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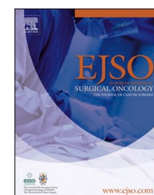

Phase I trial of phIL12 plasmid intratumoral gene electrotransfer in patients with basal cell carcinoma in head and neck region

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A R T I C L E   I N F O

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A B S T R A C T

Introduction: In the treatment of cancer, immunomodulatory approaches are developed to support the organism in fighting cancer or to enhance the immunomodulatory effects of local ablative techniques. To this end, we conducted an interventional, open-label, single-arm Phase I trial to evaluate the safety and tolerability of intratumoral phIL12 plasmid DNA gene electrotransfer as primary objectives.

Methods: The study was dose-escalating with 3 consecutive cohorts of 3 patients per phIL12 dose level (0.5 mg/ml, 1 mg/ml or 2 mg/ml) according to a matched 3 + 3 design. Recruitment of patients was staggered. The waiting period was 30 days after treatment of the previous patient, based on the expected duration of acute and subacute toxicity.

Results: The results of this phase I clinical trial in basal cell carcinoma demonstrated the feasibility and safety of the phIL12 plasmid by gene electrotransfer. We were able to demonstrate that phIL12 gene electrotransfer induced local IL-12 production, which was accompanied with IFN-γ expression. Triggering of the immune response was demonstrated by increased infiltration of immune cells and some antitumor effect. Based on these data, we would recommend the use of a concentration of 2 mg/ml of the plasmid in future trials.

Conclusion: The trial lays the foundation for future Phase II clinical trials in which phIL12 gene electrotransfer is used in combination with local tumor-ablative approaches, such as electrochemotherapy or radiotherapy.

1. Introduction

Cancer immunotherapy has evolved into a transformative approach in oncology that utilizes the body's own immune system to target and eliminate malignant cells. This modality includes various strategies such as immune checkpoint inhibitors, adoptive cell transfer, and therapeutic vaccines, all of which aim to enhance the immune system's recognition and destruction of tumor cells [1]. Immune checkpoint inhibitors, which block inhibitory signaling pathways that restrain T cell activity, have been shown to be highly effective in various cancers and have resulted in durable responses and prolonged survival in some patients. However, the heterogeneity of tumors and the complexity of the tumor microenvironment pose a major challenge and result in variable patient response [2]. Ongoing research aims to improve and optimize immunotherapeutic approaches to improve clinical outcomes.

Cytokines represent another category of immunotherapeutics [3].

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Interleukin-12 (IL-12) is a potent immunostimulatory soluble cytokine that exhibits notable anti-tumor efficacy by enhancing both innate and adaptive immunity, primarily through the induction of interferon-gamma (IFN-γ) [4–6]. In addition to its immunostimulatory properties, IL-12 also has antiangiogenic effects [7]. The therapeutic use of systemic applications of recombinant IL-12 has been limited due to the severe toxicity observed at the high doses required for clinical efficacy, resulting in its discontinuation in clinical trials. The solution to this limitation is localized delivery of IL-12 into the tumor tissue [8]. This can be achieved by administering an IL-12 encoding plasmid using a non-viral gene delivery method called gene electrotransfer (GET) [7,9,10]. GET utilizes electroporation, in which electrical pulses are used to transiently permeabilize the cell membrane, facilitating the intracellular transfer of large, non-permeable molecules [11]. Numerous clinical studies have demonstrated the feasibility, safety and efficacy of this non-viral gene delivery method [12–15].

GET using plasmid encoding IL-12 has been tested in clinical trials in the USA under the brand name TAVO™ (OncoSec Medical Incorporated, NJ, USA). Results from completed clinical trials with TAVO™ have demonstrated a local immune response that subsequently induced systemic effects, either as monotherapy or in combined treatment approaches [16–18].

However, the TAVO™ plasmid contains an antibiotic resistance gene that is used as a selection marker in the production process [16], a trait that is discouraged by European regulators. European Union regulators favor the use of plasmids without selection genes to prevent the possible transfer of these genes to pathogenic bacteria, which could contribute to the general problem of antibiotic resistance or cause susceptibility in certain individuals [19]. To address this problem, we have developed an IL-12 encoding plasmid, phIL12, and established a clinical grade production process in our previous studies to facilitate transfer to clinical trials in the European Union [20–22]. The phIL12, similar to TAVO™, encodes the p35 and p40 subunits of the heterodimeric human IL-12 protein, resulting in the production of functional IL-12 p70. However, the phIL12 has a different backbone that does not contain antibiotic resistance genes. The phIL12 utilizes operator repressor titration technology (ORT, Charles River, Wilmington, MA, USA), which facilitates bacterial propagation by titrating the repressor of an essential gene (dapD in the case of phIL12). This titration enables the expression of the essential gene by multiple operators present on the plasmid and thus an antibiotic-free production process of the phIL12 [23]. Since phIL12 GET is intended as an adjuvant treatment to local ablative therapies to enhance both the local and systemic responses, the IL-12 coding sequence was placed under the control of an inducible p21 (cyclin-dependent kinase inhibitor 1A, CDKN1A) promoter. This promoter can be activated by the hypoxic tumor microenvironment and/or genotoxic stress induced by local ablative therapies such as tumor irradiation or electrochemotherapy [21].

The phIL12 was extensively tested in a combined non-clinical proof-of-concept, safety and pharmacokinetic study to obtain approval for a phase I clinical trial in basal cell carcinoma (BCC) [24]. This study demonstrated the antitumor efficacy of the IL-12 transgene protein, accompanied by a notable infiltration of immune cells. Biodistribution analysis showed widespread distribution of the plasmid throughout the body, with plasmid concentration decreasing over time in all organs except the skin surrounding the tumor. Importantly, the therapy showed no detectable systemic toxicity. These findings from the non-clinical evaluation supported the safety and efficacy of phIL12 GET, and based on these results, approval was gained for the initiation of the phase I clinical trial in BCC.

In the present study, we report the results of the phase I clinical trial of phIL12 GET for the treatment of BCC in patients with operable tumors in the head and neck region. This exploratory dose-escalation study was designed with primary objectives focused on safety and tolerability of treatment. Secondary objectives included evaluating the pharmacokinetics and pharmacodynamics of the treatment, assessing the feasibility of patient recruitment, and identifying a safe dose of phIL12 that elicits biological activity for subsequent phase II study.

