Title: A phase I trial of oncolytic adenovirus ICOVIR-5 administered intravenously to cutaneous and uveal melanoma patients.

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Short title: ICOVIR-5 phase 1 melanoma trial.

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ABSTRACT

Oncolytic viruses represent a unique type of agents that combine self-amplification, lytic and immunostimulatory properties against tumors. A local and locoregional clinical benefit has been demonstrated upon intratumoral injections of an oncolytic herpes virus in melanoma patients, leading to its approval in USA and Europe for patients without visceral disease (up to stage IVM1a). However, in order to debulk and change the local immunosuppressive environment of tumors that cannot be injected directly, oncolyitc viruses need to be administered systemically. Among different viruses, adenovirus has been extensively used in clinical trials but with few evidences of activity upon systemic administration. Preclinical efficacy of a single intravenous administration of our oncolytic adenovirus ICOVIR5, an adenovirus type 5 responsive to the pRB pathway commonly deregulated in tumors, led us to use this virus in a dose-escalation phase I trial in metastatic melanoma patients. The results in 12 patients, treated with a single infusion of a dose up to 1E13 viral particles, show that ICOVIR5 can reach melanoma metastases upon a single intravenous administration but fails to induce tumor regressions. These results support the systemic administration of armed oncolytic viruses to treat disseminated cancer.

INTRODUCTION

Malignant Melanoma represents the ninth most common tumor in Europe with more than 100,000 new cases registered in 2012. [1] In advanced stages, the only approved chemotherapy is Dacarbazine whose response rate is around 10 %. [2] Recently, several treatments have been approved which are specifically targeting melanoma tumors with *BRAF* V600E or V600K mutations or blocking some forms of MEK. [3-5] Immune checkpoint blockers, ipilimumab, pembrolizumab and nivolumab, are also treatments recently approved due to their ability to improve overall survival. [6-8] In addition, Talimogene laherparepvec (T-VEC), a genetically modified oncolytic herpes simplex virus type 1, which secrets granulocyte-macrophage colony stimulating factor, is approved for local intralesional injection based on data that demonstrated shrinkage of lesions, showing the plausibility of a strategy based on oncolysis and immune system stimulation by viruses. [9]

Oncolytic adenovirus-based therapy remains a promising approach to cancer treatment, as virus can infect tumor cells and selectively replicate in them, leading to oncolysis, and release of new viruses, which conduct to local and bloodstream spread and finally, induction of an immune reaction against the tumor. [10, 11] The existing experience with intravenous administration of oncolytic virus shows that high doses of adenovirus ($\geq 10^{13}$ viral particles) in more than 300 cancer patients resulted in limited efficacy with mild toxicity characterized by a transient febrile peak, elevation of transaminases, and occasionally moderate transient nausea and flu-like symptoms.

ICOVIR-5 derives from the oncolytic adenovirus Ad-Δ24RGD [12], which contains two modifications compared to wild type Ad5: first, the E1A region responsible for pRB protein binding is deleted to abrogate the capability of E1A to release E2F from the pRB-E2F complex, characteristic of quiescent cells, contributing to the preferential replication of the virus in tumor and dividing cells. Second, an RGD sequence inserted at the HI loop of the fiber knob to target integrins at the surface of cancer cells as a primary receptor for virus entry. Two additional modifications were present in ICOVIR-5 [13]: replacement of the E1A promoter by the human E2F-1 promoter (insulated

from the E1A enhancer using the myotonic dystrophy insulator (DM-1)) and a consensus Kozak

sequence inserted before the first codon of the *E1A* gene to boost E1A mRNA translation.

The main objective of this first-in-human study was to determine the maximum tolerated dose

(MTD) of the infusion of conditionally replicating oncolytic adenovirus ICOVIR-5 in patients with

advanced malignant melanoma. Secondary objectives were recommended dose, safety, toxicity

profile, and preliminary efficacy data if any.

MATERIALS AND METHODS

This phase I trial of ICOVIR-5 was conducted at the Catalan Institute of Oncology (Barcelona, Spain) in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice, and it was approved by The Clinical Research Ethics Committee of the Bellvitge University Hospital and the Spanish Medicines and Sanitary Products Agency (AEMPS). All participants provided written informed consent. This study was registered at clinicaltrials.gov with the identifier NCT01864759.

Patients

Eligible patients had uveal or cutaneous metastasic malignant melanoma. Patients could have received any prior treatment for metastatic disease. Inclusion criteria were histological proof of cancer, age ≥ 18 years, performance status 0 to 2 (Eastern Cooperative Oncology Group [ECOG] scale), absolute neutrophil count (ANC) greater than or equal to 1,500/μL, platelet count greater than or equal to 100,000/μL, normal function of the liver (bilirubin level < the upper limits of normal [ULN], AST/ALT level ≤ 2.5 times the ULN, alkaline phosphatase level < 2.5 times the ULN) and kidney (creatinine clearance ≥ 50 ml/min), and negative HIV serology. Patients with previous participation in studies with adenovirus, viral syndrome diagnosed two weeks before inclusion, concomitant chronic immunosuppressant medication, concomitant malignant hematology disease, Li Fraumeni Syndrome or germ known deficit pRb pathway were excluded.

Study Design

The study was conducted using an accelerated escalating phase I design with cohorts of 1 patient if no grade >2 toxicity was found, and then with 3-6 patient cohorts after the first grade 3 toxicity finding. A Dose-Limiting Toxicity (DLT) observation period of 4 weeks was established before the entry of the first patient at the next dose level. If one out of three patients experienced DLT during the treatment cycle, at least one more additional patient was to be treated at this dose

level and up to 6 patients could be sequentially treated at this dose level. The Maximum Tolerated Dose (MTD) was defined as the dose level at which two of the three to six treated patients experienced DLT and the recommended dose (RD) was the highest dose level at which less than 2 of 6 patients experienced DLT. Toxicity was classified according to the Common Toxicity Criteria of the National Cancer Institute (NCI CTC, version 3.0). Dose Limiting Toxicity was defined as: ANC <0.5 x 10^9 / L for more than 5 days, febrile neutropenia as defined by the NCI CTC 3.0, platelet count <25 x 10^9 / L, any other non-hematologic grade 3-4 toxicity except for alopecia, nausea/vomiting unresponsive to specific treatment, or any toxicity grade 2 unrecovered at 6 weeks of treatment.

Treatment

ICOVIR-5 was administered as a single infusion in an inpatient regimen. Before administration, ICOVIR-5 was thawed and diluted in 50ml 0.9% NaCl for intravenous administration. Patients were monitored in a specific isolation room at the hospital until acute events were solved.

Dose levels: 1a, $1x10^{11}$ viral particles (vp); 2a, of $3.3x10^{11}$vp; 3a, of $1x10^{12}$vp; 4a, of $3.3x10^{12}$vp and 5a, of $1x10^{13}$vp. A dose level of $1x10^{10}$ (-1a) was planned if dose 1a became limiting.

Study assessments

All patients were evaluated by physical examination, ECG and chest X-ray. Complete blood cell (CBC) count, liver, and kidney function tests and urine analysis were obtained before treatment, on days 1, 2, 3, 5, 12, 19, and at the end of the study (day 26). Study of extent of disease was performed by computed tomography (CT) or magnetic resonance imaging (MRI) of the chest, liver and abdomen, and bone scan with X-ray if hot spots were observed. Assessments of tumor lesions were made at 4 weeks of treatment applying the criteria recommended for evaluation of response in solid tumors (RECIST 1.1). Tumor biopsies were obtained if feasible at the beginning of the study and at day 5 post-treatment.

