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Phase I study of anti-GD2 Chimeric Antigen Receptor-Expressing T cells in pediatric and young adult patients affected by relapsed/refractory central nervous system tumors: preliminary data

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Index

1. Introduction……………………………………………………………..1

1.1 Central Nervous System Tumors……………………………………………........1

1.2 Chimeric Antigen Receptor…………………………………………………......2

1.3 CAR-T cells in solid tumors……………………………………………………..5

1.4 Limitations and Challenges of CAR-T Cell Therapy in CNS Tumors……………7

1.4.1 Trafficking through the blood-brain barrier…………………………...........7

1.4.2 Administration strategies for CAR-T cell immunotherapy in CNS tumors…9

1.5 GD2-CAR-T cell immunotherapy in solid tumors and CNS………..…………..10

1.6 Toxicity…………………………..……………………………………….......13

1.6.1 Cytokine Release Syndrome (CRS)……………………………………......13

1.6.2 Immune effector cell-associated neurotoxicity syndrome (ICANS)…………16

1.6.3 Tumor Inflammation Associated Neurotoxicity (TIAN)…………………......19

2. Materials and Methods…………………………………………………..23

2.1 Objective of the study……………………………………………….…………23

2.2 Clinical Trial Design……………………………………………………………23

2.3 T Cell Apheresis ………………………..……………………………………......25

2.4 Production and trasduction of T cells………………………………………………26

2.5 Lymphodepleting regimen and iC9-GD2-CAR-T cell infusion………………….26

2.6 Clinical trial eligibility and exclusion criteria…………………………….…..…..27

2.7 Clinical and laboratory evaluation of the patients…………………………......30

2.8 Disease assessment……………………………………………………………......30

2.9 Immune-monitoring………………………………………………………………....31

2.10 Toxicity assessment and management…………………………………………….31

2.11 Neuromonitoring……………………………………………………………….....35

2.12 Statistical Analysis………………………………………………………………...35

3. Results………………………………………………………………..36

3.1 Patient Characteristics……………..………………………………………...36

3.2 Manufacturing of GD2-CART01……………………………………….………...38

3.3 GD2-CART01 In Vivo Expansion and Persistence, cytokine profiling…………38

3.4 Safety of infusion……………………………………………………………..46

3.5 Radiological responses after GD2-CART01 Infusion and clinical outcome……57

4. Discussion…………………………………………………………….....59

5. Conclusion…………………………………………………………….....67

6. References…………………………………………………………….....68

1. Introduction

1.1 Central Nervous System Tumors

Central Nervous System (CNS) tumors are the most frequent solid tumors in childhood, accounting for 20-25% of cancers affecting children. Second in incidence only to leukemia, with an average annual age-adjusted incidence rate of 25.34 per 100,000 population between 2017 and 2021 (1). Most brain tumors occur before the age of 10, with a peak incidence around 5 years of age and a higher prevalence in males (2). Nevertheless, they can affect individuals of all age groups.

Pediatric CNS tumors are highly heterogeneous and differ from adult brain tumors both histologically and biologically (3). This difference, long recognized from histological and prognostic perspectives, now also finds molecular explanations. In the past 20 years, significant advancements have been made in the biological characterization of CNS tumors, thanks to next-generation sequencing techniques and methylation profiling analysis (4). These advancements have led to a radical revision of the classification of CNS tumors, moving from a purely histological definition of neoplasms to an integrated classification that consider molecular characteristics. This major shift allowed the 2021 World Health Organization (WHO) classification to define a clear distinction between pediatric and adult tumors (4).

Although significant progress has been made in treating these pathologies and understanding the molecular mechanisms underlying them, CNS tumors remain the leading cause of disease-related death in pediatric patients and morbidity among long-term survivors (1). The survival rate of patients with CNS tumors is globally around 70% (1). Many tumors, such as high-grade gliomas (HGG) and diffuse midline glioma (DMG) continue to have a poor prognosis, with 5-year overall survival less than 20% and 5%, respectively (2).

Overall, these findings underscore the need to identify innovative treatment strategies to improve the outcome of children affected by high-grade CNS tumors, sparing them from

the burden of long-term sequelae. Immunotherapies exploiting the immune system to attack cancer have become the focus of a wide stream of cancer research. The advances in cancer immunotherapy have improved outcomes for several human cancers, and in some cases, have produced dramatic responses in patients highly refractory to all conventional treatments (5). There are an increasing number of clinical trials with promising results for various oncological and hematological diseases (6–8). However, studies in the pediatric population are still limited, and those involving CNS tumors in children are even more scarce and heterogeneous (9–11).

1.2 Chimeric Antigen Receptor

Chimeric Antigen Receptors (CARs) are synthetic antigen receptors that can be transduced into a T lymphocyte to direct its cytotoxicity toward a specific tumor antigen. Unlike T-cell receptors (TCRs), CARs directly identify antigens expressed on the surface of tumor cells without the restriction of the Major Histocompatibility Complex (MHC), and they can recognize a variety of antigenic structures such as proteins, carbohydrates, and lipids (12). Originally devised by Eshhar et al. in 1989, CAR T-cell immunotherapy involves engineering T-cells to express a CAR resulting from the fusion of the variable regions of the heavy and light chains of a monoclonal antibody (mAb) with the cytotoxic zeta chain (ζ) of a TCR (figure 1) (13).

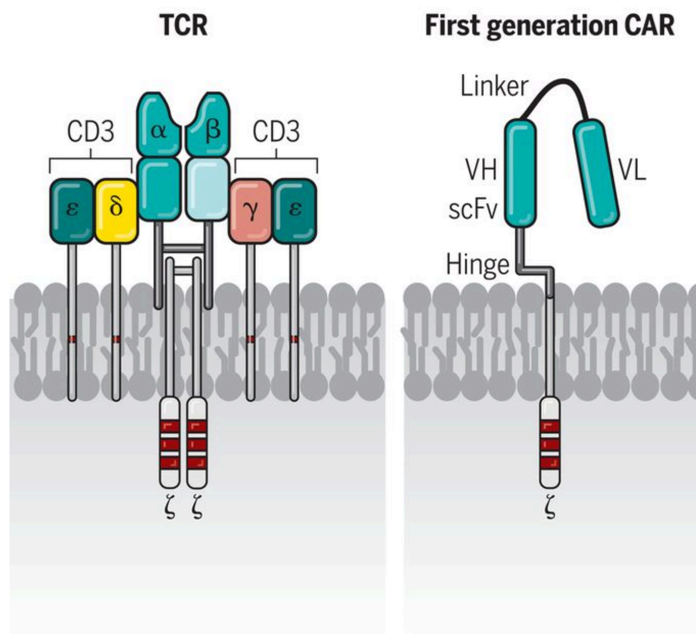

Figure 1. First generation CAR T.

Chimeric receptors are structured with three main domains: an extracellular domain for antigen recognition, a transmembrane domain to anchor the receptor to the cell membrane, and a cytoplasmic domain for intracellular signaling. The extracellular domain consists of two variable regions from a monoclonal antibody (VH from the heavy chain and VL from the light chain) connected by a flexible glycine-serine linker. The cytoplasmic domain includes an Immunoreceptor Tyrosine-based Activation Motif (ITAM) sequence that initiates T cell signaling upon ligand binding, leading to tumor cell lysis. When CARs engage their tumor-specific ligands, phosphorylation of the ITAM motifs triggers T cell activation, targeting tumor cells via perforin and granzyme. However, to ensure full CAR-T cell activation and proliferation, second-generation CARs (figure 2) incorporate costimulatory domains (e.g., CD28, OX40, 4-1BB) in addition to the CD3-ζ chain, allowing for both effective tumor cell killing and sustained CAR T-cell expansion (14–16).

However, it is well known that, in relation to solid tumors, second-generation CARs do not always expand adequately after infusion in patients. Additionally, it is well established that effective antitumor activity requires adequate expansion and persistence in vivo (17). Understanding how receptor structure affects these properties is therefore

crucial for future CAR T-cell designs. There is growing evidence of the primary role of T cell exhaustion in limiting the antitumor efficacy of the cells when exposed chronically to antigen (18,19). Recently, the central role of the CAR structure in promoting T cell activation and chronic exhaustion has been demonstrated, showing that CD28 costimulation increases, while 4-1BB costimulation reduces, exhaustion induced by persistent CAR signaling (20). In the search for an optimal CAR design, even more efficient constructs have been developed, containing two costimulatory domains (figure 2).