2. Materials and methods

2.1. Trial design

This trial was conducted according to the guidelines of the Declaration of Helsinki, the protocol was approved by the Institutional Review Board of Institute of Oncology Ljubljana (ERIDNPVO-0051, September 10th, 2021) and National Ethics Committee of the Republic of Slovenia (0120–524/2020-12, July 14th, 2021). The trial was approved by Agency for Medicinal Products and Medical Devices of the Republic of Slovenia (1050–65/2021-10, September 23rd, 2021). Trial identifiers: EudraCT 2021-000852-21, ISRCTN15479959, ClinicalTrials NCT05077033.

The trial was designed as an interventional, open-label, single-arm phase I to evaluate the safety and tolerability of the intratumoral phIL12 GET as primary objectives (Fig. 1). Secondary objectives included evaluating the pharmacokinetics and pharmacodynamics of the treatment, assessing the feasibility of patient enrollment, and identifying a safe dose of phIL12 that elicits biological activity for subsequent confirmatory studies (Fig. 1). The detailed protocol of the trial is available in a separate publication [25]. Briefly, the phase I trial was conducted on operable basal cell carcinoma of the head and neck. The study was dose-escalating with 3 consecutive cohorts of 3 patients per phIL12 dose level (0.5 mg/ml, 1 mg/ml or 2 mg/ml) according to an adapted 3 + 3 design. Recruitment of patients was staggered. The waiting period was 30 days after treatment of the previous patient, based on the expected duration of acute and subacute toxicity.

Inclusion criteria were: (i) Histologically or cytologically confirmed, previously untreated cutaneous basal cell carcinoma located in the head and neck region; (ii) Solitary tumors with a largest diameter of up to 3

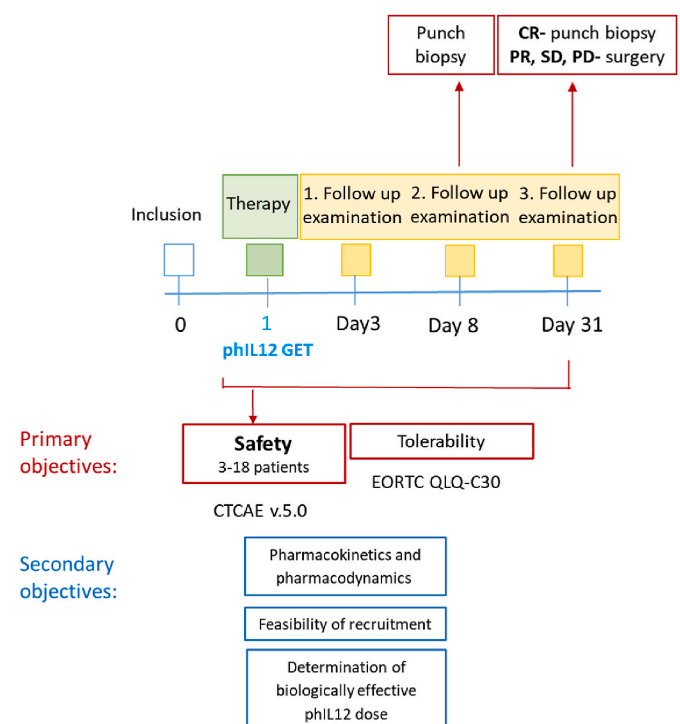

Fig. 1. Clinical trial design. CTCAE v.5 = Common Terminology Criteria for Adverse Events version 5.0; CR = complete response; EORTC QLQ-C30 = European Organization for the Research and Treatment of Cancer Quality of Life Questionnaire C30; PR = partial response; PD = progressive disease; SD = stable disease. With permission of Radiology and Oncology [25].

cm in the region where curative (R0) surgery is possible; (iii) Age 18 years or older; (iv) Life expectancy >3 months; (v) Physical performance in accordance with the Karnofsky scale ≥70 or < 2 according to the World Health Organization (WHO) scale; (vi) The patient must be able to understand the treatment procedure and possible adverse events which may occur during treatment; (vii) The patient must be able to sign the informed consent form to participate in the clinical trial (voluntary and informed consent after education); (viii) Prior to enrollment in the trial, the patient must be presented in a multidisciplinary advisory team meeting.

Exclusion criteria were: (i) Other malignancy at the time of inclusion; (ii) Lesions not suitable for treatment with GET (invasion of bone, large vessel infiltration); (iii) Life-threatening infection and/or severe heart failure and/or liver failure and/or other life-threatening systemic diseases; (iv) Significantly reduced lung function requiring determination of DLCO; (v) Treatment with immunosuppressants, steroids and other drugs that would impair wound healing or would have immunomodulatory effects.; (vi) Age under 18 years; (vii) Severe disorders of the coagulation system (not responding to standard therapy – vitamin K replacement or fresh frozen plasma); (viii) Chronic deterioration of renal function (creatinine >150 μmol/L); (ix) Epilepsy; (x) Pregnancy and breastfeeding; (xi) Inability of the patient to understand the purpose or procedure of the trial or not consenting to participate in the trial; (xii) Patients unwilling or unable to comply with protocol requirements and scheduled visits.

The timeline of clinical trial is presented in Fig. 1.

Preliminary tumor response was assessed according to RECIST v1.1 criteria. The measurements of tumor diameters were recorded in millimeters using caliper. Baseline measurements were conducted on the same day as the treatment, prior to the initiation of the treatment procedure. The skin lesions were also documented by color photography including a ruler at each measurement. The responses to be measured were: Complete response (CR): disappearance of all target lesions; partial response (PR): at least 30 % decrease in the sum of the diameters of the target lesions, taking the sum of the initial diameters as reference; progressive disease (PD): at least 20 % increase in the sum of the diameters of the target lesions, in addition to the relative increase of 20 %, the sum must also show an absolute increase of at least 5 mm (the appearance of one or more new lesions is also considered progression); stable disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, using as reference the smallest sum of diameters during the study.