ICOVIR-5

ICOVIR-5 has been previously described. [13] It derives from the oncolytic adenovirus Ad-Δ24RGD [12], currently in clinical trials against glioblastoma under the name DNX-2401 (by DNAtrix Therapeutics, Houston Tx). ICOVIR-5 was constructed by homologous recombination in bacteria and transfection in HEK293 cells. Then, it was plaque-purified and amplified in A549 cells, and purified using a CsCl gradient. The viral genomic structure was verified by restriction analysis. The DM-1 insulator, E2F-1 promoter, Kozak sequence, E1A-Δ24 deletion, and RGD fiber were sequenced. The clinical batch of ICOVIR-5 was manufactured at the Center for Cell & Gene Therapy at Baylor College of Medicine (Houston, TX, USA) under GMP conditions using A549 cells. Virus titer was adjusted to 1×10^{12} vp/ml in 20 mM Tris pH 8.0, 25 mM NaCl, 2,5% glycerol, aliquoted in sterile vials, and stored at -80 ^{o}C with continuous monitoring of temperature. Functional virus titer was 5.33×10^{10} plaque-forming units (PFU) / ml, which corresponds to 18.7 virus particle / functional particle bioactivity ratio. No wild type Ad5 or other adventitious viruses were detected by PCR.

Virus pharmacokinetics

Immediately after the end of virus administration (time 0), and at 30min, 1h, 2h, 4h, 6h, and 24h after virus administration, blood samples were taken using 4ml Lithium Heparin Vacuette tubes (Greiner Bio-One, Monroe, NC, USA) and total DNA was isolated by the QIAamp DNA Mini and QIAamp DNA Blood Mini Kits (QIAGEN, Valencia, CA) in triplicate according to the manufacturer protocol. Four microliters of DNA were used to quantify genome copy number by Quantitative Real Time-PCR using ICOVIR-5 specific primers (ICO5F2-5'-GAT TTG GCG CGT AAA AGT G -3' which overlaps E2F1 promoter and the reverse ICO5R2-5'-CGG CCA TTT CTT CGG TAA TA -3' which overlaps with the E1A gene, generating a 126 bp amplicon). Real Time PCR consisting of 10 min at 95 ^{o}C and 40 cycles (95 ^{o}C, 15 s; 60 ^{o}C s; 72 ^{o}C, 10 s) was performed on the LightCycler® 480 II

(Roche, Basel, Switzerland), using LightCycler 480 SYBR Green I Master (Roche) and analyzed with LightCycler® 480 Software release 1.5.0 SP4 (Roche). A control PCR without DNA was included to monitor reagent contamination.

For genome copy quantification, blood from a donor was spiked with a 10-fold serial dilution of ICOVIR-5 to generate a standard curve from $1x10^{10}$ vp/ml to $1x10^2$ vp/ml. This curve and negative controls of blood without virus were processed with the same protocol as patient samples and analyzed in the same Real Time PCR to quantify genome copies. Precision, accuracy, and limits of detection (LOD) and quantification (LOQ) were established using the standard curve in a previous experiment. LOD, the lowest concentration of genomes in the standard curve that can be detected, was $1x10^3$ genomes/ml. LOQ, the lowest concentration of genomes in the standard curve which maintains linearity with the standard curve (slope 0.8-1.2), was $1x10^4$ genomes/ml.

Analysis of virus shedding

Blood, urine, sputum, and stool samples were collected before administration and on days 1, 2, 5, 12, 19, and 26 post-treatment. Samples were analyzed when available due to compliance of protocols. Among them, sputum and stool presented a higher frequency of lack of compliance. DNA was extracted with the following kits: QIAamp DNA Mini and QIAamp DNA Blood Mini Kits for blood; QIAamp Viral RNA Mini kit for urine after concentration with Millipore Amicon Ultra Centrifugal Filters Ultracel 50k (Millipore; Darmstadt, Germany); QIAampMinElute Virus Spin for sputum; and QIAamp DNA Stool for stool (all kits from QIAGEN). All samples were processed in triplicate.

A 10-fold serial dilution standard curve for each type of sample was generated diluting ICOVIR-5 in the corresponding sample obtained from non-treated donors. Standard curves and negative controls (samples without virus) were processed as patient samples.

Isolated DNA (4 microliters) were used to quantify the genome copy number using the same Quantitative Real Time-PCR program described above for pharmacokinetics.

The LOQ and LOD were previously determined for the different samples. For blood, LOQ and LOD were the same described above for pharmacokinetics. For urine, LOQ and LOD were established as 1.25×10^4 genomes/ml. For sputum, LOD 1×10^3 genomes/ml and LOQ was 1×10^4 genomes/ml. For stool LOD was 1×10^3 genomes/g and LOQ was 1×10^4 genomes/g.

ICOVIR-5 genomes in tumor biopsies

Patient tumor biopsies were obtained when possible by core needle biopsy (CNB) from accessible skin lesions at day 5 post-treatment to detect ICOVIR-5. Samples were digested with proteinase K and DNA extracted using QIAamp DNA Mini and QIAamp DNA Blood Mini Kits according to the manufacturer instructions. A 10-fold serial dilution standard curve was generated diluting ICOVIR-5 with sterile phosphate buffered saline (PBS, Life Technologies, Carlsbad, CA, USA) and processed as tumor samples.

Isolated DNA (4 microliters) were used to quantify the genome copy number using the same Quantitative Real Time-PCR program as above for pharmacokinetics. The LOD was 10^3 genomes/sample and the LOQ 10^4 genomes/sample.

Analysis of cytokines in blood.

Cytokines IL-6 and IL-10 were chosen for the analysis as possible predictors of immunologic response and toxicity. Basal and post-treatment levels (6h, 24h, 48h, day 5, day 12, day 19, and day 26) of cytokines IL-6 and IL-10 in patient serum were quantified using ELISA assays (R&D; Minneapolis, MN, USA). Patient peripheral blood was collected in Vacuette Z Serum Sep Clot Activator tubes (Greiner Bio-One, Monroe, NC, USA), allowed to coagulate for 30 minutes and centrifuged at 1500 g for 10 minutes. Serum samples were harvested and stored at $-80\,^{\circ}C$ until analysis. ELISA assays were carried out according to manufacturer's protocols. Each sample was analyzed in triplicate.

Anti-ICOVIR-5 neutralizing antibodies

Neutralizing antibodies in serum against adenovirus type 5 were analyzed before treatment and at day 19 after ICOVIR-5 administration. Samples of serum were heated at 56 °C for 30 minutes in order to inactivate complement. A 2-fold serial dilution of inactivated samples from 1/10 to 1/5120 was performed in Dulbecco's modified Eagle's medium in 96-well plates (final volume 50 µl) in quadruplicates. Serum dilutions were incubated for 1h at room temperature with 2.5×10^4 transducing units (TU) of AdTL, an adenovirus type 5 E1-deleted vector expressing luciferase and enhanced green fluorescent protein (EGFP). [14] Afterwards, 1×10^5 HEK-293 cells per well were added (to obtain a proportion or multiplicity of infection of 0.25 TU/cell) and cultured at 37 °C and 5% CO2. After 24h of infection, media was removed and cells washed once with PBS. Finally, the level of luciferase in each sample was quantified using a Victor X5 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). The percentage of neutralization was calculated with the formula (% neutralization = [1-((LUsample-LUnegative control)/(LUpositive control-LUnegative control))] x 100.

As a negative control we used the luciferase units (LU) of a non-infected well and as a positive control we used the LU of an infected well without serum. The neutralizing antibody titers were expressed as the reciprocal of the serum dilution closest to 50% neutralization of the infection. Every serum sample was quantified in quadruplicate.

Statistical analysis

Descriptive statistics were used for safety analyses for all patients who received one dose of ICOVIR-5. Categorical and continuous data were summarized with frequencies and percentages. The efficacy population included all patients with a baseline assessment and a post-baseline tumor assessment. Fisher´s exact test was used to correlate neutralizing antibodies with clinical responses and linear regression analysis to correlate viral doses with cytokine levels.

