparation. A technical data sheet will also be provided with injection procedure, general waste disposal and emergency protocol in case of accidental splashes.

Any preparation transfer will be carried out in sealed containers. Before ICOVIR-5 administration, the virus solution will be prepared according to standard clinical procedures for injecting solutions.

Moreover, clinical personnel will adopt current Hospital policy referring to the manipulation of life vaccines.

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

- Areas where ICOVIR-5 is manipulated, stored or transported will be equipped with disinfectant, such as bleach, that must be available.
- In case of ICOVIR-5 splashes, personnel involved in cleaning must adopt available specific procedures.
- All contaminated areas must be properly cleaned and decontaminated.
- Infected clothes must be discarded into biosecurity containers. If not disposable, they must be cleaned according to available specific procedures for infectious materials.

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread

- Areas where ICOVIR-5 is manipulated, stored or transported will be equipped with disinfectant, such as bleach, that must be available.
- In case of ICOVIR-5 splashes, personnel involved in cleaning must adopt available specific procedures.
- All contaminated areas must be properly cleaned and decontaminated.
- Infected clothes must be discarded into biosecurity containers. If not disposable, they must be cleaned according to available specific procedures for infectious materials.

4. Plans for protecting human health and the environment in the event of an undesirable effect

ICOVIR-5 does not result in productive infections in non-human hosts, is not integrative and its viral cycle is deeply attenuated with respect to HAd5 in non-tumor cells. The probability that ICOVIR-5 disrupt population dynamics in the environment is negligible.

ICOVIR-5 infection in non-intended individuals (non-treated humans) can not be absolutely excluded but is highly improbable. Moreover, tumor-specific DM-K-E2F1 promoter presence in ICOVIR-5 genome provokes that this virus is attenuated in terms of viral cycle, host interaction, pathogenicity, toxical or allergological effects even in immuno-compromised patients. Consequently, viral load to which general population (non-intended population) is exposed is significantly lower with respect to patients systemically administered with ICOVIR-5.