2.2. phIL12 GET treatment protocol

Plasmid phIL12 was manufactured under GMP (Good Manufacturing Practices) conditions at JAFRAL d.o.o. (Ljubljana, Slovenia) and supplied in sterile vials with a final concentration of 2 mg/ml and stored at −80 °C. Prior to use, the plasmid was thawed and diluted in sterile saline to the desired concentration (0.5, 1.0 or 2.0 mg/ml) as required.

All patients underwent analgosedation using propofol, fentanyl and paracetamol before the start of the treatment procedure. The phIL12 injection volume was calculated using formula $P = V/4$, where P is the plasmid injection volume and V is the tumor volume. The tumor volume was estimated using formula $V = ab^2\Pi/6$, where a is the longest diameter and b is the perpendicular diameter to a. A single dose of phIL12 at the appropriate concentration was injected intratumorally and 5 min later the electric pulses were applied to the tumor using the CLINIPORATOR™ electrical pulse generator (IGEA, s.p.A., Carpi, Italy) according to updated standard operating procedures [26]. Parallel needle electrodes were used to treat all patients, and the number of pulse applications was adjusted to the tumor volume to ensure that the entire tumor was covered by the electric field. After the treatment, when the patients have regained consciousness, the post-procedure pain level was assessed in accordance with the visual analog scale (VAS). The pain was assessed in all subsequent follow-up examinations (day 3, day 8 and day 31) [25].

2.3. Determination of serum IL-12 cytokine levels

Serum IL-12 concentrations were measured using a commercially available kit for the detection of IL-12 (Millipore, Darmstadt, Germany) for the Luminex platform on a Magpix instrument (Luminex, Austin, TX, USA) according to the manufacturer's instructions. Data analysis was performed using Milliplex Analyst 5.1 software. All values outside the upper and lower ends of the standard curve were considered maximum and minimum values, respectively.

2.4. Histology and immunohistochemistry of tumors

The tumor samples were taken before treatment (denoted as day 0) and day 8 and day 31 after the treatment. For patient SMG 02 the primary biopsy could not be obtained. Histological and immunohistochemical stainings were performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections (2–4 μm thickness) of tumor punch biopsies (day 8) or excised tumors (day 31). The tissue sections were stained for hematoxylin-eosin and for different immunohistochemical markers (Supplementary Table 1). Immunohistochemical staining of all antibodies used except for IL-12 was performed using the fully automated Ventana Benchmark Ultra IHC system (Ventana ROCHE Inc.; Oro Valley, Arizona, USA). Epitope retrieval was achieved onboard through heat-mediated epitope retrieval using a high pH "Cell Conditioning Solution 1" (catalog number 950–124, Ventana ROCHE Inc.). The retrieved epitope was then detected using different antibodies diluted in DAKO REAL™ antibody diluent (catalog number S2022; DAKO Agilent Technologies Inc.; Santa Clara, California, USA). Specific binding of the primary antibody was visualized using the OptiView DAB IHC Detection Kit (catalog no. 760–700; Ventana ROCHE Inc.) with or without amplification using the OptiView Amplification Kit (catalog no. 760–099; Ventana ROCHE Inc.), followed by hematoxylin counterstaining.

For IL-12 immunohistochemistry staining, antigen retrieval was performed in a sodium citrate buffer (10 mM, pH 6) at 95 °C for 30 min. Sections were incubated overnight at 4 °C with goat anti-IL-12 (ab124635, Abcam; 1:250). A peroxidase-conjugated streptavidin-biotin system (CTS008, Anti-Goat HRP-DAB Cell & Tissue Staining Kit, R&D Systems, Mi, USA) served as a chromogenic reagent, followed by hematoxylin counterstaining.

The stained slides were scanned using a NanoZoomer 360 Digital Slide Scanner (Hamamatsu Photonics, NY, NJ, USA). The extent of lymphocyte infiltration and the positivity of immunohistiolochemical staining were assessed by three independent researchers who were blinded to the experimental groups. To collectively evaluate the immune cell infiltration, an immunoscore was used, where each of three different parameters; immune cell infiltration estimated on HE stainings, IL-12 and IFN-γ positivity scored one point.

2.5. Flow cytometry

Blood samples taken on days 0, 3, 8 and 31 were analyzed by flow cytometry. Flow cytometry measurements were performed to determine the changes in immune populations (T lymphocytes, B lymphocytes and natural killer (NK) cells) after therapy. From the blood samples, the cells were counted and a suspension of 0.4×10^6 cells per test tube was prepared. Red blood cells were lysed using BD FACS Lysing Solution (BD Biosciences, Franklin Lakes, NJ, USA). An antibody cocktail (all from BD Biosciences) was then added to each test tube (4 μl CD4 FITC, 3 μl CD56 PE, 5 μl CD19 PerCpCy5.5, 5 μl CD5 PE-Cy7, 2 μl CD7 APC, 2 μl CD8 APC-Cy7, 3 μl CD3 V450, 2 μl CD45 V500). The samples were incubated for 20 min in the dark at room temperature. The samples were then analyzed using a FACSCanto 10-color flow cytometer (BD Biosciences) with three lasers (405, 488 and 633 nm) and the software FACSDiva

8.0.2 (BD Bioscience). The flow cytometer was routinely set up and calibrated using the FACSDiva™ CS &T IVD beads (BD Biosciences). The analysis was then performed in FACSDiva 8.0.2 (BD Bioscience) software. First, the debris and non-haemopoietic cells were excluded using the SSC-A/CD45 dot plot, followed by the removal of the doublets using the FSC-W/FSC-A dot plot and the removal of the non-lymphocyte population using the SSC-A/FSC-A dot plot. From the lymphocyte population the T lymphocytes ($CD3^+$, $CD19^-$), B Lymphocytes ($CD3^-$, $CD19^+$) and NK cells ($CD3^-$, $CD56^+$) were gated on. Further, from the T lymphocyte population the Cytotoxic T cells ($CD8^+$) and Helper T cells ($CD4^+$) were gated on. For each of the above populations, the percent of positive cells from the parent population was determined.