RESULTS

Patient characteristics, tolerability, and efficacy

Thirteen patients were enrolled in the study and twelve were treated at five dose levels (Table 1). Patient #05 was screened but not treated due to early progression. All 12 treated patients were evaluable for toxicity. Patients' characteristics were as follows: 7 males and 5 females; the median age was 51 (range 40-80) and the ECOG performance status was 0 (5 patients) or 1 (7 patients). All patients had evaluable or measurable disease. Six patients (50%) had received at least one prior chemotherapy regimen for metastatic melanoma, and 4 (33%) patients had received previous immunotherapy (supplementary table 1). Six patients had uveal melanoma and six cutaneous melanoma. All patients received one treatment injection and were observed for 4 weeks, except for one (#02) who did not complete one cycle of observation due to early progression. This patient #02, following the protocol, was replaced, although he was considered evaluable for toxicity. The patient, in the context of a rapid progression, presented a disseminated intravascular coagulation (DIC) not related to the treatment but to the progression of the underlying disease.

Table 2 lists the most significant all-cycle non-hematological toxicities observed in the study. At dose levels 1a to 3a no relevant toxicity was observed. The grade 2 toxicity observed at level 4a allowed to proceed to dose escalation. Acute toxicity was mainly a flu-like syndrome with fever, chills, arthromyalgia, headache, nausea and vomiting, and diarrhea, which began in the first 4-6 hours after infusion and during 2-4 days. The first patient at the level 5a (patient #08) experienced toxicity as transaminitis grade 3 at day +1. In addition, this patient presented grade 3 thrombocytopenia at day +4. On day +2, transaminitis was recovered to grade 1 and thrombocytopenia lasted to day +12. This dose level was then expanded to 6 patients. Patient #13 presented the second DLT as hepatic grade 3 toxicity (grade 3 AST elevation which lasted two days and recovered to grade 0 at day +12). Both patients had normal transaminases level before treatment, and only patient #08 had metastatic liver involvement at the beginning of the study.

According to protocol, this level (5a: 1×10^{13}) was declared MTD, and the recommended phase 2 dose was defined at the inferior dose level 4a (3.3×10^{12}). Other significant toxicities at this dose level included: grade 2 neutropenia in patient #08 and grade 2 AST elevation in both patients.

With regard to efficacy, the eleven patients who had received treatment and had undergone at least one disease evaluation (performed day 26) were evaluated. No objective responses, partial or complete, were observed. At the lower dose levels two patients had stable disease, patient #03 until day 100 post-treatment and patient #06 until day 433 after treatment. At the highest dose level (1×10^{13} vp) stable disease was observed in 5 out of 6 patients, patient #09 until day 56, patient #010 until day 94, patient # 11 until day 131, patient # 12 until day 84, and patient #013 until day 112 post-treatment (Table 3). Comparing the survival of cutaneous melanoma patients that never received targeted therapy or anti-PD1 antibodies after participating in the trial versus uveal melanoma patients, the survival probability was 3.7 times longer for the uveal melanoma patients (Supplementary figure 1. Median 271 days for uveal vs 73 days for cutaneous, HR 0.15; 95% CI 0.026-0.85).

Neutralizing antibodies

The levels of Anti-Ad5 neutralizing antibodies were analyzed before treatment and day 19 post-treatment in sera (Table 3). All patients developed an antiviral immune response as shown by the increase of neutralizing antibodies after virus administration regardless of the basal level and the dose of ICOVIR-5 administered. The increase of titer did not correlate with the dose of ICOVIR-5 administered. Only the three patients with lower basal levels of NAbs did not reach the upper limit of quantification (>5120). Finally, for the highest dose cohort, where stable diseases were seen, no correlation was observed between the pre-existence of basal neutralizing antibodies and the stabilization of the disease using Fisher's exact test (p=1).

Virus pharmacokinetics

Pharmacokinetics of ICOVIR-5 in patients' blood was monitored for the first 24h post-administration by Quantitative Real Time-PCR using the specific set of primers for the ICOVIR-5 genome. The level of ICOVIR-5 in blood correlated with the dose administered (Fig 1). Patient #01 treated with $1x10^{11}$vp and patient #03 treated with $3.3x10^{11}$vp had circulating viral genomes up to 1h ($1.69x10^4$genomes/ml) and 6h ($2.85x10^4$genomes/ml) post-administration, respectively (patient #01 had detectable but not quantifiable at 6h), with no evidence of viral genomes in blood after 24h post-administration. Patient #06 treated with $1x10^{12}$vp had quantifiable viral genomes at 6h post-administration ($1.56x10^5$genomes/ml) and detectable levels at 24h post-administration. Patient #07 administered with $3.3x10^{12}$vp showed quantifiable viral genomes at 24h post-treatment ($3.61x10^4$genomes/ml). Finally, 5 out of the 6 patients of the last cohort (treated at $1x10^{13}$vp) had quantifiable virus genomes at 24h post-treatment ($9.18x10^5$genomes/ml $\pm$ $8.67x10^5$ genomes/ml), with only one patient (patient #08) with detectable but not quantifiable levels at this time point.

Virus shedding

In all patients from the lower dose levels (one patient per each dose of $1x10^{11}$, $3.3x10^{11}$, and $1x10^{12}$vp) ICOVIR-5 genomes could not be detected in any of the samples analyzed (blood, urine, sputum, and stool).

In patient #07 treated with $3.3x10^{12}$vp virus DNA was detected in blood (although not quantifiable) and in sputum ($3.96x10^3$ genomes/ml) at 48h post-treatment. Both types of samples became negative for the presence of virus in the next time point analyzed, at 5 days post-treatment, and remained negative.

In patients treated at the highest dose level ($1x10^{13}$vp) an increase in the virus shedding was detected (Fig 2). In blood, 24h post-treatment 5 out of the 6 patients presented quantifiable viral genomes ($9.18x10^5$genomes/ml $\pm$ $8.67x10^5$genomes/ml) and one patient (patient #08) presented detectable but not quantifiable levels. At 48h post-treatment, 4 out of 5 patients had quantifiable

virus genomes (2.90×10^5genomes/ml ± 3.70×10^5genomes/ml) and one patient (patient #08) had detectable but not quantifiable levels. At 5 days post-treatment, 4 out of 6 patients had quantifiable viral genomes (8.64×10^5genomes/ml ± 9.94×10^5genomes/ml) and the remaining 2 patients (patients #08 and #10) had detectable but not quantifiable levels. Virus genomes could be detected up to day 6 (patients #08 and #11) or day 8 (patients #09 and #10) after virus administration. Surprisingly, in the remaining 2 patients (patients #012 and #013), virus genomes were detected until the end of the treatment (day 26 after virus administration). In urine, virus DNA was detected only in patient #09. This patient had 1.16×10^6genomes/ml at 24h, 1.51×10^5genomes/ml at 48h, and 2.16×10^5genomes/ml at day 5 after treatment. In stool, 24h post-administration virus genomes were detected in 4 out of 4 patients analyzed (patients #09, #010, #011, and #012, 5.89×10^4genomes/g ± 4.35×10^4genomes/g); 48h post-administration virus was detected in 2 (#09 and #012) out of 3 (patients #09, #010 and #012) analyzed (6.98×10^4genomes/g ± 3.90×10^4genomes/g); and at day 5 post-administration virus was detected in 1 (patient #013) out of 3 (patients #09, #012 and #013), but did not reach a quantifiable level. Finally, in sputum at 24h post-administration viral genomes were detected in 2 (patients #09 and #010) out of 3 (patients #08, #09 and #010) samples analyzed (6.09×10^4genomes/ml ± 5.87×10^4genomes/ml); at 48h post-administration in 2 (patients #010 and #012) out of 4 (patients #08, #09, #010 and #012) samples analyzed (3.43×10^4genomes/ml and one not quantifiable); and at day 5 post-administration in 1 (patient #012) out of 5 (patients #08, #09, #010, #012, and #013) samples analyzed (3.91×10^4genomes/ml). All samples from days 12, 19, and 26 were negative.