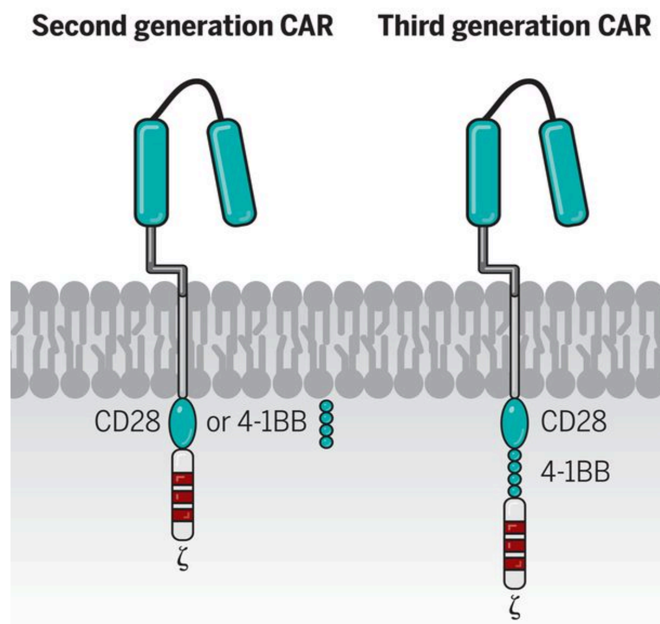

Figure 2. Second and third generation CAR T.

These so-called third-generation CARs provide more robust proliferation upon encountering tumor antigens compared to first- or second-generation CARs, and there is also greater resistance of T-cells to various tumor evasion strategies (21).

CAR design has undergone progressive refinement to enhance efficacy. Fourth-generation CARs also termed T-cells redirected for universal cytokine-mediated killing, build upon second-generation constructs by introducing an inducible transgenic protein,

like interleukin-12 (IL-12), to amplify antitumor effect (22). The fifth generation of CARs is currently under development, featuring a novel design based on second-generation constructs but with the addition of a truncated cytoplasmic receptor and a binding motif for transcription factors like STAT3/5 (23).

1.3 CAR T-cells in solid tumors

To effectively eradicate solid tumors, CAR T-cells must be able to migrate and infiltrate tumor tissues, proliferate, and persist long enough to exert therapeutic effects, selectively identifying and destroying cells that express the antigen within the tumor microenvironment. However, the ability of CAR T-cells to achieve these goals has been impeded by several factors that make solid tumors significantly different from hematological ones.

One of the greatest challenges for the successful application of CAR T technology to solid tumors is the selection of valid antigenic targets. Solid tumors present a variety of mutations that promote uncontrolled cellular growth and proliferation, leading to the creation of tumor epitopes that differ depending on the neoplasms and even within the same ones. As a result, solid tumors consist of highly heterogeneous cellular subpopulations from a molecular point of view, expressing different and overlapping profiles of unique tumor-associated antigens (TAAs) (24). Therefore, the current pool of potential antigens for CAR development in solid tumors consists of suboptimal targets that lack uniformity of expression or tumor exclusivity, introducing further biases for the efficacy and safety of this type of treatment. This molecular heterogeneity is difficult to replicate in preclinical studies on tumors derived from human cell lines, limiting the ability of these studies to faithfully mimic human disease and predict patient responses to treatment (25). Moreover, many of the potential targets for immunotherapy are also expressed in healthy tissues. To avoid attacking healthy tissues, CARs must ideally be designed exclusively against antigens expressed by neoplastic cells.

In addition to the challenge of identifying a specific antigen expressed only by neoplastic cells, another issue arises: the majority of mutations responsible for the antigenic

peculiarities of the neoplasm occur through the processing of intracellular proteins, which are presented in the context of major histocompatibility complex class I molecules (MHC-I) and are therefore inaccessible to CAR recognition. The identification of a tumor-specific antigen target is further complicated by the often inconsistent and incomplete expression of unique tumor epitopes throughout the tissue. Consequently, a subpopulation of neoplastic cells lacking the tumor antigen target will escape destruction by CAR T-cells (25–28). Targeting multiple TAAs through bi-specific CAR products has the potential to increase overall specificity for tumor cells, reduce the risk of off-target effects, and diminish the emergence of antigen-loss variants and tumors resistant to therapy (29).

Another obstacle to the effectiveness of CAR T immunotherapy in solid tumors is related to the profound immunosuppression induced by the tumor. Through the production of immunosuppressive cytokines and other soluble mediators, the recruitment of inhibitory leukocytes, and the activation of immune checkpoints, tumors create a microenvironment that inhibits effector cell activity and resists immune-mediated destruction. This multitude of immunosuppressive influences is responsible for the rapid loss of effector function observed after CAR T-cells enter tumor sites (30–32). Moreover, the presence of inhibitory cells within the tumor microenvironment, characterized by regulatory T lymphocytes (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs), suppresses the proliferation and function of antigen-specific cytotoxic T lymphocytes (CTLs) (33).

In contrast to the simplicity and ease of encountering neoplastic cells in hematological tumors, CAR T-cells for solid tumors face the additional challenge of having to migrate and infiltrate tumor sites. With a reduced ability to proliferate and a relatively short overall duration, CAR T immunotherapy in solid tumors becomes a "race against time," as the adoptively transferred cells have limited time to reach and destroy the neoplastic cells.

In summary, while CAR T-cell therapy holds great promise for solid tumors, the unique challenges posed by the tumor microenvironment, antigen heterogeneity, and immune

evasion mechanisms make it much more difficult to achieve the same success seen in blood cancers.

1.4 Limitations and Challenges of CAR T-Cell Therapy in CNS Tumors

CNS tumors present additional peculiarities that differentiate them from other types of solid neoplasms, both in terms of molecular characteristics and disease location. In recent years, our understanding of the molecular models of pediatric CNS tumors has increased, thanks to high-resolution genomic profiling, epigenetics, and transcriptomics, allowing for a more specific classification of these tumors (4). Interestingly, large-scale sequencing studies of pediatric tumors have identified new driver genetic mutations, but pediatric brain tumors generally exhibit few somatic mutations (34,35).

This low mutational burden is believed to be the reason for the lower immunogenicity shown by pediatric tumors compared to those in adults (36,37), as well as the less promising results obtained with checkpoint inhibitors (37). Additionally, some of the most clinically aggressive pediatric brain tumors, including DMG, HGG and medulloblastoma (MB), do not appear to have a highly immunosuppressive or inflammatory immune microenvironment, making them immunologically "cold" tumors (38,39).

There are other several specific challenges in treating CNS tumors with CAR T-cell immunotherapy, such as the peculiar location, the presence of the blood-brain barrier, and the risks associated with the onset of an inflammatory reaction in the CNS.

1.4.1 Trafficking through the blood-brain barrier

The blood-brain barrier (BBB) is a permeability barrier formed by tight junctions between endothelial cells that line the brain capillaries (40). Its primary function is to protect the brain from pathogens and maintain homeostasis (41,42). The mechanisms by which T cells penetrate brain tissue are complex. Resting T-cells do not cross the BBB; instead, they travel from meningeal blood vessels into the cerebrospinal fluid (CSF), where they access the brain via the pia mater or choroid plexus. In contrast, activated T-cells can cross the BBB's capillary tight junctions (43). There are three primary entry routes for

lymphocytes and antigen-presenting cells in the CNS: 1) through post-capillary venules into the perivascular space; 2) by extravasation through the choroid plexus into the CSF; or 3) via superficial leptomeningeal vessels into the subarachnoid space (figure 3) (44). These findings suggest that T-cells administered via systemic infusion can access tumors through the CSF and choroid plexus, challenging the notion of the brain as an immune sanctuary (45,46). This evidence supports promising results in neuroimmunology using CAR T-cells, vaccines, and other immunotherapies (46,47), which can target tumor tissue while sparing normal brain parenchyma. CAR T-cells are particularly appealing for brain tumors because they a) do not require a functional systemic immune response due to their intrinsic antitumor cytotoxicity, and b) their efficacy is not correlated with tumor mutational burden, relying solely on target antigen expression (27,46). Numerous trials with intravenously infused CD19-directed CAR T-cells have demonstrated their ability to cross the BBB, as they have been detected in the CSF through flow cytometry and immunofluorescence post-treatment, indicating their potential for neuro-oncology applications (40,46).

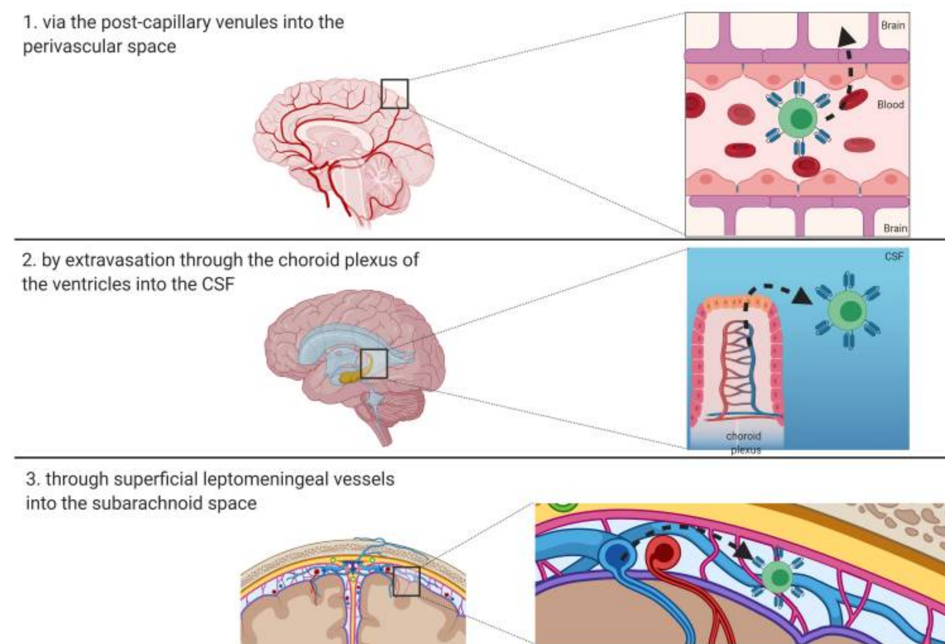

Figure 3. Route of entry of T-cells and CAR-T cells in the brain. 1) via the post-capillary venules into the perivascular space; 2) by extravasation through the choroid plexus of the ventricles into the CSF; 3) through superficial leptomeningeal vessels into the subarachnoid space.