2.6. Determination of plasmid DNA copy number in tumor tissue, saliva samples and skin swabs

DNA was isolated from formalin-fixed, paraffin-embedded tumor tissue using the MagMAX™ FFPE DNA/RNA Ultra Kit (ThermoFisher Scientific, MA, USA). The GenElute Plasma/Serum Cell-Free Circulating DNA Purification Kit (Sigma-Aldrich, Merck, Rahway, NJ, USA) was used to isolate plasmid DNA from saliva samples and skin swabs. Plasmid copy number was determined from the isolated DNA samples by quantitative real-time polymerase chain reaction (qRT-PCR) as previously described [20].

For saliva and skin swab samples, the results were normalized to the baseline value of each patient (values on day 0). This was not possible for tumor samples that were collected only at day 31 by surgical excision. For these samples, a threshold value of 10^4 plasmid copies/μg DNA was set as the Lower Limit of Quantification (LoQ). Therefore, only an increase in values above this threshold was considered positive for the presence of plasmid DNA.

2.7. Statistical analysis

The study was exploratory and used a standard 3 + 3 dose-escalation design, therefore only descriptive statistics was used. GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) was used for graphical visualization.

3. Results

3.1. Patient characteristics and treatment data

Nine patients were enrolled in 3 dose cohorts between September 2021 and November 2023. The protocol proved feasible, since all patients received treatment as planned with no major protocol violations. Patient characteristics and treatment data are shown in Table 1.

Minor problems were encountered during patient recruitment, mainly due to the required strict follow-up schedule, which was challenging for potential participants. However, once patients were enrolled, there were no significant problems with the treatment procedures or the follow-up program. Importantly, the study had a solid retention rate, and no patients were lost to follow-up, ensuring the integrity and reliability of the data collected.

3.2. Safety of the phIL12 GET

The assessment of adverse events was performed according to the CTCAE v5.0 criteria. No serious adverse events were identified. None of the 9 patients reported pain immediately after the procedure. One patient experienced mild pain two days after treatment and another patient experienced mild edema in the treatment area two days after treatment (Fig. 2).

Blood parameters examined included complete blood count (CBC) and differential blood count i.e., sodium, potassium, chloride, glucose, creatinine, urea, uric acid, inorganic phosphate, calcium, alkaline phosphatase, gamma-glutamyl transferase (gamma-GT), bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), C-reactive protein (CRP), total proteins and albumin. No safety concerns were observed as there were no noticeable changes from the reference values of blood parameters during treatment. Similar results were obtained when determining the immune cell populations using flow cytometry. In all three dose cohorts, there were no clinically relevant deviations from the pre-treatment baseline values within the observation period of one month (Fig. 3).

Table 1
Patient characteristics and treatment data.

Cohort		Patient characteristics			Tumor characteristics		Treatment				
	Patient	Age	Sex	ECOG	Volume before GET (mm^3)	Location	Concentration of phIL12 (mg/ml)	Injected volume of phIL12 (1/4 of tumor volume) (μl)	Injected amount of phIL12 (μg)	No of electric pulse applications	Maximal current (A)
1	SMG 01	87	M	0	785	Nose	0.5	200	100	8	4
	SMG 02	85	M	0	335	Ear	0.5	85	42.5	4	4
	SMG 03	80	F	0	170	Ear	0.5	43	21.5	13	3
2	SMG 04	74	F	1	78	Nose	1	19.5	19.5	4	2
	SMG 05	45	M	0	151	Nose	1	40	40	4	2
	SMG 06	59	M	1	1077	Cheek	1	270	270	8	3
3	SMG 07	74	M	0	38	Neck	2	10	20	11	3
	SMG 08	68	M	0	207	Ear	2	52	104	11	3
	SMG 09	58	M	0	697	Nose	2	174	348	13	4

Abbreviations: M, male; F, female; ECOG, Eastern Cooperative Oncology Group performance status; GET, gene electrotransfer.

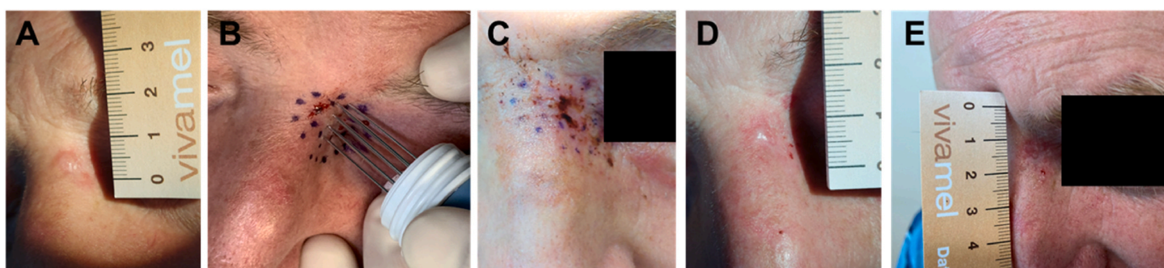

Fig. 2. Edema present in the treatment area two days post-GET in patient from 2 mg/ml cohort. (A) Before treatment, (B) application of electric pulses during treatment, (C) 2 days after treatment, (D) 7 days after treatment, (E) 1 month after treatment.

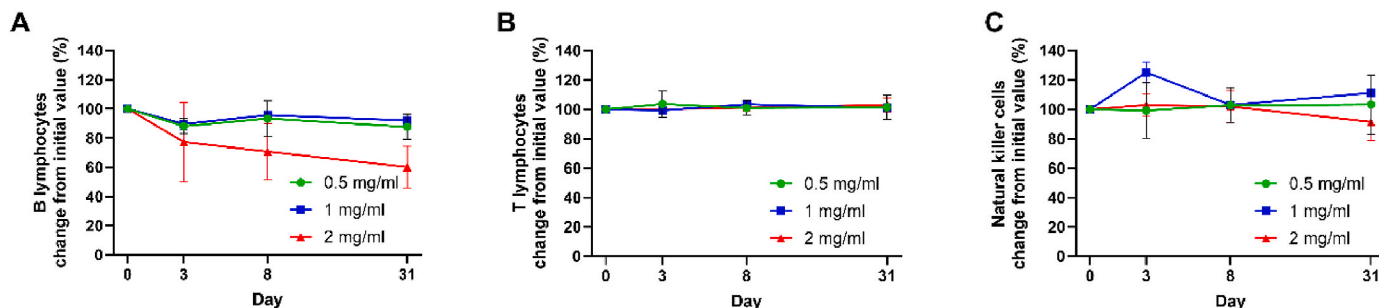

Fig. 3. Populations of (A) B lymphocytes, (B) T lymphocytes and (C) Natural killer cells in the peripheral blood of patients in the first month after the treatment. The change from initial value (%) and standard deviation for each dose cohort are presented.