Cytokine analysis

Analysis of serum IL6 and IL10 levels was performed before and after treatment (6h, 24h, 48h, day 5, day 12, day 19, day 26 post-ICOVIR-5 administration) (Fig 3). An increase of IL6 levels was detected for all patients in the first 24h post-administration, likely reflecting an early innate immune response against the virus. At 6h post-administration, there was a correlation between

the viral dose used and the IL6 level detected (22.6 pg/ml for $1x10^{11}$vp, 28.8 pg/ml for $3.3x10^{11}$vp, 31.5 pg/ml for $1x10^{12}$vp, 49.9 pg/ml for $3.3x10^{12}$vp, and a mean of 303 pg/ml for $1x10^{13}$vp; lineal regression IL6 concentration versus amount of ICOVIR-5 administered, r=0.97 p<0.005). The peak of IL6 level observed for most patients was lower than 100 pg/ml. Only 3 patients showed a peak higher than 200 pg/ml (patients #01, #08, #010). Among them, only in patient #08 ($1x10^{13}$vp) this higher IL6 elevation was accompanied with a dose-limiting toxicity, as a transaminitis grade 3 at 24h post-treatment. Serum IL10 was also measured as it is another biomarker of inflammation commonly detected upon systemic adenovirus administrations, although with anti-inflammatory properties contrary to the pro-inflammatory IL6. A peak of IL10 was detected in all patients, reaching higher levels with higher doses. As previously reported, the peak of IL10 (24h) was delayed compared to IL6 (6h). [15] At 24h post-administration, there was a correlation between the viral dose and the IL-10 level detected (lineal regression IL10 concentration versus amount of ICOVIR-5 administered, r=0.81; p<0.05). Patient #013 ($1x10^{13}$vp) had the highest IL10 peak (530 pg/ml) and showed a dose-limiting toxicity as a transaminitis grade 3 at 24h post-treatment. There was a correlation of IL6 levels at 6h and IL10 at 24h (r=0.93; p<0.05).

Detection of ICOVIR-5 in tumor biopsies

One core needle biopsy (CNB) was obtained from skin lesions of cutaneous melanoma patients and liver lesions of uveal melanoma patients. Biopsies were obtained at day 5 post-treatment in patients treated with $1x10^{11}$vp, $3.3x10^{11}$vp, $3.3x10^{12}$vp, and 3 (patients #09, #011 and #013) out of the 6 patients from the highest dose of $1x10^{13}$vp. DNA was extracted from biopsies and the presence of viral genomes analyzed by qPCR using specific primers for ICOVIR-5. Virus DNA in metastasis was not detected in the patient treated with the lower $1x10^{11}$vp dose. In contrast, virus DNA was detected in patients treated with $3.3x10^{11}$vp and $3.3x10^{12}$vp, and in 2 (patients #09 and #011) out of 3 patients from the highest $1x10^{13}$vp dose (Table 3). In these 2 patients the presence of virus DNA in blood at the time of biopsy (day 5 post-administration) precludes a clear

conclusion on tumor transduction. However, in the 2 patients with lower virus doses (3.3×10^{11}vp and 3.3×10^{12}vp) no viral DNA was detected in peripheral blood at day 5 post-administration suggesting that the detected virus derives from tumor cells and not from blood.

DISCUSSION

The use of oncolytic viruses to treat cancer, known as virotherapy, is an old idea born after the observation of cancer remissions upon viral infections. [16] After up and downs, the concept has been revisited in the last 20 years using viruses, such as Vaccinia Virus, Herpes Simplex Virus (HSV), Measles, and Adenoviruses (Ad), designed to target and replicate more selectively in cancer cells, and wild type viruses with a natural selectivity for tumor cells such as Reovirus, Parvovirus, or Coxsackievirus A21, among others. Intratumoral injection of these viruses in patients has proven the concept that when a virus reaches a tumor mass it can cause tumor lysis. [17] However, tumor targeting upon systemic administration is still a challenge.

This first-in-humans phase 1 study shows that the intravenous administration of the conditionally replicative adenovirus ICOVIR-5 is well tolerated. A dose of 1×10^{13} vp of ICOVIR-5 induced transaminitis in 2 out of 6 patients, and thrombocytopenia in one of them, while a dose of 3.3×10^{12} vp can be safely administered without DLT. The primary objective of the trial was to recommend a phase II dose. According to the protocol, the dose level in which no DLT was observed would be taken as a safe dose level and therefore the recommended phase II dose. Our results indicate that for ICOVIR5 this dose is 3.3×10^{12} vp. Mild liver toxicity has been previously described with similar intravenous administered viruses. [15, 18, 19] ICOVIR-7, an oncolytic adenovirus similar to ICOVIR-5, produced up to grade 2 liver transaminases elevation in 13 of 21 patients, although most of the patients received treatment by intratumoral injection and only 3 intravenously. [20] For ICOVIR-5 transaminitis was the main toxicity limiting the dose escalation beyond the 1×10^{13} vp dose. This transient transaminitis observed in 2 patients, started at days 1 and 5 post-treatment, and persisted for 1 and 7 days, respectively.

Systemic administration has been attempted in clinical trials using Newcastle Disease Virus HSV, reovirus, picornavirus, measles, Vaccinia Virus [21] and adenovirus. [19] Out of eight evaluated

patients in a recent trial of an oncolytic Vaccinia Virus administered systemically, two proved positive for virus in biopsy. [21] Still, the inadequate viral delivery was identified as a main factor for insufficient antitumor efficacy. Adenovirus has been extensively used in gene therapy and virotherapy clinical trials. Among several clinical trials with systemically administered Onyx015 only occasional and transient responses were observed in some colorectal carcinoma patients. [22, 23] With CG7870, an oncolytic adenovirus designed for prostate carcinoma with E1A under the probasin promoter and E1B under the PSA promoter, the serum PSA level dropped 25% to 49% transiently in 5 out of the 23 intravenously treated patients, 4 of them at the highest doses ($6x10^{11}$ to $6x10^{12}$ vp). [19] Focusing on tumor targeting, occasional evidence for systemic tumor targeting has been reported. Reid et al. injected ONYX-015 intra-arterially in 11 patients with liver metastases of colorectal cancer and biopsies at day 4 post-infusion resulted negative for virus. [22] Similar results were obtained in the phase II trial after infusing $2x10^{12}$ vp as a maximum feasible dose on multiple days (1, 8, 22, 50, and 78). [23] It was concluded that improving delivery and replication efficiency would be needed to obtain evidences of intratumoral replication. Hamid et al. infused intravenously ONYX-015 to 18 patients with liver metastases of colorectal carcinoma. [18] The autopsy of one patient that died 56 h after virus administration showed very few cells positive for virus in the tumors. Nemunaitis et al. infused ONYX-015 intravenously in 10 patients with lung metastases reaching much higher doses (up to $2x10^{13}$ vp). One analyzed biopsy in a patient treated with $2x10^{12}$ vp resulted positive for virus presence 5 days post-infusion. [15] A more potent virus, CG7870, where E1A and E1b were regulated by the probasin and the PSA promoters respectively, was infused intravenously in prostate carcinoma patients up to a dose of $6x10^{12}$ vp, identified as the MTD, but no biopsies were analyzed for tumor targeting. [19] Lack of antitumor efficacy in these clinical trials led to abandon the systemic approach with oncolytic adenoviruses based on adenovirus serotype 5 with an unmodified capsid. After an interval of several years, a survey of clinical trials ongoing in 2014 identified ICOVIR-5, ColoAd1, and VCN01 (all of them with capsids different from Ad5-wild type) as oncolytic Ads used intravenously, and no additional