1.4.2 Administration strategies for CAR T-cell immunotherapy in CNS tumors

Despite the established ability of intravenously (i.v.) administered CAR T-cells to cross the BBB, targeted delivery of T-cells to the CNS presents an attractive strategy to reduce systemic toxicity and enhance CAR T-cell homing and concentration at tumor sites. Alternative approaches for direct delivery of CAR T-cells to the CNS include spinal intrathecal infusion, intraventricular infusion, intra-tumoral injection, and disruption of the BBB using focal ultrasound.

Research using patient-derived orthotopic xenograft models showed that CAR T-cell trafficking into the tumor site is not affected by the route of administration. However, loco-regional delivery provides greater antitumor activity and lower systemic pro-inflammatory cytokine production in these models (48). Preclinical studies comparing delivery routes for glioblastoma (GBM) have demonstrated that local delivery of CAR T-cells is more effective than systemic delivery. Intratumor administered IL13Rα2-CAR T-cells resulted in long-term survival in orthotopic GBM models, whereas i.v. delivery provided no significant benefit over control (49). Furthermore, when comparing locoregional delivery routes in a multifocal GBM model, intraventricular infusion exhibited improved targeting of multifocal disease (49).

The optimal application route may depend on the molecular target. Antigens with broad expression in normal tissues (e.g., HER2, B7H3) may be associated with reduced toxicity after intracerebral administration (50). Current evidence supports loco-regional administration, as demonstrated by second-generation IL13Rα2-CAR T-cells, which are safe and well-tolerated with no significant toxicities (49,50). Conversely, systemic administration of HER2 CAR-T cells has been linked to severe pulmonary toxicities, while loco-regional administration showed safety in the BrainChild01 trial (10) such as B7H3 CAR T-cell (11). Moreover, in one trial involving GD2 CAR T-cells for pediatric and young adult DMG, intraventricular delivery resulted in lower systemic toxicity and higher cytokine levels compared to i.v. delivery (9).

The choice of delivery route may also be influenced by the type of cell product. While autologous CAR T-cells are currently the primary product used in brain tumors, other products, such as irradiated CAR-NK cells, are being explored. Although CAR-NK cells have lower toxicity, they face challenges like short lifespan and reduced in vivo cytotoxicity (51). Their characteristics may favor loco-regional delivery, although some clinical evidence has shown promise (52).

1.5 GD2 CAR T-cell immunotherapy in solid tumors and CNS

Disialoganglioside GD2 is a glycosphingolipid predominantly expressed on the surface of certain tumor cells, including neuroectodermal tumors like neuroblastoma and brain tumors. The expression of the disialoganglioside GD2 in normal tissues is primarily limited to the central nervous system, peripheral sensory nerve fibers, dermal melanocytes, lymphocytes, and mesenchymal stem cells, making it an attractive target for immunotherapeutic strategies aimed at treating brain tumors (53).

In cancer the disialoganglioside GD2 contributes to increased proliferation, motility, migration, adhesion, and invasion of cancer cells, depending on the type of tumor, and confers resistance to apoptosis (54).

GD2 is notably overexpressed in various tumors including neuroblastoma and brain tumors, such as high-grade gliomas, ependymomas, and medulloblastomas (55,56).

CAR T-cells targeting GD2 has shown extremely promising results in the treatment of patients with relapsed or refractory high-risk neuroblastoma. In our academic Phase I/II trial (NCT03373097), we observed an overall survival (OS) rate of 60% at three years among patients treated with the recommended dose. This treatment demonstrated a low toxicity profile and no long-term sequelae (7).

Moreover, CAR T-cell therapy targeting GD2 has shown promising results in preclinical models of brain tumors (55,56). Notably, Majzner et al. have demonstrated that intraventricular or intrathecal administration of GD2 CAR T-cells can lead to effective tumor control with minimal systemic toxicity, a crucial factor given the sensitivity of the CNS (9).

Table 1 provides an overview of all CAR T-cell trials for brain tumors in pediatric and young adult patients, while table 2 highlights the currently published clinical trial data.

Table 1. CAR T-cells clinical trial in pediatric patients and young adults

Study Number	Antigen target	Disease	Site	Status
NCT05298995	GD2	Recurrent and refractory pediatric and young adults brain tumors	Bambino Gesù Hospital and Research Institute, Rome	Recruiting
NCT06221553	B7H3 IL-7Ra	DMG	Chulalongkorn University	Recruiting
NCT04099797	C7R-GD2.CART	- Diffuse Intrinsic Pontine Glioma - High Grade Glioma - Embryonal Tumor - Ependymal tumor	Baylor College of Medicine	Recruiting
NCT04510051	IL13Ralpha2	Recurrent and refractory pediatric brain tumors	City of Hope Medical Center	Recruiting
NCT03170141	Antigen-specific IgT cells	GBM	Shenzhen Geno-Immune Medical Institute	Enrolling by invitation
NCT03500991	HER2	Recurrent and refractory pediatric brain tumors	Seattle Children's Hospital	Active, not recruiting
NCT03638167	EGFR806	Recurrent and refractory pediatric brain tumors	Seattle Children's Hospital	Active not recruiting
NCT04185038	B7-H3	Recurrent and refractory pediatric brain tumors	Seattle Children's Hospital	Recruiting
NCT02442297	HER-2	Recurrent and refractory pediatric brain tumors	Baylor College of Medicine	Active, not recruiting
NCT04196413	GD2	DMG	Stanford University	Recruiting
NCT01109095	HER.CAR CMV-specific CTLs	GBM	Baylor College of Medicine	Completed
NCT05768880	B7-H3, EGFR806, HER2, And IL13-Zetakine (Quad)	- DMG - Recurrent and refractory CNS Tumor	Seattle Children's Hospital	Recruiting

Table 2. Clinical trial data published on pediatrics patients affected by CNS tumors

Study number	NCT03500991	NCT04196413	NCT04185038
Author Years	Vitanza NA et al., 2021	Majzne RG et al., 2022	Vitanza NA et al., 2023
Number of patients	3 (children, young adults)	4 (children, young adults)	3 (children, young adults)
Brain tumors	1 anaplastic astrocytoma 2 ependymoma	DMG	DMG
Antigen target	HER2	GD2	B7H3
Construct	Second generation	Second generation	Second generation
Route of administration	Locoregional	Intravenously and locoregional for patients who exhibited clinical benefit.	Locoregional
Response	Not reported	Three of four patients exhibited clinical and radiographic improvement.	2 PD 1 PR through 12 months on study.
Toxicity	No associated dose-limiting toxic effects.	CRS and TIAN (reversible in all cases).	No associated dose-limiting toxic effects.

1.6 Toxicity

1.6.1 Cytokine Release Syndrome (CRS)

Cytokine Release Syndrome (CRS) is an acute systemic inflammatory response resulting from the rapid and excessive release of cytokines by immune cells.

Although the elevation of inflammatory cytokines is predictable and often produces mild flu-like symptoms, there have been reports of dysfunction followed by multiorgan failure and death.

The clinical manifestations of CRS are categorized as follows (figure 4):

- Constitutional: fever, malaise, anorexia, asthenia, myalgia, arthralgia, headache;
- Cutaneous: rash;
- Gastrointestinal: nausea, vomiting, diarrhea;
- Respiratory: tachypnea, hypoxemia, acute respiratory distress syndrome;
- Hepatic: hypertransaminasemia, hyperbilirubinemia, acute liver damage;
- Cardiovascular: tachycardia, hypertension, hypotension, increased cardiac output (early phase), decreased cardiac output (late phase), cardiac arrest;
- Coagulative: elevation of D-dimer, hypofibrinogenemia, bleeding, disseminated intravascular coagulation;
- Renal: acute kidney damage;
- Neurological: confusion, delirium, difficulty in speaking or frank aphasia, hallucinations, tremor, dysmetria, seizures.