3.3. Tolerability

The treatment proved to be excellently tolerated, as shown by the results of a quality-of-life questionnaire (EORTC QLQ-C30 version 3.0) presented by the patients. Throughout the study, participants reported minimal adverse effects that had little to no impact on their daily activities and general well-being. Scores remained stable over the course of treatment, indicating that patients were able to maintain a high quality of life during therapy (Table 2).

3.4. Pharmacokinetics, pharmacodynamics and determination of biologically effective phIL12 dose

Serum levels of the cytokine IL-12 were monitored at each patient's visit to detect a possible increase in transgenic IL-12 concentration in the blood due to IL-12 release. Baseline IL-12 levels prior to phIL12 GET showed fluctuations between patients (Day 0; Fig. 4A). After treatment, serum IL-12 concentrations remained low and within a safe range, with no trend toward increasing serum concentrations in any of the three treated cohorts with increasing plasmid dose (days 3, 8, 31; Fig. 4A).

Plasmid DNA shedding was investigated using skin swabs from the treated area and saliva. Samples were taken before treatment (day 0), immediately after treatment (day 1) and at each follow-up visit (days 3, 8 and 31). Immediately after treatment (day 1; Fig. 4B), an increase in plasmid copy number was observed in skin swabs from the treatment application site. Plasmid copy number gradually decreased during subsequent follow-up examinations and returned to baseline by day 31 post-treatment. In contrast, a trend in excretion of phIL12 in saliva samples was not detected as plasmid copy number mainly remained below LoQ (Fig. 4C).

The presence of phIL12 in the tumor samples collected at the final follow-up visit (day 31) from surgically excised tumor tissue was mostly below LoQ, demonstrating that plasmid DNA was degraded until the last follow-up visit (Fig. 4D).

3.5. Tumor lymphocyte infiltration and tumor IL-12 and IFNγ expression

Histopathological analysis of tissue biopsies demonstrated an increased infiltration of immune cells in all three dosage groups both 8 and 31 days after treatment compared to pre-treatment levels (Fig. 5A). This infiltration peaked 8 days after treatment in the groups receiving 1 mg/ml and 2 mg/ml plasmid DNA. At the same time, IL-12 expression was highest 8 days after treatment, with no substantial differences observed between the dosing groups (Fig. 5B). Notably, IFN-γ expression

Table 2
Health and quality of life of patients assessed via EORTC QLQ-C30 version 3.0 quality of life questionnaire. Minimal value 1, maximal value 7.

Cohort		Health			Quality of life		
	Patient	Before treatment	Day 7	Day 31	Before treatment	Day 7	Day 31
1 phIL12: 0.5 mg/ml	SMG 01	6	6	6	6	6	6
	SMG 02	4–5	4–5	5	5	5	6
	SMG 03	6	6	6	6	6	6
2 phIL12: 1 mg/ml	SMG 04	5	5	7	5	5	7
	SMG 05	5	7	7	6	7	7
	SMG 06	4	4	4	4	4	4
3 phIL12: 2 mg/ml	SMG 07	5	6	6	7	7	7
	SMG 08	5	5	4	5	5	4
	SMG 09	7	7	7	7	7	7
	Median	5	6	6	6	6	6

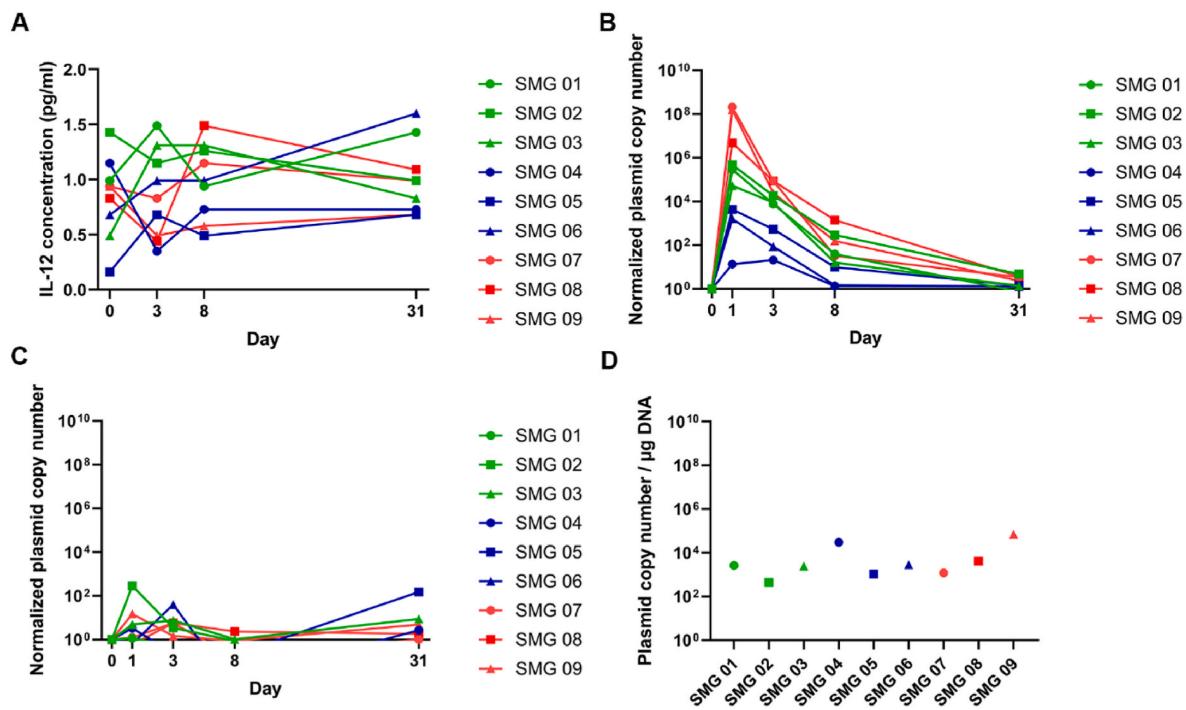

Fig. 4. Serum IL-12 concentration (A) and plasmid copy number detected in skin swabs from the treatment application site (B), saliva samples (C) or excised tumor tissue at day 31 (D).