adenoviruses were added to this list in 2015. [24, 25] However, the Ad-D24RGD oncolytic adenovirus, with the same RGD-modified capsid as ICOVIR-5, has been used intratumorally [26], intraperitoneally [27], and in a combination of intratumoral and intravenous routes [20, 28]. Evidence of systemic tumor targeting in non-injected tumors distant from injected tumors was observed in an autopsy of one patient (C200) that died 19 days after injection in multiple tumor sites. [28] With regard to oncolytic adenoviruses with other capsid modifications, metastatic tumor targeting has been demonstrated in a patient treated intravenously with a serotype 5/3 chimeric fiber. [28] The biodistribution analysis in patient autopsy samples indicated a fair capability of tumor targeting of oncolytic adenoviruses even when injected intratumorally. [28] This consistent detection of virus in occasional patient autopsies suggests that the small samples that can be analyzed in a biopsy may underestimate the tumor targeting potential of the virus. In this context it is noteworthy our detection of viral genomes in four out of seven analyzed biopsies, in two cases with a negative viremia. The same can be concluded from the more frequently reported observation of secondary peaks of viremia compared to positive biopsies, which suggest virus replication in tumors. We did not observe secondary peaks of viremia even in the four patients where the virus had been detected in the tumor at day 5. In two patients (#012 and #013) viremia levels were positive until the end of the study (day 26). Among them, one (patient #013) had a tumor biopsy analyzed at day 5 and it resulted negative. Although preliminary, our results indicate that the presence of virus in the tumor does not necessarily leads to secondary peaks of viremia.

In our study, pharmacokinetics analysis indicates that there is a correlation between exposure and the administered dose, since larger doses lead to longer time to virus elimination. In addition, the study shows that the virus shedding is also dose-dependent: the presence of the virus is higher in the last dose level, but at the recommended dose level it is found in blood and sputum at 48 hours, indicating effective spread of the virus. The analysis shows that neutralizing antibody titers were not dose-dependent and pretreatment levels did not correlate with tumor response.

All patients had an early onset of innate immune response, with a correlation between dose and levels of IL6. As reported, the peak of IL10 was delayed compared to IL6. IL6 and IL10 levels correlated with the dose and between them. Patients with grade 3 transaminitis (patients #08 and #013) showed highest peaks of IL6 and IL10. This toxicity was very short in time and no systemic inflammatory response syndrome was observed.

As a most relevant finding, viral genomes were detected in the biopsy of metastatic lesions of four patients, indicating that oncolytic adenovirus can achieve tumor targeting of metastases upon intravenous administration. With regards to clinical activity, no tumor responses were observed, but 7 stabilizations of disease were seen out of 11 patients, 5 of them among the 6 patients treated at the highest dose. Despite this low antitumor activity, the longer survival of uveal melanoma patients compared to cutaneous melanoma patients was intriguing as the expected survival before the emergence of targeted therapy (BRAF/MEK inhibitors) and anti-PD1 antibodies for those patients should be the same. [29] Although the number of patients is very small and definitive conclusions cannot be taken, this trend suggests a superior activity of systemic ICOVIR5 against metastatic uveal melanoma compared to metastatic cutaneous melanoma. It could be speculated that this could be related to the shared hepatotropism of adenovirus and uveal melanoma metastases or/and that uveal melanoma liver metastases are more prone to be controlled by the immune system once infected by the virus. Low antitumor efficacy has been previously reported with oncolytic adenoviruses. By intratumoral administration head and neck patients ONYX-015 produced 3 partial and 2 minor responses and 8 disease stabilizations. [30] In 23 pancreatic cancer patients produced six minor responses and 10 stabilizations. [31] In the phase 1 study of intratumoral Telomelysin in various solid tumors, one partial and seven stable disease responses were reported. [32] By intraperitoneal injection of Ad5-Δ24-RGD, 15 disease stabilizations were observed among 21 malignant gynecologic patients. [27] Finally, among 35 non-invasive bladder cancer patients receiving CG0070 intravesically, 17 showed complete responses. [33] By

intravenous administration, ONYX-015 induced one mixed response and 9 disease stabilizations in 10 patients. [15] In a subsequent phase II trial, seven out to 18 patients experienced stable disease. [18] Systemic administration of CG7870 in 23 prostate cancer patients induced some decrease in serum PSA in 5 patients, with no complete or partial PSA responses. [19] With ICOVIR7 [20], out of two evaluated patients that were treated only by the intravenous route, one head and neck tumor patient had a 10% decrease of tumor size.

There is little experience with oncolytic adenoviruses applied to melanoma, and none after systemic administration alone. When oncolytic adenovirus Ad5/3-D24-GMCSF was administered to 9 patients with melanoma (50% of dose intratumoral and 50% intravenous), one out of four evaluable patients showed a minor response (15% size decrease) in an injected lesion. [34]

Our study adds interesting information to the development of virotherapy with oncolytic adenoviruses. One point of growing interest is the potential combination of oncolytic viruses with immunotherapy, as the virus-induced immune response may favor the infiltration of lymphocytes in tumors. In this setting, it is imperative for the virus to reach all tumor sites systemically to promote the infiltration on "cold" tumors characterized by the lack of tumor-infiltrating lymphocytes. Our results indicate that systemic tumor targeting of metastases with oncolytic adenoviruses is feasible using the intravenous administration route. Although armed oncolytic adenoviruses may be needed to obtain sufficient tumor debulking by oncolysis before antiviral immunity neutralizes the virus, our results encourage further studies by systemic administration. We are currently exploring the use of a hyaluronidase-armed oncolytic adenovirus (VCN-01) with a slightly different capsid than ICOVIR-5 (RGD in the fiber shaft instead of the HI-loop) by systemic administration in cancer patients.