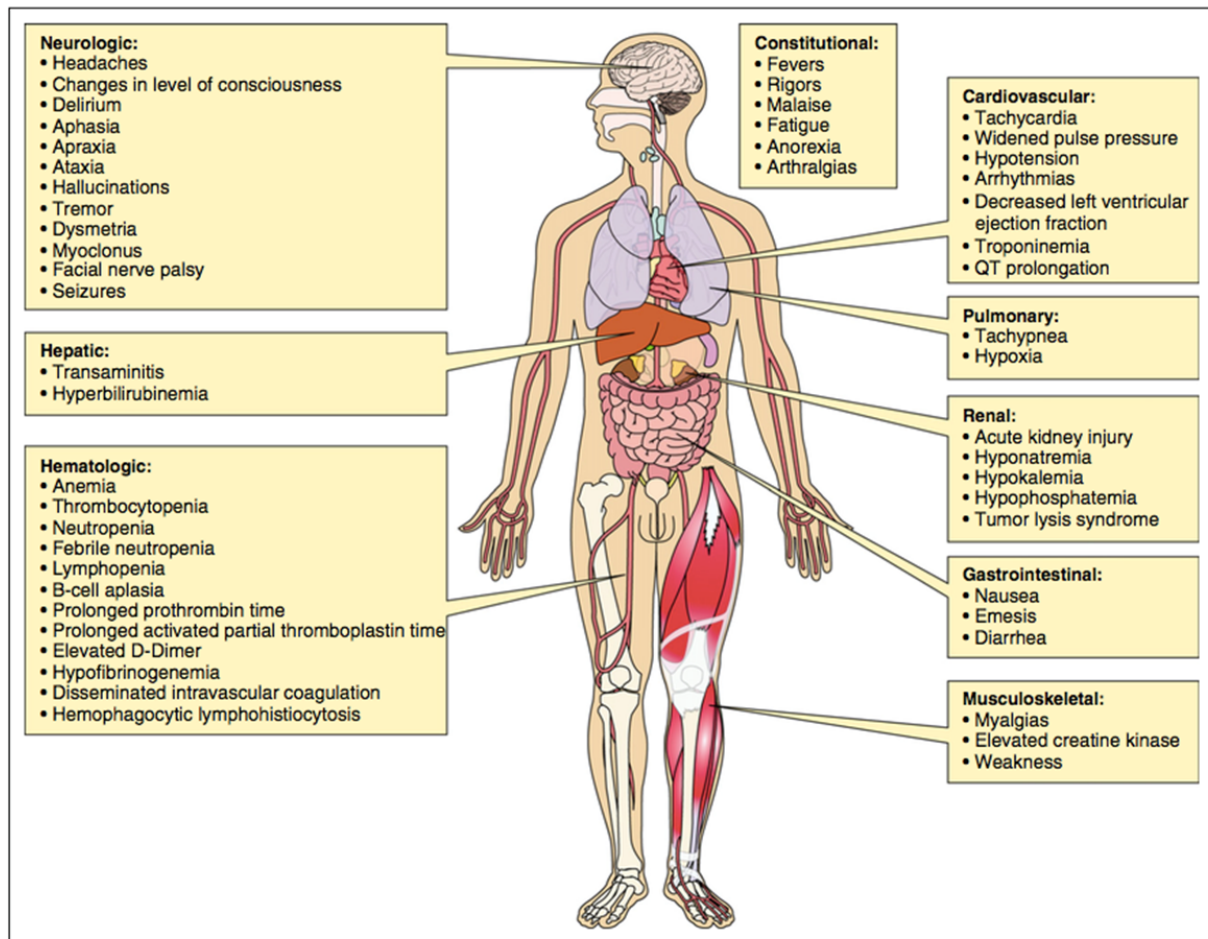

Figure 4. Manifestations of CRS

However, the above signs and symptoms are not pathognomonic for CRS. Therefore, absolute caution is necessary when diagnosing CRS, excluding all other possible causes of fever, hypotension, hemodynamic instability, and/or respiratory distress, such as a documented infection. A reasonable temporal relationship with cellular therapy must be present. Although CRS associated with effector immune cells may have a delayed onset, it rarely occurs more than 14 days after the start of therapy. Patients presenting symptoms consistent with CRS outside this time window should be carefully evaluated for other causes.

The diagnostic criteria for clinically significant CRS secondary to CAR T-cell immunotherapy are:

- Fever (>38°C) for at least three consecutive days;

- Two cytokines with a maximum fold change of 75 or one cytokine with a fold change of 250;
- At least one clinical sign of toxicity such as: a. hypotension (requiring at least one intravenous vasopressor) or, b. hypoxia (pO2 <90%) or, c. neurological disturbances (including changes in mental status, lethargy, and seizures).

However, proper management of CRS requires classification based on the severity of clinical manifestations:

- **Grade 1:** signs and symptoms not life-threatening (fever, nausea, fatigue, headache, myalgias, malaise) requiring only symptomatic treatment (antipyretics and antiemetics);
- **Grade 2**: signs and symptoms that require and respond to moderate intervention (oxygen therapy with an O2 requirement <40%, fluids, or low doses of vasopressors), organ toxicity of grade 2;
- **Grade 3:** signs and symptoms that require aggressive intervention (oxygen therapy with an O2 requirement of 40%, fluids, and high doses of vasopressors, immunosuppressants such as Tocilizumab - anti-IL6 antibody, or steroids), organ toxicity of grade 3 or grade 4 hypertransaminasemia;
- **Grade 4:** potentially lethal symptoms (need for ventilatory support, in conjunction with the above treatment for grade 3 CRS), organ toxicity of grade 4 (excluding hypertransaminasemia);
- **Grade 5:** patient death.

The management of CRS is based on the severity grade and is summarized in the figure 5.

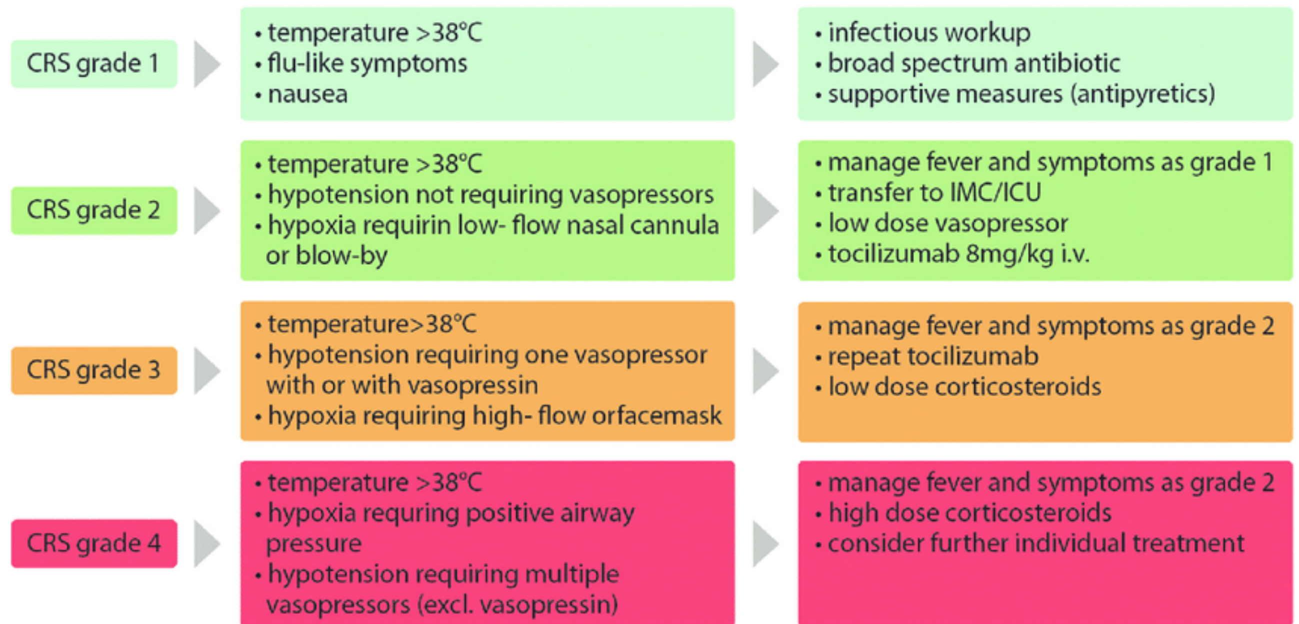

Figure 5. CRS management.

Despite the availability of multiple treatment modalities, CRS can still be fatal and must be recognized early. Any neurotoxicity that occurs concomitantly with or following the CRS period does not affect the grade of CRS but is considered separately in the neurotoxicity scale (57).

1.6.2 Immune effector cell-associated neurotoxicity syndrome (ICANS)

Neurotoxicity is another complication that has recently been defined as immune effector cell-associated neurotoxicity syndrome (ICANS) and is the second most common adverse event following CAR T-cell infusion. It is a disorder characterized by a pathological process involving the CNS following any immunotherapy that results in the activation or engagement of endogenous or infused T cells and/or other immune effector cells. The clinical manifestations are various, as toxicity does not affect a specific region of the CNS and include: encephalopathy (confusion or delirium), expressive aphasia or speech disturbances, motor weakness, myoclonus or tremor, headache, seizures, and altered consciousness. In rare cases, patients may rapidly develop diffuse cerebral edema. Expressive aphasia seems to be a typical symptom. It is initially characterized by object

naming difficulties, paraphasic errors, hesitant speech, and verbal perseveration, progressing to global aphasia, which is characterized by expressive and receptive difficulties. Patients with global aphasia may appear fully awake but are mute and unable to follow commands.