was increased in the 0.5 mg/ml and 2 mg/ml groups, while it was comparatively lower in the 1 mg/ml group. The results shown in the representative images of patient SMG 08 on day 8 illustrate a notable infiltration of immune cells (Fig. 5D, yellow arrows), along with high levels of IL-12 (Fig. 5E) and IFN-γ (Fig. 5F, black arrows). In addition, numerous other immunostainings were performed, such as CD3, CD4 and CD8, supporting the HE staining and immune cell infiltration data (Supplementary Fig. 1). Furthermore, an immunoscore was calculated based on the estimation of all three parameters—immune cell density on HE stained sections and positivity of IL-12 and IFN-γ staining. The analysis showed that the 2 mg/ml groups exhibited the highest immunoscore on day 8 compared to the other two groups (Supplementary Table 2).

3.6. *Clinical response*

The preliminary objective response of the tumor was evaluated according to the RECIST v1.1. criteria, considering 2 tumor diameters, where diameter a represents the largest diameter and diameter b represents the diameter perpendicular to a. According to the change in the sum of the diameters, 3/9 responses represented PR and 6/9 responses represented SD (Table 3, Fig. 6A). No CR or PD were detected. Treatment of the tumors with 0.5 mg/ml phIL12 led to a reduction in tumor size in 1/3 of the tumors. In 1/3 of the tumors the size remained unchanged and in 1/3 the size increased (Fig. 6B). Treatment with 1 mg/ml or 2 mg/ml led to a reduction in tumor size in 2/3 of the tumors, and the size remained unchanged in 1/3 of the tumors (Fig. 6B).

4. Discussion

This phase I exploratory clinical study was the first in human gene therapy trial in Europe, using plasmid DNA with a transgene coding for IL-12. Three doses of intratumoral application of phIL12 plasmid were tested in the study. The delivery system used was electroporation – GET and patients with BCC were treated. The results show that this therapeutic approach, i.e. phIL12 GET is feasible and safe.

BCC is one of the most common non-melanoma skin cancers, making it the most prevalent malignant tumor affecting the skin [27]. Despite its typically slow progression and low metastatic potential, BCC can present significant therapeutic difficulties, particularly in cases of aggressive growth or insufficient initial treatment. These challenges are often exacerbated by the anatomical complexity of facial regions such as the nose, orbital area, and ears. Standard treatment options, including surgery, radiotherapy, and electrochemotherapy, generally provide similar rates of local control for small, superficial lesions [27,28]. However, relapses can occur due to inadequate initial tumor evaluation or underestimation of the disease's extent. Additionally, repeated interventions, whether surgical or radiotherapeutic, tend to have diminished effectiveness and can compromise both the functionality and cosmesis. These limitations underscore the pressing need for innovative therapeutic strategies.

BCC was chosen for this clinical study due to its unique biological and clinical characteristics. First, due to its slow growth and non-metastatic nature, this was the only tumor type for which the observation period of one month was ethically acceptable and patients could still receive standard treatment. BCC was also selected due to its practicality for sequential biopsies, which facilitate the monitoring of tumor immune responses. Based on these features, we designed the proposed clinical study, which incorporates phIL12 GET into the tumor. This approach aims to stimulate a robust local immune response to eradicate the tumor. If proven safe, this method could offer improved aesthetic outcomes compared to surgery or radiotherapy. Furthermore, this treatment with immune stimulation of the tumor bed could have the potential for cancer surgery.

We used an IL-12 encoding plasmid in the study. This cytokine was selected based on encouraging preclinical and clinical results. IL-12 is a well-known immunostimulatory cytokine that enhances both arms of the immune response and also exhibits anti-angiogenic activity. Many preclinical data using IL-12 in different formulations, as recombinant protein or as gene therapy, have demonstrated its antitumor activity in preclinical models [9,21,29]. In addition, our data suggest that IL-12 gene therapy works well in combination with electrochemotherapy or radiation [30,31]. Regarding the route of administration, clinical data suggest that systemic administration of recombinant IL-12 can lead to