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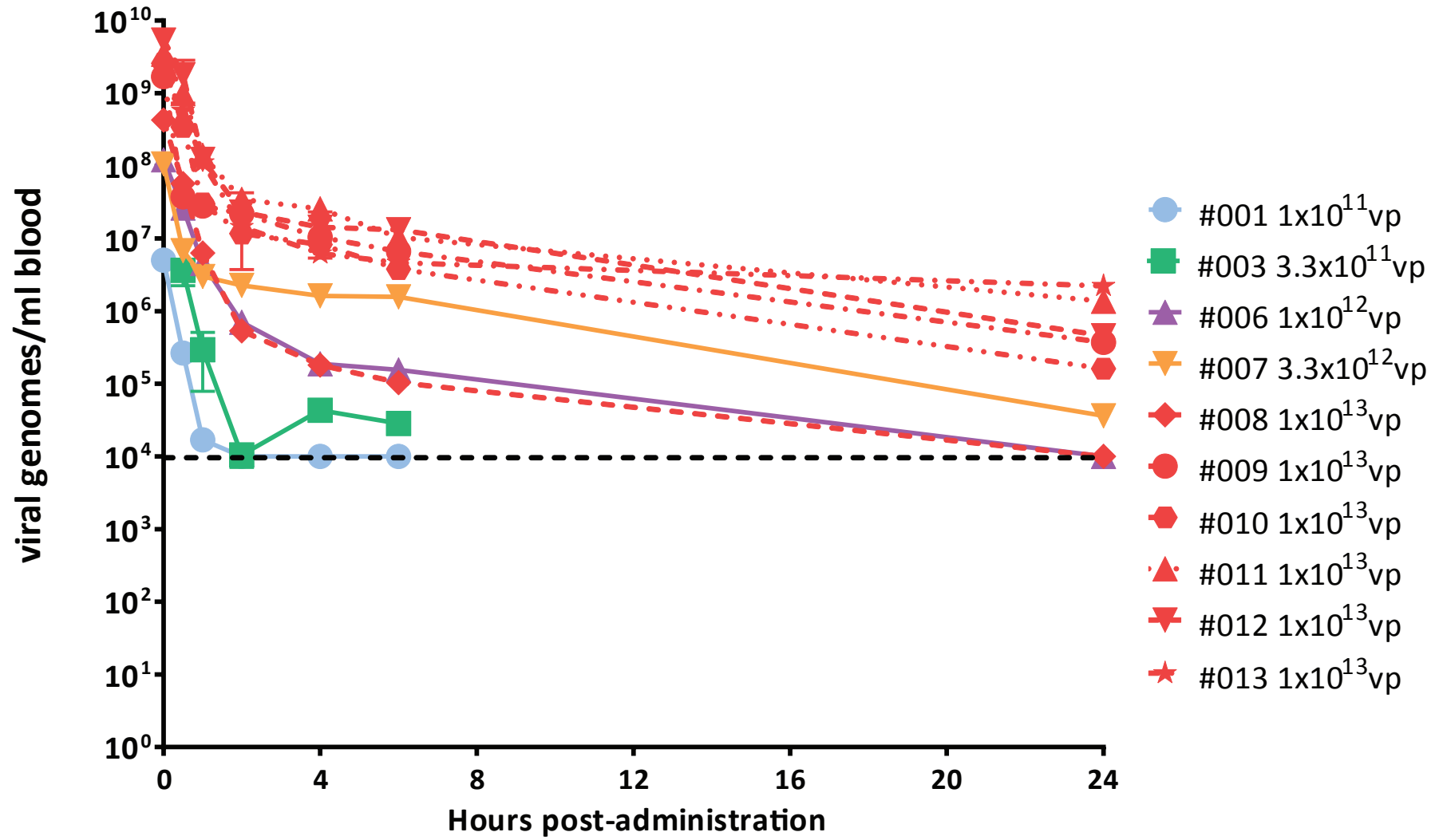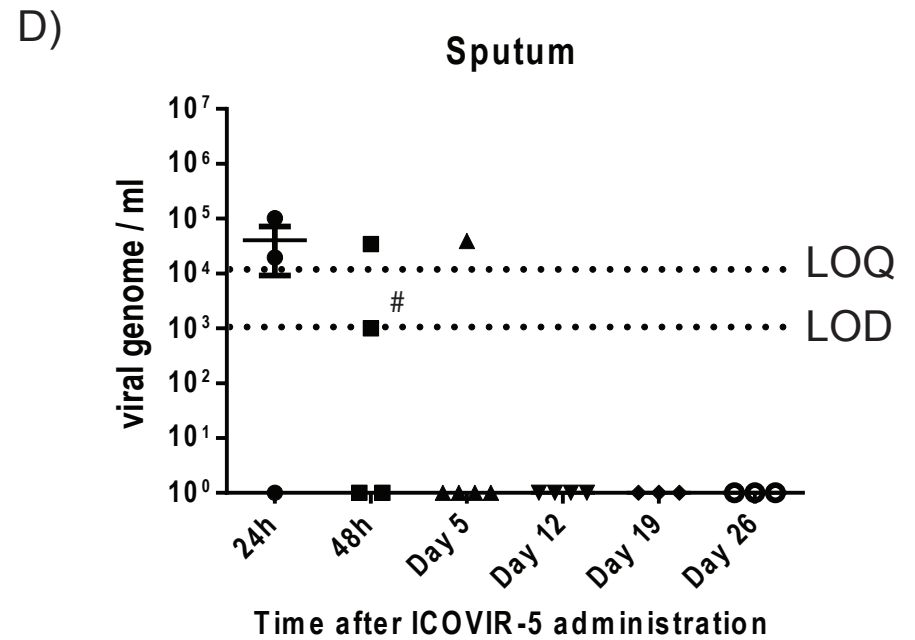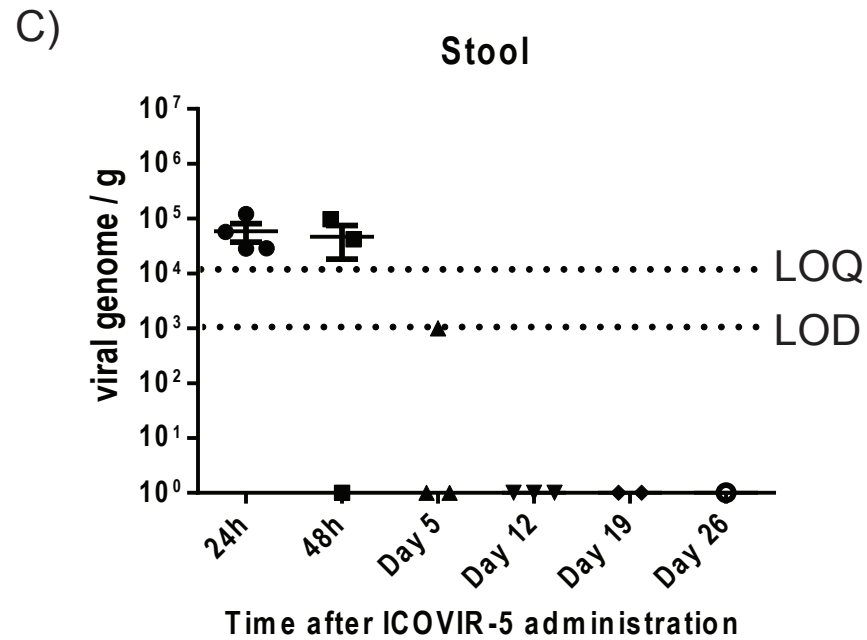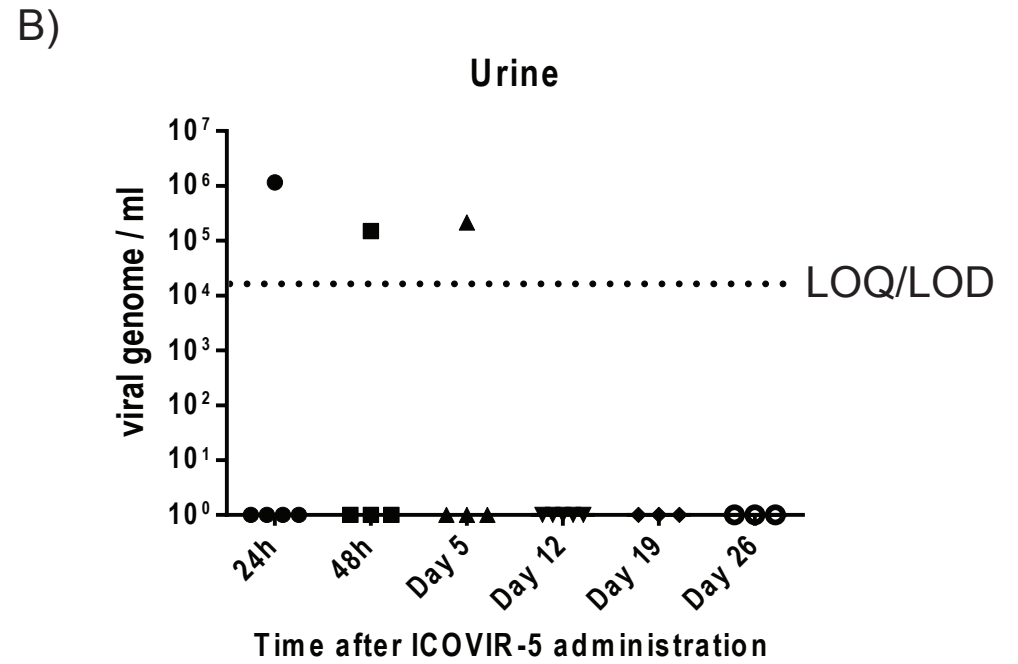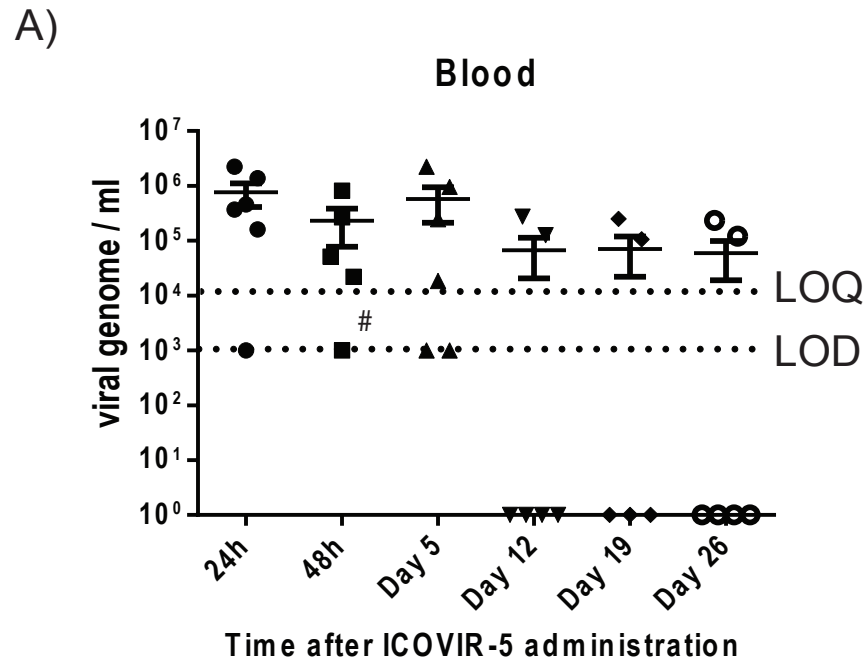
Figure legends