The onset of ICANS can vary from a few hours to three or four weeks after CAR T-cell infusion. ICANS is usually self-limiting, and most symptoms resolve within three or four weeks, with persistent abnormalities being uncommon. CRS may impact neurotoxicity and complicate its assessment.

In recent years, several scoring systems have been proposed for the assessment of neurotoxicity related to cell therapy that are summarized below (table 3) (57).

Table 3. Encephalopathy Assessment Tools for Grading of ICANS

<table>
<tr><th>CARTOX-10</th><th>ICE</th></tr>
<tr><td>• Spatial-temporal orientation (year, season, month, day, country, region, city, hospital…): 5 points</td><td>• Spatial-temporal orientation: 4 points</td></tr>
<tr><td>• Naming: ability to name 3 objects (e.g., point to a watch, pen, button): 3 points</td><td>• Naming: ability to name 3 objects (e.g., point to a watch, pen, button): 3 points</td></tr>
<tr><td>• Writing: ability to write a standard sentence (e.g., "Our national bird is the bald eagle"): 1 point</td><td>• Attention: ability to count backward from 100 to 10: 1 point</td></tr>
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<tr><td>• Attention: ability to count backward from 100 to 10: 1 point</td></tr>
</table>

In the classification system, the final grade of ICANS is determined by the most severe event among the various domains:

- **Grade 1:** defined as a score of 7 to 9 on the ICE assessment. A patient with grade 1 ICANS may present with a delay in response or disorientation regarding time or place, mild inactivity with difficulty counting backwards, or writing impairment. Drowsiness may be present, but the patient awakens spontaneously and, if prompted, should be able to complete most of the ICE assessment.
- **Grade 2:** defined as a score of 3 to 6 on the ICE assessment. Patients with grade 2 ICANS often exhibit some expressive and/or receptive aphasia, which limits their ability to communicate spontaneously. Additionally, early signs in grade 2 include paraphasic errors (production of unwanted syllables and words during attempts to speak) and verbal perseveration (repetition of the same words continuously). Patients may have a depressed level of consciousness but are responsive to voice, and responses may be delayed.
- **Grade 3:** defined by a score of 1-2 on the ICE assessment. Patients with grade 3 ICANS present with severe global aphasia and do not speak or follow commands even when fully awake and therefore may not be able to respond to any of the ICE questions. Alternatively, they may have excessive drowsiness and require tactile stimulation to pay attention to the examiner. Any clinical seizure, whether simple partial, complex partial, or generalized, and any electrographic seizure meet the criteria for grade 3 ICANS.
- **Grade 4:** defined by a score of 0 on the ICE assessment. Stupor and coma may be observed. Some patients may require intubation to protect the airway. Additionally, any patient presenting with prolonged or repetitive clinical or subclinical electrographic seizures without a return to baseline between episodes or who presents with deep focal motor weakness, such as hemiparesis or paraparesis, should be considered to have grade 4 ICANS. Patients with signs and symptoms of ICP, such as projectile vomiting with headache, depressed consciousness, cranial nerve VI palsy, papilledema, Cushing's triad (bradycardia, hypertension, and respiratory depression), decerebrate or decorticate

posture, and diffuse cerebral edema on imaging, are also considered to have grade 4 ICANS.

- o **Grade 5:** defined as death due to ICANS.

The treatment of ICANS is diversified based on the grade:

- ❖ **Grade 1 ICANS:** supportive care, IV hydration, neurological consultation, EEG/MRI, consider antiepileptic medication;
- ❖ **Grade 2 ICANS:** supportive care, consider transfer to intensive care, consider antiepileptic medication (if not yet started), low-dose corticosteroids;
- ❖ **Grade 3 ICANS:** supportive care, transfer to intensive care, continuous infusion of corticosteroids (e.g., dexamethasone 10 mg every 6 hours) and antiepileptic medications, repeat MRI;
- ❖ **Grade 4 ICANS:** supportive care, specific neuro-intensive treatment with high-dose corticosteroids (status epilepticus, cerebral edema), consider further individualized treatment (57).

1.6.3 Tumor Inflammation Associated Neurotoxicity (TIAN)

In brain tumor patients, neurotoxicity can be a more challenging side effect due to the location of the disease. Thus, treating brain tumors with CAR T-cells requires careful consideration of these specific toxicity scenarios. Tumor tissue infiltration by immune cells is an expected and desired outcome of treatment. In fact, a transient increase in tumor volume, a phenomenon known as pseudo-progression, has been widely reported with immunotherapies (58). The intracranial volume reserve (i.e., the capacity to tolerate an expanding mass within the skull) is limited, especially in patients with CNS malignancies, who already experience increased intracranial volume related to the tumor mass itself and surrounding edema. Unfortunately, the available literature provides limited data to accurately define the intracranial volume reserve in a specific patient. Post-mortem studies suggest it accounts for about 5% of total cranial volume in young adults, approximately 65-75 ml, with lower volumes in women (59). The volume reserve could be significantly lower in children and patients with pre-existing intracranial

lesions. Most of the compensatory volume, following the presence of a growing intracranial mass, is due to the redistribution of CSF. The capacity for intracranial pressure compensation depends not only on the volume itself but also on the volume-to-time change: a large, slow-growing mass is clinically much better tolerated than a smaller, fast-growing lesion.

The tumor's location warrants careful assessment when considering CAR T-cell therapy in brain tumors. The intracranial mass effect will be potentially more concerning for deep lesions (thalamic, midline, brainstem, and posterior cranial fossa tumors), where the risk of trans-falcine and transtentorial pressure peaks, leading to herniations and hydrocephalus, is higher (60) (figure 6).

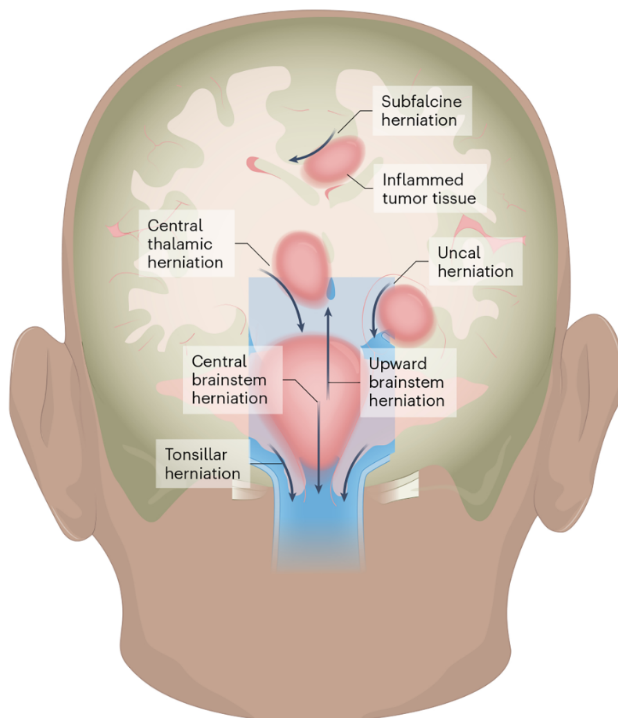


Figure 6. Herniation syndromes.

The ideal and opposite scenario would be one of leptomeningeal spread, with a lower potential for increased ICP development. While CNS inflammation risk also involves adults, it is a particular problem in pediatrics due to the different intracranial volume reserve. Specifically, in younger children, unclosed cranial sutures allow for greater reserve, which, on the one hand, offers better adaptation to increased intracranial pressure but, on the other hand, is responsible for the lack of symptoms associated with

the onset of intracranial hypertension. These factors make the identification of a more sophisticated and early monitoring system particularly important in pediatrics.

Two categories of TIAN are currently described. Type 1 TIAN primarily represents neurological symptoms and signs due to mechanical factors, such as elevated intracranial pressure (related to edema within the space constraints of the cranium). Type 2 TIAN primarily reflects local neural dysfunction. Distinguishing between these two categories is important for appropriate clinical management, but because the two mechanistic categories can be related, the grading system is singular (60).

TIAN grading scale is summarized below:

- **Grade 1:** Headaches associated with fevers or mild worsening of existing neurological clinical signs and symptoms from baseline, resulting in minor functional deficits for which only observation or symptomatic management is needed.
- **Grade 2:** Moderate changes in the neurological exam from baseline that substantially affect function
- **Grade 3:** Severe neurological clinical signs and symptoms that may affect critical cardiorespiratory functions or clinical signs and symptoms of increased intracranial pressure (>20 mmHg) that are responsive to intervention*
- **Grade 4:** Life-threatening, clinically significant elevated ICP (>20 mmHg) refractory to CSF drainage with no improvement in clinical symptoms in response to CSF drainage, possibly warranting urgent escalation of neurosurgical intervention (such as with emergent EVD or VPS placement)** or concerning clinical signs and symptoms of impending/early herniation or severe medullary dysfunction requiring endotracheal intubation for airway protection and/or mechanical ventilation (60).

*In patients with spinal cord tumors, grade 3 TIAN can occur when there is risk of debilitating loss of cord function.

**Emergently accessing an existing device for CSF drainage does not necessarily qualify as grade 4 TIAN, if such drainage successfully manages ICP.