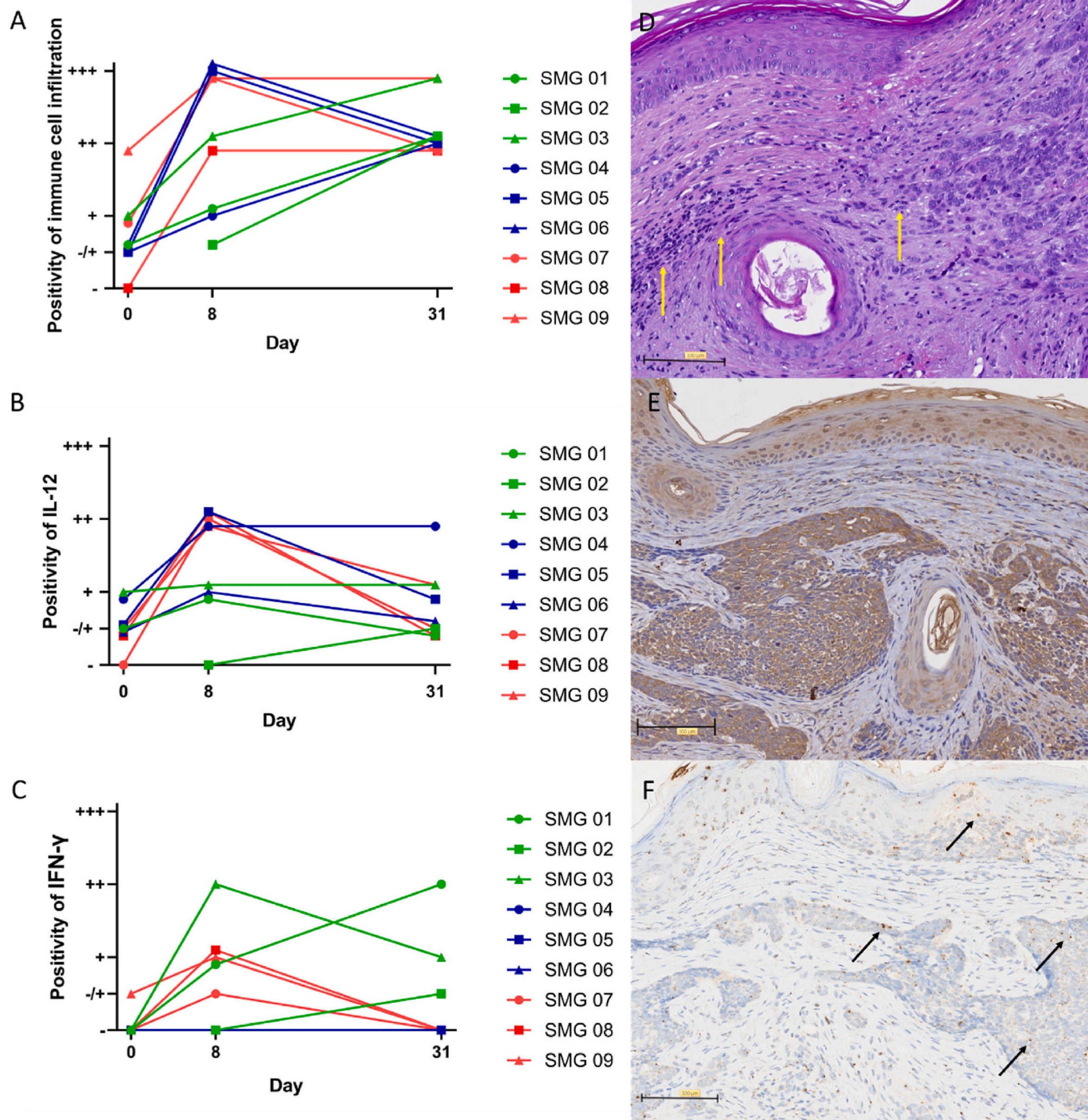

Fig. 5. Graphs showing the evaluation of immune cell infiltration (A), IL-12 (B) and IFN-γ (C) staining together with the representative figures of patient SMG 08 on day 8 after the treatment (D,E,F; Yellow arrows presenting infiltration of immune cells; black arrows showing the positive cells for IFNγ). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Tumor size and response rate.

Cohort	Patient	Tumor size before treatment (day 1)			Tumor size after treatment (day 31)			Change from baseline (%)	Response rate
		a (mm)	b (mm)	Sum (mm)	a (mm)	b (mm)	Sum (mm)		
1	SMG 01	15	10	25	11	8	19	−24	SD
	SMG 02	10	8	18	10	8	18	0	SD
	SMG 03	9	6	15	10	8	18	20	SD
2	SMG 04	6	5	11	4	3	7	−36	PR
	SMG 05	8	6	14	5	4	9	−36	PR
	SMG 06	17	11	28	16	12	28	0	SD
3	SMG 07	8	3	11	7	4	11	0	SD
	SMG 08	11	6	17	10	5	15	−12	SD
	SMG 09	11	11	22	2	2	4	−82	PR

Abbreviations: PR, partial response; SD, stable disease.

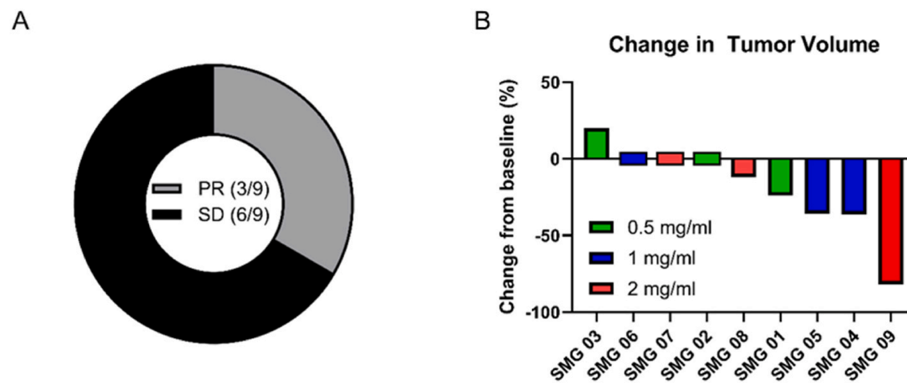

Fig. 6. Response rate (A) and change in tumor volume (B) after treatment with phIL12 GET.

severe side effects, whereas local administration has been shown to be much safer [29]. In the case of the plasmid, intratumoral administration is the only possibility, with the transgene produced and released in a paracrine manner, resulting in a minimal systemic release [32].

Our study found that no elevation of IL-12 concentration was detected in the patients' blood after intratumoral phIL12 GET, at any of the three doses tested. In addition, we also measured the plasmid in the patients' saliva after GET into the cutaneous tumors in the head and neck region. Plasmid copy number remained under LoQ in the saliva, indicating that gene transfer was limited to the treated area. We were able to detect an increased number of plasmid copies in the treated area, which was to be expected as the swabs were taken from the treated area immediately after treatment. However, decreasing amounts were detected on day 3 and day 8 post-therapy. On day 8, almost no plasmid was detectable, while on day 31 the amount of phIL12 was in the lower limit of detection threshold range.

Interestingly, the amount of plasmid in the tumor tissue on day 31 was also low and did not differ between the three doses administered. These data suggest that the transgene is transcribed in the first 8 days and thereafter, due to tumor cell death and dilution of the plasmid copy number during cell proliferation, its replication is limited to a few days after GET. This also underlines the safety of intratumoral GET of plasmid DNA. The safety of phIL12 GET into the skin was demonstrated also in a porcine animal model, where the plasmid was detected in different organs, but decreased to minimal values 28 days after GET [33].

While skin swabs were collected in this study to evaluate the shedding of plasmid DNA from the application site, the potential for assessing protein levels, such as IFN-gamma, was not explored. Total DNA was extracted from the swabs as part of our methodological approach, which was specifically designed for DNA analysis. The evaluation of proteins from skin swabs would require the development and validation of specialized methods, as proteins are susceptible to degradation [34]. Although not included in the current study, this represents an intriguing direction for future research, as it could provide valuable insights into the local immune response and confirmation of transgene action.