Figure 1. Quantification of ICOVIR-5 pharmacokinetics in blood by Real-Time PCR. In dose levels 1a, 2a, 3a and 4a only viral dose are indicated. For dose level 5a, patient number identification is also indicated. The dashed lines represent the lower limit of quantification of ICOVIR-5 genomes in blood. Samples were analyzed in triplicates.

Figure 2. Quantification of ICOVIR-5 genomes in high-dose patients (1x10^{13}vp) by Real-Time PCR in: A) Blood, B) Urine, C) Stool and D) Sputum. The dashed lines represent the lower limit of quantification (LOQ) and detection (LOD) of ICOVIR-5 genomes. Samples were analyzed in triplicates. # : Sample detectable but not quantifiable.

Figure 3. Analysis of IL6 and IL10 in serum samples by ELISA. Analysis of IL6 (A) and IL10 (B) in serum samples on days 0, 1, 2, 5, 12, 19 and 26 post-ICOVIR-5 administration. Samples were analyzed in triplicates.

Figure 1. García et al

Legend:
- #001 1x10^{11}vp
- #003 3.3x10^{11}vp
- #006 1x10^{12}vp
- #007 3.3x10^{12}vp
- #008 1x10^{13}vp
- #009 1x10^{13}vp
- #010 1x10^{13}vp
- #011 1x10^{13}vp
- #012 1x10^{13}vp
- #013 1x10^{13}vp

X-axis: Hours post-administration

Y-axis: viral genomes/ml blood

Figure 2. García et al

A) Blood

B) Urine

C) Stool

D) Sputum

Figure 3 García et al
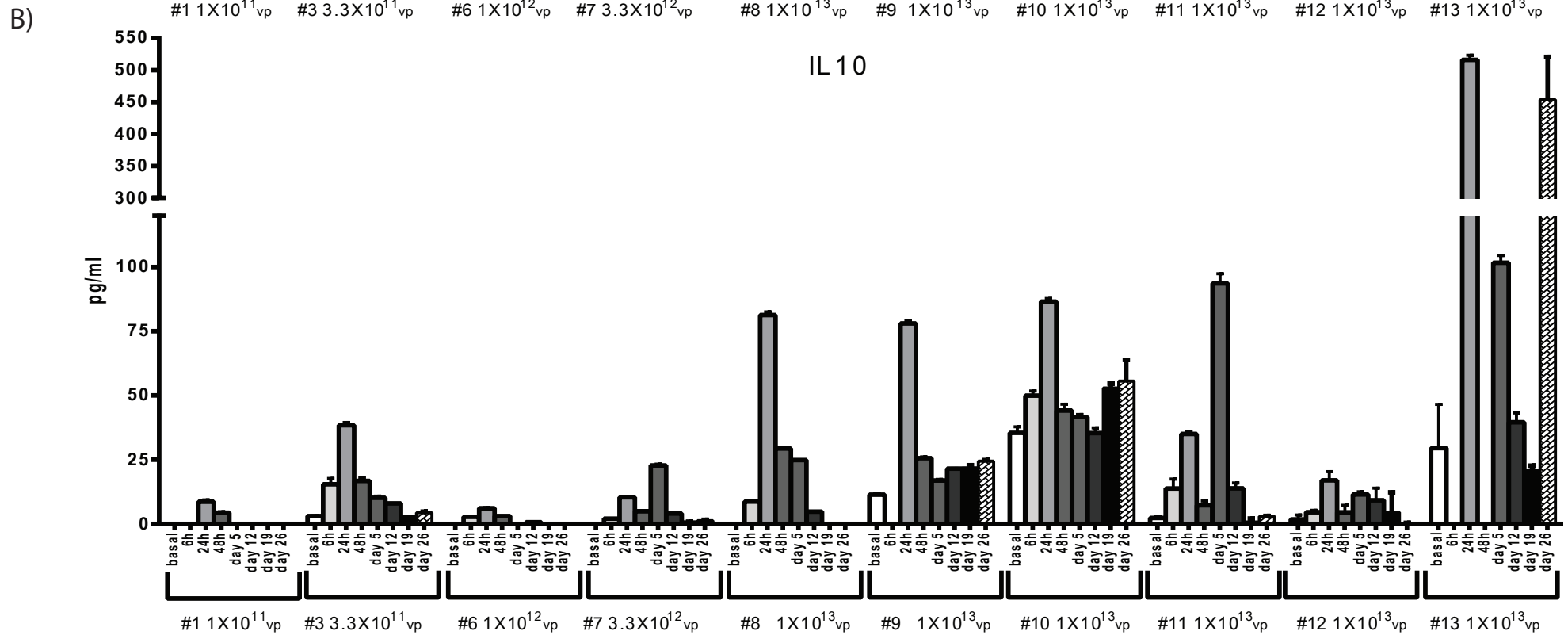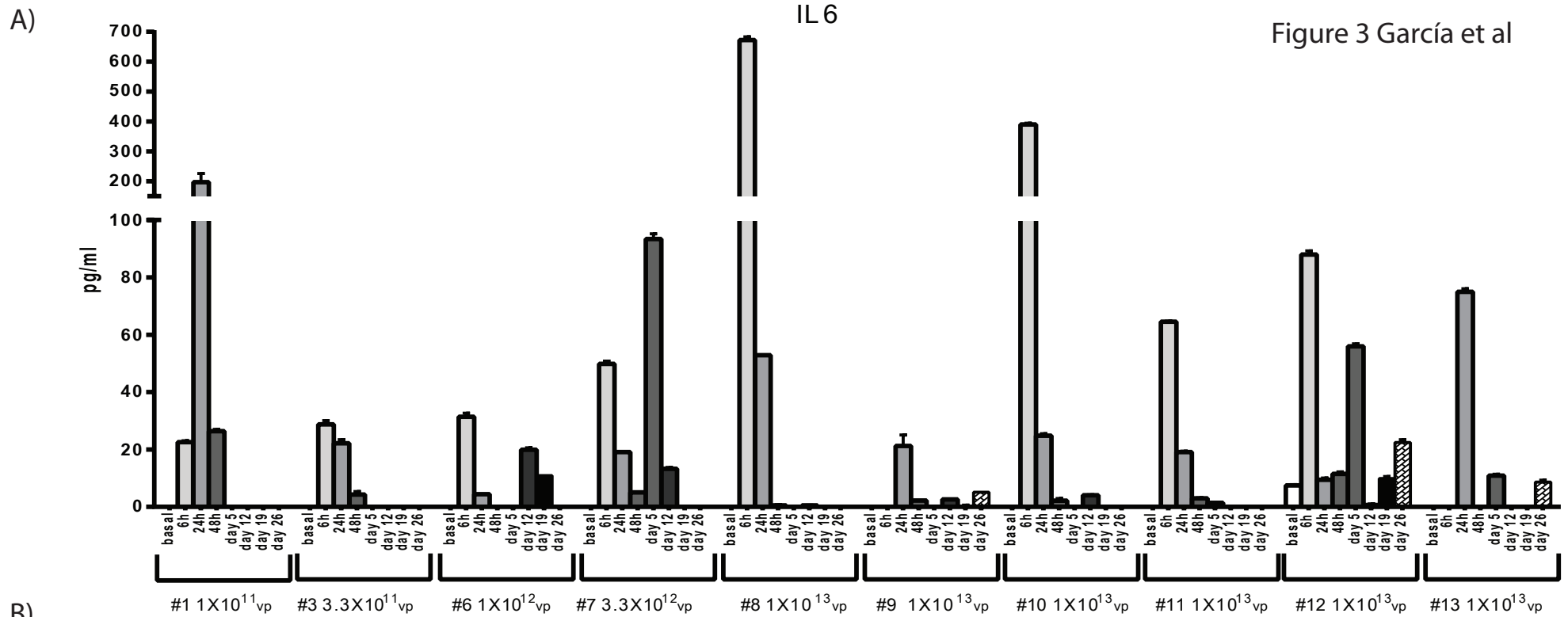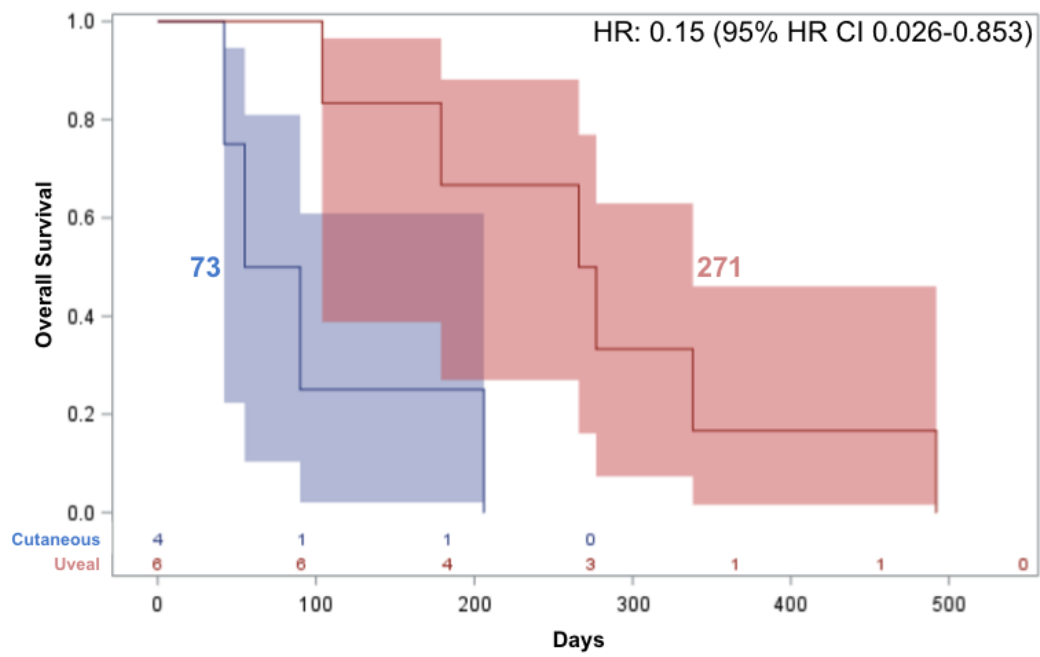
Supplementary figure 1. Kaplan-Meier survival analysis of cutaneous (n=4) vs uveal (n=6) melanoma patients.