Various strategies can be hypothesized to manage this potentially severe side effect of treatment. Despite the limited options for non-invasively determining compensatory CSF volume, evidence in the literature highlights the predictive value of invasive analysis of intracranial pressure waves. A specific wave morphology may suggest a reduction in compensatory volume, prompting appropriate therapeutic countermeasures (61).

A ventricular access device could be inserted before CAR T-cell infusion and used both to directly assess intracranial pressure waves and to remove certain volumes of CSF if appropriate. Moreover, when a high risk of decompensation secondary to CAR T-cell therapy is suspected, based on a significant mass effect of a CNS lesion, preventive surgical debulking may be an option in selected cases. Finally, at the extreme of aggressive ICP management, decompressive surgical procedures could be considered. However, as is well known from neurotrauma and malignant stroke literature, functional outcomes may be unacceptable, raising ethical concerns for invasive procedures in fragile patients with an overall poor prognosis (62,63).

Other strategies may be considered to reduce the risk of life-threatening severe intracranial pressure increases secondary to pseudo-progression. Sequential administration of low doses of CAR T-cells may result in less tumor infarction and a relatively slow, progressive lysis, reducing the risk of sudden intracranial hypertension. Additionally, the use of a different cell platform, with lower persistence over time and a reduced inflammatory profile upon activation, such as NK cells, could reduce the risks associated with the infusion of these cells. Similarly, the use of transiently expressed CAR T-cells, generated via RNA electroporation, could represent a viable risk mitigation strategy. Finally, the introduction of a suicide gene (inducible caspase 9 or Herpes Simplex Virus-1 thymidine kinase), capable of rapidly inducing apoptosis in CAR T-cells and mitigating inflammation and pseudo-progression, represents a significant enhancement of the safety profile of this novel therapeutic approach (64,65).

2. Materials and Methods

2.1 Objective of the study

The GD2CAR02 is a phase I, open label, non-randomized study. The primary objective of the phase I dose-finding study is to evaluate the safety and feasibility of intravenous injections of autologous iC9-GD2-CAR T-cells (GD2-CART01) in patients with refractory/relapsed malignant brain tumors. The secondary objectives of the protocol will be aimed at characterizing the kinetics of expansion and distribution of the infused GD2-CART01 both in peripheral blood (PB) and in LCR, document CRS features and obtain preliminary data on the efficacy of the treatment.

2.2 Clinical Trial Design

Considering the peculiar potential risks associated with the treatment of CNS tumors, the study has been designed to enroll patients in 3 different arms depending on the histology and location of the disease. This model of enrollment is aimed at testing the safety sequentially, starting from categories of patients at lower risk of severe intracranial hypertension first, and subsequently proceeding with patients at proportionally increased risk. This strategy will also allow defining possible different MTD/RDs depending on the diagnosis of the patient, thus providing a more tailored treatment with an optimized benefit/risk ratio for children with brain tumors. In particular, the three arms explored will be relapsed or refractory:

- Arm A: MB/other embryonal tumors
- Arm B: Hemispheric HGG
- Arm C: Thalamic HGG, DMG and other rare CNS tumors not included in arm A and B.

For each arm, the study design will consist of 5 dose levels (DL) to treat 3-6 patients each who will be treated following a 3+3 dose escalation/de-escalation schema. The 5 dose levels included in the present study are: 0.25 x 10^6 (DL1), 0.5 x 10^6 (DL2), 1.0 x 10^6 (DL3), 3.0 x 10^6 (DL4) and 6.0 x 10^6 (DL5) cells/kg recipient total body weight of transduced

(“bioactive”) CAR+ T-cells. The use of the escalation/de-escalation design is supported by the lack of effective therapies for patients with refractory/relapsed CNS tumors, recognizing that starting at sub-therapeutic dosing would very likely lead to a rapid progression of this aggressive disease and inability to determine if any efficacy was present.

The initial 3 subjects (DL3) will be enrolled sequentially, with a 4-week toxicity evaluation between each consecutive patient in this first cohort. The first patient of the next DL will be infused no sooner than 4 weeks after the last patient in DL3 has been treated. All subsequent patients in the dose escalation will be enrolled following the usual dose-finding 3+3 design: if none of the 3 patients in a cohort experiences a DLT, another 3 patients will be treated at the next higher DL. All patients within a DL need to complete a 4-week toxicity evaluation follow-up after the cell infusion for the assessment of DLT before enrolling a patient in the next higher DL. Except for the initial DL3, between consecutive patients in the same cohort a 2-week interval will be maintained for observation of the patient. If one of the patients experiences a DLT, 3 more patients need to be treated at the same DL. A maximum of 6 patients per DL will therefore be treated.

Clinical Trial will be carried out following a sequentially parallel enrollment for each arm. In details, when the enrollment of the DL3 cohort of patients of arm A will be concluded, in the absence of DLTs, a parallel enrollment will be enabled as follow: DL4 cohort of patients in arm A and DL3 cohort of patients in arm B. The same strategy will be applied for arm C, at the end of the enrollment of the DL3 of arm B.

The Clinical Study Team will review the emerging safety data from each arm and each cohort to determine if dose escalation will occur. Alternatively, an intermediate dose level between the non-tolerated dose level and the previously tolerated dose level may be explored and declared the MTD if < 2 out of 6 subjects experience a DLT at that dose.

DLTs are defined as any of the following toxicities occurring within 4 weeks of the infusion of the GD2-CART01 and not due to infection or to underlying malignancy:

- Grade 3 or 4 non-hematological toxicity attributable to GD2-CART01 and non-responsive to AP1903 infusions

• Grade 4 reactions related to infusion
• Death related to GD2-CART01 or to AP1903 infusions.

The following toxicities will not be considered DLTs:
• Abnormal electrolytes responding to supplementation
• Hypoalbuminemia
• Hypogammaglobulinemia
• Liver dysfunction resolving to grade ≤2 within 14 days
• Transient (<72 hours) grade 4 hepatic enzyme abnormality
• Grade 3 or 4 fever or neutropenic fever
• Grade 4 cytopenia following the lymphodepleting chemotherapy.

The MTD is defined as the highest dose level at which < 33% of patients (no more than 1 out of 6) experience DLT. The protocol may be amended to investigate an additional dose level if no DLT is observed in Phase I, or if DLT occurs at the actual DL1, upon competent authority approval of the amendment.

2.3 T Cell Apheresis

Leukapheresis will be performed using a cell separator (OPTIA SPECTRA from Terumo BCT), which utilizes a centrifugation system to obtain a buffy coat of mononuclear cells (MNC). The procedure involves the use of two venous access points for processing whole blood: peripheral venous access and/or a dual-lumen central venous catheter (CVC PROLINE). The collected whole blood is separated using a centrifuge, and the MNCs are collected using discontinuous phases.

The Sepax Cell Processing Instrument will be used to prepare starting material for the production of CAR T-cells. Sepax C-Pro can be part of a robust CAR T-cell manufacturing workflow that is adaptable for Good Manufacturing Practice (GMP) compliance in production of clinically relevant doses. In our process, Sepax can enrich mononuclear cells fraction via a density gradient medium. This procedure reduces also

the hematocrit of the sample by reducing the risk of erythrocyte lysis upon thawing and improving the culture conditions for CAR T generation.

2.4 Production and transduction of T cells

The starting material for GD2-CART01 production is a patient-derived leukapheresis collection processed through Sepax gradient mononuclear cell purification. This cell product will be shipped frozen to OPBG GMP manufacturing facility. Upon receipt and after verification of the acceptability of the starting material, the cell product will be transferred to the manufacturing cleanroom and processed. Then, an aliquot of $750x10^6$ cells will be further processed. The cells are washed and treated with CD3+/CD28+ antibodies, in the presence of IL7 (10 ng/mL) and IL15 (5 ng/mL). After 24hrs, stimulated cells will be exposed to the clinical released OPBG-91.7 retroviral virus. Post-transduction, the cells are cultured in vitro until day+15 (±2 days), then the product will be frozen to allow the completion of tests for the release.

Patient-specific GD2-CART01 lot production must meet the following criteria to be released under GMP regulation for human use: vitality >80%, CD3+ cells >80%, CD3+ CAR+ cells ≥20%, CD3+ CAR+ antitumor activity >60% in functional co-culture assay at an Effector: Target ratio 1:1, apoptotic CAR+ cells upon AP1903 exposition >20%, RCR negativity, Vector Copy Number ≤10, sterility, endotoxin free.

2.5 Lymphodepleting regimen and iC9-GD2-CAR T-cell infusion

Patients will receive a lymphodepleting regimen before T cell infusion. They will be given Fludarabine 25 mg/m^2 per day on days -5, -4 and -3 + Cyclophosphamide 500 mg/m^2 per day on days -5, -4 and -3.

T-cell infusion will be performed i.v. over 10-30 minutes on day 0. A 7-day delay will be permitted for resolution of intercurrent clinical conditions. The cell product will be thawed in a 37±2 °C water bath and administered over 10-30 minutes through either a peripheral or a central line. The patient will be pretreated with paracetamol and chlorphenamine according to institutional standards.

Monitoring during infusion will follow institutional standards for administration of blood products.