Reversible electroporation is a physical phenomenon that enables enhanced transport or diffusion of drugs with impeded membrane transport. It is known that different electric pulse parameters are required for electrochemotherapy than for gene transfer. In the case of electrochemotherapy, smaller molecules are transported than in the case of the plasmid GET. Electrochemotherapy uses shorter electric pulses with a higher amplitude than GET. However, the short and higher electric pulses used in electrochemotherapy can also induce GET [20]. Therefore, we selected these pulses for GET. The first reason was that the first study on plasmid DNA encoding IL-12 GET in melanoma patients used these types of pulses, which were obviously clinically effective [13]. The second reason was that in the future we plan to test in preclinical setting and design a clinical trial in which we would simultaneously administer both the chemotherapeutic agent for electrochemotherapy and the plasmid for GET intratumorally. Such treatment approach with only single pulse application for drug and plasmid delivery have already been successfully used in veterinary oncology for treatment of mast cell tumors. The results of the veterinary study showed that local tumor control was significantly better in the electrochemotherapy group in combination with intratumoral GET compared to electrochemotherapy alone. In addition, the disease-free interval and progression-free survival were also significantly longer in the group with the combined-therapy [35].

The local plasmid transfection did not induce a systemic response of the organism as measured by blood parameters or increased B or T lymphocytes or NK cells. The immune response was elicited locally, as shown by biopsies of the tumors and increased infiltration of immune cells in all three doses of the plasmid day 8 after the therapy. The downstream cytokine induced by IL-12 is IFNγ, which was also elevated on day 8. These data support the notion that immune stimulation can be expected 8 days after treatment. The higher immunoscore showed the highest efficacy of GET at 2 mg/ml. As a secondary objective, we also measured the tumor response. Since the tumors were excised one month after treatment and we did not observe any complete responses, it is difficult to evaluate the antitumor efficacy of phIL12 GET. Since this is an immunomodulatory approach and the basal cell carcinomas are slow-growing tumors, a further antitumor effect could be expected after the observation period of one month, as in this study. Nevertheless, we observed that one third of the tumors responded with a PR and two thirds with SD. However, the highest dose of 2 mg/ml had the greatest reduction in tumor size. According to RECIST 1.1 guidelines, clinical lesions are considered measurable when their diameters are equal to or exceed 10 mm. In this study, this threshold was not met in all cases, as 4 out of 9 patients presented with lesions smaller than 10 mm in diameter. As the evaluation of response rate was not the primary objective of this phase I study and was conducted in an exploratory setting, response assessments were performed based on RECIST 1.1 criteria that were agreed upon and approved by the ethics committee, although this presents a limitation of this study. For the planning of the phase II study, in which the assessment of clinical response rate will be a primary objective, we intend to include a modified RECIST or WHO criteria to account for smaller lesions, ensuring a more comprehensive assessment of tumor response.

Based on this observation, we would recommend the 2 mg/ml dose as safe in future studies. In addition, the results of this study confirmed that the treatment procedure is feasible and has no serious local or systemic side effects. Only two patients experienced mild pain or edema. This is primarily due to the application of electric pulses, which cause pain. In electrochemotherapy, local anesthesia or even general anesthesia is used for larger tumors [36]. However, attempts are being made to develop electrodes that do not penetrate the tissue with a set of pulses that cause less pain, so that anesthesia is not required [37].

This study was a phase I study, in which primarily the feasibility and

safety were tested. However, it was intended to be the study that will continue with a phase II study on skin tumors in combination with one of the ablative techniques. The concept of tumor ablation in combination with immunostimulation is currently being extensively researched [38, 39]. There is data on the combination of immunotherapy and radiotherapy with positive results [38]. Our preclinical studies also indicate a beneficial combination of radiotherapy or electrochemotherapy with IL12 GET [30,31,40]. The combination of radiotherapy with single intratumoral IL-12 GET resulted in decrease of tumor control dose from 29.8 Gy (irradiation alone) to 13.8 Gy in combination group [30]. In addition, the results suggest that IL-12 GET enhance the effect of electrochemotherapy, even with some abscopal effect in a preclinical model on murine tumor models as well as in homologues model in primary tumors in companion dogs [35,40]. In recent retrospective human clinical study, the systemic efficacy of combined electrochemotherapy and the immune checkpoint inhibitor pembrolizumab demonstrated a prolongation of progression-free survival and even overall survival of the combined treatment in melanoma patients [41]. This could also be the reason for the combination of electrochemotherapy with intratumoral GET of phIL12, or possibly the combination with local tumor irradiation. Furthermore, future studies could extend this approach to investigate its efficacy in other immunogenic tumors, such as malignant melanoma or triple-negative breast cancer, or explore its potential as an immunostimulatory treatment of the tumor bed in surgical oncology. These are further steps we want to take to bring phIL12 into clinical trials in combined treatment regimens.

5. Conclusion

The results of this phase I clinical trial on basal cell carcinoma have demonstrated the feasibility and safety of the phIL12 plasmid using GET for delivery. We were able to demonstrate that phIL12 GET induced local IL-12 production, which was accompanied with IFN-γ expression. Triggering of the immune response was demonstrated by increased infiltration of immune cells and some antitumor effect. Based on these data, we would recommend the use of a concentration of 2 mg/ml of the plasmid in future combined modality therapies.

CRediT authorship contribution statement

Primoz Strojan: Conceptualization, Methodology, Investigation, Writing – review & editing. **Tanja Jesenko:** Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Masa Omerzel:** Methodology, Investigation, Formal analysis, Visualization. **Crt Jamsek:** Investigation. **Ales Groselj:** Conceptualization, Methodology. **Ursa Lampreht Tratar:** Investigation, Formal analysis, Writing – original draft, Visualization. **Bostjan Markelc:** Investigation. **Gorana Gasljevic:** Investigation. **Alojz Ihan:** Investigation. **Frenk Smrekar:** Investigation, Resources. **Matjaz Peterka:** Investigation, Resources. **Maja Cemazar:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Project administration. **Gregor Sersa:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejso.2025.109574.

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