Table 1. Dose levels and DLTs

Patient	Dose (vp)	Level	Toxicity	DLT	Decision taken by investigators' committee
#01	1×10^{11}	1a	Grade 3 lymphopenia Grade 2 neutropenia	No	Grade 3 toxicity not associated to treatment Decision: escalation to the next level
#02	3.3×10^{11}	2a	Grade 3 asthenia	No	The patient did not complete the cycle. Decision: replacement of patient
#03	3.3×10^{11}	2a	Grade 1 toxicity	No	Decision: escalation to the next level
#04	1×10^{12}	3a	Grade 2 thrombocytopenia	No	Grade 3 thrombocytopenia out of the cycle. By relevance, the decision is replacement of patient.
#06	1×10^{12}	3a	Grade 3 neutropenia	No	Transient (<24 hours) grade 3 toxicity. Decision: escalation to the next level
#07	3.3×10^{12}	4a	< Grade 2 toxicity	No	Grade 2 unrelated asthenia Decision: escalation to the next level
#08	1×10^{13}	5a	Grade 3 transaminitis Grade 3 thrombocytopenia	Yes	Expansion of cohort by DLT
#09	1×10^{13}	5a		No	
#10	1×10^{13}	5a		No	
#11	1×10^{13}	5a		No	
#12	1×10^{13}	5a		No	
#13	1×10^{13}	5a	Grade 3 transaminitis	Yes	End of study due to two DLT. MTD declared level

Vp: viral particles, DLT: dose limiting toxicity; MTD: maximum tolerated dose

Table 2. Number of patients with nonhematologic toxicity, considering the worst grade toxicity for each patient (n=12).

Symptoms	Toxicity (grade)			
	1	2	3	4
Fever	8	1	0	0
Stomatitis	1	1	0	0
Myalgia/arthralgia	2	1	0	0
Headache	1	0	0	0
Asthenia	4	2	1	0
Rash	2	0	0	0
Diarrhea	1	0	0	0
Constipation	1	0	0	0
Hypertension	1	1	0	0
Edema	1	0	1	0
Nausea/ Vomiting	2	0	0	0
Renal failure	0	1	0	0
Chills	2	0	0	0
Transaminitis	0	1	2	0
Hypocalcemia	0	1	0	0
Hypophosphatemia	0	1	0	0
Hypomagnesemia	1	0	0	0
Hypokalemia	1	0	0	0

Table 3. Anti-Ad5 neutralizing antibodies, clinical responses, and viral genomes in biopsies.

Patient	Dose	Basal NAbs	NAbs after treatment (d19)	Clinical response	ICOVIR5 in biopsy	ICOVIR5 in biopsy (genomes/mg)	ICOVIR5 in blood (day 5) (genomes/ml)
#001 (CM)	1×10^{11} vp/ml	1/1280	>1/5120	PD	negative		negative
#003 (UM)	3.3×10^{11} vp/ml	1/10	1/2560	SD (100)	positive	1.04×10^{3}	negative
#004 (CM)	1×10^{12} vp/ml	1/1280	>1/5120	PD	negative		negative
#006 (UM)	1×10^{12} vp/ml	1/80	>1/5120	SD (433)	NA		
#007 (CM)	3.3×10^{12} vp/ml	1/160	>1/5120	PD	positive	3.89×10^{2}	negative
#008 (UM)	1×10^{13} vp/ml	1/80	>1/5120	PD	NA		
#009 (CM)	1×10^{13} vp/ml	1/10	1/1280	SD (56)	positive	1.47×10^{5}	$1,84\times10^{4}$
#010 (UM)	1×10^{13} vp/ml	1/320	>1/5120	SD (94)	NA		
#011 (UM)	1×10^{13} vp/ml	1/320	>1/5120	SD (131)	positive	1.65×10^{2}	9.65×10^{5}
#012 (UM)	1×10^{13} vp/ml	1/640	>1/5120	SD (84)	NA		
#013 (CM)	1×10^{13} vp/ml	1/10	1/1280	SD (112)	negative		2.23×10^{6}

Patient: CM, cutaneous melanoma; UM, uveal melanoma. Nabs: anti-adenovirus neutralizing antibodies. >1/5120 upper limit of detection, Vp: viral particles, PD: progressive disease, SD: stable disease (until day indicated); NA: not available.