2.6 Clinical trial eligibility and exclusion criteria

Procurement eligibility

Inclusion Criteria

All the patients must meet the following eligibility inclusion criteria at the time of procurement.

1. Histological diagnosis of relapsed/refractory CNS tumors, including:

a. Medulloblastoma (MB)/other embryonal tumor (arm A)

b. Hemispheric high-grade glioma (HGG) (arm B)

c. Thalamic HGG, Diffuse Midline Glioma (DMG) and other rare CNS tumors not included in arm A and B (arm C)

2. Eligibility according to GD2 expression:

- GD2-positivity: the patient will be considered eligible and will be enrolled in the present protocol since there is not any other effective treatment to be explored

- GD2-negativity: the patient will be considered not eligible for the treatment: an alternative treatment of rescue, whenever possible, or palliation will be proposed to the patient in this case

- Impossibility of obtaining tumor samples; the patient will be considered eligible and will be enrolled in the present protocol since there is not any other effective treatment to be explored, considering the available scientific data published on GD2 expression for the majority of highly malignant brain tumors.

3. Age: 6 months – 30 years

4. Adequate venous access for apheresis or eligible for appropriate catheter placement, and no other contraindications for leukapheresis

5. Written and signed informed consent from patients, parents or legal guardians. For subjects < 18 years old their legal guardian must give informed consent. In addition,

pediatric subjects will be included in age-appropriate discussion and written informed assent will be obtained for those greater than or equal to 7 years of age, when appropriate

6. Karnofsky/Lansky ≥ 60

<u>Exclusion Criteria</u>

1. Severe, uncontrolled active infections
2. HIV or active HCV and/or HBV infection
3. Concurrent or recent prior therapies, before apheresis:

a. If receiving glucocorticoids, patient must be on a stable or weaning dose for at least 7 days prior to apheresis. Recent or current use of inhaled/topical/non-absorbable steroids is not exclusionary. Subjects receiving steroid therapy at physiologic replacement doses only are allowed provided there has been no increase in dose for at least 2 weeks prior to starting apheresis

b. Systemic chemotherapy in the 3 weeks preceding apheresis collection

c. Immunosuppressive agents in the 2 weeks preceding apheresis collection

d. Radiation therapy must have been completed at least 6 weeks prior to apheresis

Treatment eligibility

<u>Inclusion criteria</u>

1. Imaging assessments performed within 14 days of start of treatment
2. Age: 6 months – 30 years
3. Measurable or evaluable disease on at least 2 dimensions on Magnetic Resonance Imaging (MRI) at the time of treatment enrollment
4. Karnofsky/Lansky ≥ 60
5. Recover from the toxic effects of previous radiation and chemotherapies: grade 4 and or 3 non-hematologic toxicities must have resolved to grade ≤ 2; in presence of chronic complications (e.g., treatment-associated thrombocytopenia), patient must be clinically stable, according to the opinion of the treating physicians, and meet all other eligibility criteria

6. Positioning of an implantable intraventricular access device
7. Written and signed informed consent from patients, parents or legal guardians. For subjects < 18 years old their legal guardian must give informed consent. In addition, pediatric subjects will be included in age-appropriate discussion and written informed assent will be obtained for those greater than or equal to 7 years of age, when appropriate
8. Patients of childbearing or child-fathering potential must be willing to practice birth control from the time of enrollment on this study and for six months after receiving the preparative regimen
9. Females of childbearing potential must have a negative pregnancy test because of the potentially dangerous effects on the fetus

<u>Exclusion criteria</u>

1. Pregnant or lactating women
2. Severe, uncontrolled active infections
3. HIV or active HCV and/or HBV infection
4. Rapidly progressive disease with life expectancy < 6 weeks
5. History of grade 3 or 4 hypersensitivity to murine protein-containing products
6. Hepatic function: inadequate liver function defined as total bilirubin > 4x upper limit of normal (ULN) or transaminase (ALT and AST) > 6 x ULN based on age and laboratory specific normal ranges
7. Renal function: serum creatinine > 3x ULN for age
8. Blood oxygen saturation < 90%
9. Cardiac function: left ventricular ejection fraction lower than 45% by ECHO
10. Bone marrow function: absolute neutrophils count (ANC) lower than 500/mm3 and/or platelets lower than 20.000 (not reached by transfusion)
11. Congestive heart failure, cardiac arrhythmia, psychiatric illness, or social situations that would limit compliance with study requirements or in the opinion of the principal investigator (PI) would pose an unacceptable risk to the subject
12. Concurrent or recent prior therapies, before infusion:

a. If receiving glucocorticoids, patient must be on a stable or weaning dose for at least 7 days prior to infusion. Recent or current use of inhaled/topical/non-absorbable steroids is not exclusionary. Subjects receiving steroid therapy at physiologic replacement doses only are allowed provided there has been no increase in dose for at least 2 weeks prior to starting apheresis

b. Systemic chemotherapy in the 3 weeks preceding infusion

c. Immunosuppressive agents less than or equal to 30 days

d. Radiation therapy must have been completed at least 6 weeks prior to enrollment

e. Other anti-neoplastic investigational agents currently or within 30 days prior to start of protocol therapy

13. Patient-derived GD2-CART01 production failure: vitality <80%, CD3+ cells <80%, CD3+ CAR+ cells <20%, CD3+ CAR+ antitumor activity <60% in functional co-culture assay at an Effector: Target ratio 1:1, viable CAR+ cells upon AP1903 exposition >20%, Replication Competent Retrovirus (RCR) positivity, Vector Copy Number >10, non-sterility, endotoxin contamination (> 1 EU/ml).

2.7 Clinical and laboratory evaluation of the patients

Clinical and laboratory testing, including immune-monitoring, were performed before infusion, on day 0, day 3-5 and at weeks 1, 2, 4, 6, 8, 10 and 12 after infusion; subsequent evaluation was performed every 3 months for the first year and then every 6 months until 18 months and then yearly.

2.8 Disease assessment

Disease assessment will be performed according to RAPNO (Response Assessment in Pediatric Neuro-Oncology) criteria (66,67).

Radiological investigations will be undertaken for disease evaluation of the primary lesion, the metastatic sites (target and non-target lesions) and the index lesions to determine the tumor burden.

Brain tumor assessments will be conducted by brain and spine MRI with and without contrast (e.g., gadolinium), using standard MRI sequences. The neuro-radiologist may select the appropriate sequence that best highlights the tumor. The tumor burden will be recorded and evaluated according to the most recently updated criteria based on the primary tumor.

Four RAPNO working groups (DMG, MB and other leptomeningeal-seeding tumors and HGG) were specifically formed to address the several issues intrinsic to pediatric tumors and to develop a consensus on recommendations for response assessment that can be prospectively evaluated in clinical trials. For other rare brain tumor radiological assessment will be performed according to our Institutional standard practice.

Tumor response is categorized into Complete Response (CR), Partial Response (PR), Stable Disease (SD), and Progressive Disease (PD), based on predefined thresholds for changes in tumor size and patient symptoms.

2.9 Immune-monitoring

Flow-cytometry and immune-monitoring after infusion Flow-cytometry acquisition will be performed with a BD LSR FortessaX20 (BD Biosciences). Data analysis will be performed using FACs DIVA 8.0.1. Percentage of transduced cells and detection of GD2-CART01 after infusion will be determined by staining with a specific anti-idiotype antibody (1A7), as previously described (20). The following antibodies will be used for phenotypic analysis of GD2-CART01: CD45, CD3, CD4, CD8, CD56, CD45Ro, CCR7, PD-1, TIM-3, LAG-3, CD69 (BD Bioscience, USA). Healthy donor peripheral blood will be used to verify expression thresholds and gates, as well as isotype antibodies to set the fluorescence gating for each fluorochrome. To assess GD2 expression on LCR and tumor biopsies, the anti-GD2 antibody, clone 14G2a (BD Bioscience, USA) will be used.

2.10 Toxicity assessment and management

We will collect PB and LCR before and after infusion of CAR GD2 T-cells for cytokine monitoring and CAR T detection at different timepoints, and continue to clinically

monitor subjects, admitted as inpatients, for at least the first 2 weeks after infusion. Subsequently, patients will be monitored by physical examination (including detailed neurological assessment) and blood tests in the outpatients setting by their treating physicians for evidence of incipient "cytokine storm" or neurotoxicity and treated promptly.

Management of CRS is strictly dependent on the grading score (figure 7).

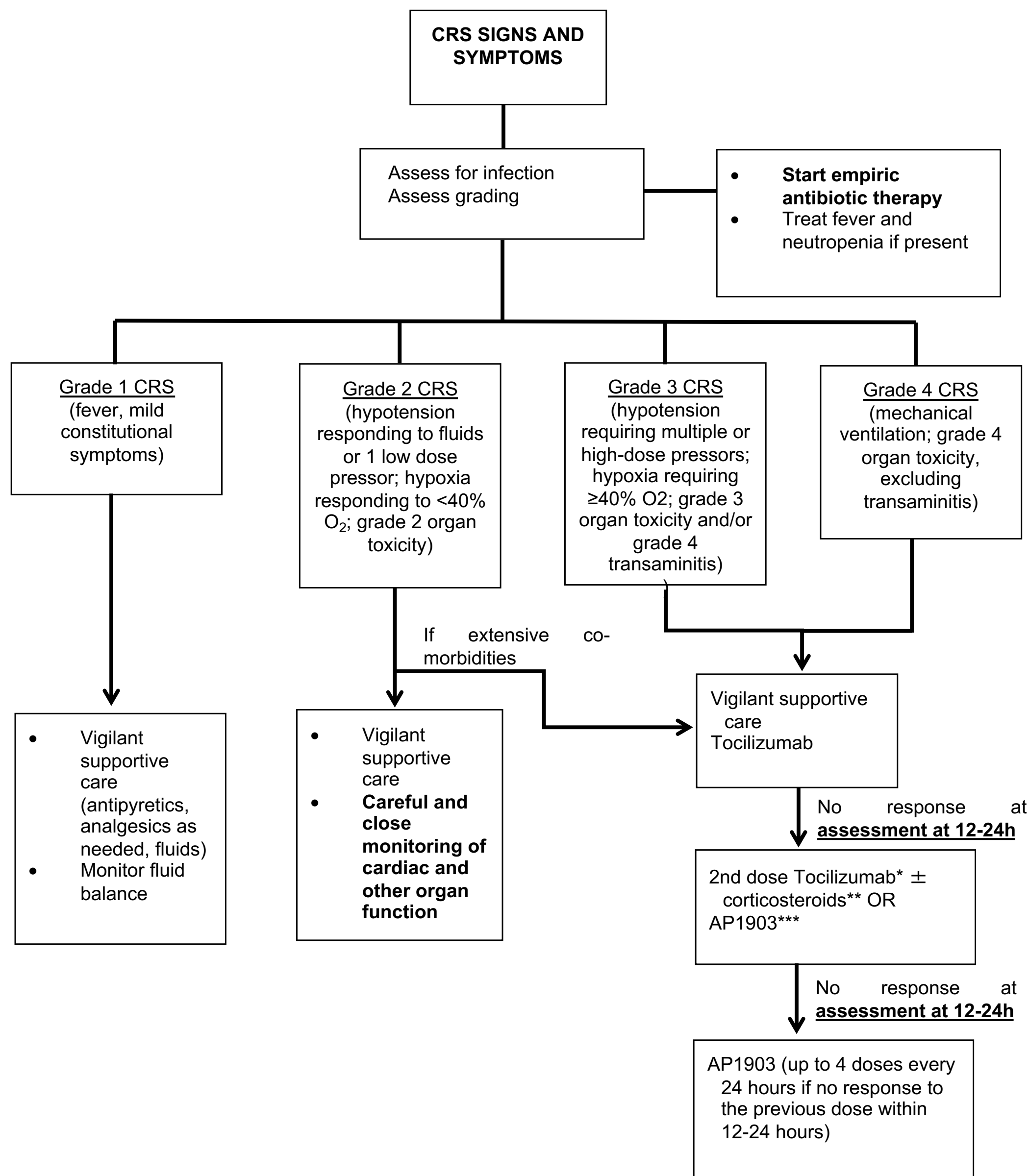


Figure 7. Management of CRS

The treatment of the syndrome includes supportive care for the symptomatic treatment (oxygen, i.v. fluids, inotropic agents), immunosuppressive agents (IL-6 receptor antibody Tocilizumab, 4-8- mg/kg; IL-1 receptor antibody Anakinra, 5-10 mg/kg, dexamethasone, 0.1-0.2 mg/kg/day) and/or AP1903, 0,4 mg/kg.

We recommend infusion of AP 1903 in the following conditions:

Severe CRS (grade 3-4) that worsen within the first 12-24 hours after Tocilizumab or Anakinra (± corticosteroids);

Severe CRS (grade 3-4) not fully controlled after a second infusion of Tocilizumab or Anakinra (± corticosteroids).

The response will be assessed at 12-24 hours. If there is no response or worsening of the CRS, then up to 3 additional doses of AP1903 may be given at 24 hours intervals.

Regardless of the treatment, patients will receive close clinical monitoring and a cytokine profiling every other day until complete resolution of the symptoms.

Within 4 hours prior to initiation of AP1903 infusion and at 24, 72 and 96 hours after initiation of infusion of each dose of AP1903, research blood samples will be taken to analyze the effects of the dimerizer drug on circulating gene modified T cells and on the cytokine profile.

The management of ICANS based on severity grade is summarized below (table 4).

Table 4. ICANS consensus grading for children and management

ICANS grade 1	-Awakens spontaneously -fatigue -ICE: 2-9 points	-Supportive care -IV hydratation -neurology consultation -EEG/MRI -consider antiepileptic drug
ICANS grade 2	-Awakens to voice -delirium/somnolence -ICE: 3-6 points	-supportive care as grade 1 -consider ICU transfer -consider antiepileptic drug, if not started -low dose corticosteroids
ICANS grade 3	-Awakens to tactile stimulus -ICE: 0-2 points -local edema on imaging -seizure, that resolves with intervention	-supportive care as grade 2 -ICU transfer -continous corticosteroids /I.e dexamethasone 10 mg every 6 hours) and antiepileptic drugs -repeat MRI
ICANS grade 4	-Comatose -ICE: 0 -Cerebral edema -Life threatening (>5 min) seizure -motor weakness	-supportive care as grade 3 -high dose corticosteroids specific neurointensive treatment (status epilepticus, brain edema) *consider further individual treatment

2.11 Neuromonitoring

Monitoring intracranial pressure via a telemetric device allows to assess real-time changes in brain pressure, crucial for detecting early signs of cerebral edema, elevated ICP, or other neurological complications. For all patients enrolled in the clinical trial a ventricular catheter connected to a Rickham reservoir and a Miethke M.scio flat telemetric ICP monitoring device will be implanted prior to the infusion. ICP will be monitored at least six times daily or according to clinical needed.

Intracranial hypertension will be treated according to our Institutional standard practice.

2.12 Statistical Analysis

All the characteristics of the patients and trial outcomes were described with usual summary statistics (i.e., counts and percentages for categorical factors and the mean, median, or range for continuous factors).

Student t-test was used to compare CAR T-cells and cytokine levels between different dose levels and arms of treatment. Student t test was conducted as a two-sided unpaired test with a confidence interval of 95%.

Data were visualized and analyzed using GraphPad Prism software.

3. Results

3.1 Patient Characteristics

Between November 2023 and October 2024, 12 patients met the eligibility criteria and were enrolled in the GD2CAR02 trial. Of these, two patients did not receive the product due to advanced progressive disease with a high disease burden. Ten patients remained eligible for treatment, and to date, eight of them have received the experimental product (figure 8).

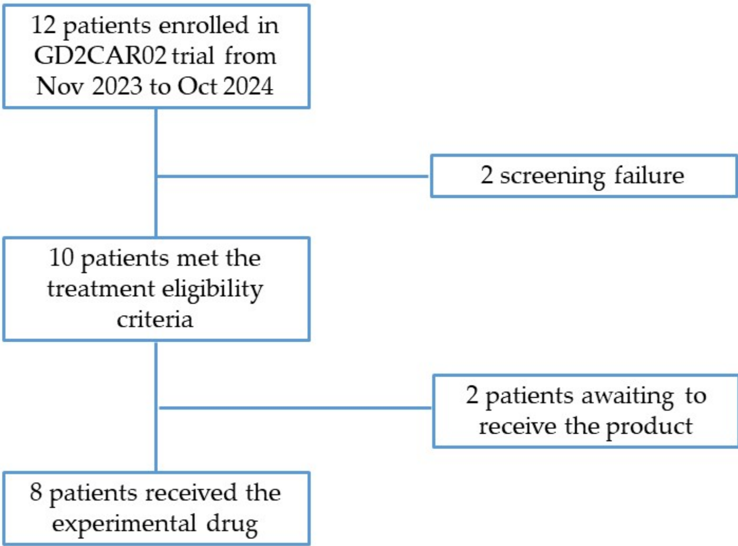


Figure 8. CONSORT diagram to describe the patient flow

The baseline characteristics of the treated patients are shown in table 5.

Table 5. Characteristics of the patients at baseline

Pt ID	Sex	Age (y)	Disease	Arm	Disease status at infusion	Neurological examination	Dose Level	Treatment lines pre-infusion	Time to diagnosis (y)
GD2CNS-OPBG-001	F	23	MB classic type G4	A	Two lesion site: surgical tumor bed and intraventricular	Negative	DL3	4	9,73
GD2CNS-OPBG-002	F	10	AT/RT	A	Multiple lesions: Linear and nodular leptomeningeal involvement in intracranial and perimedullary regions, ventricular areas, and larger nodules in the subtentorial region	Negative	DL3	1	1,37
GD2CNS-OPBG-003	M	13	MB classic type G4	A	Multiple lesions: Linear and nodular leptomeningeal involvement in supratentorial and infratentorial regions	Slowed motor function and speech, ocular convergence deficit, dysmetria, tremor in voluntary movements of the right upper limb. Weakness in the right upper limb and limited movement in the lower limbs, with hyperactive tendon reflexes in the right lower limb.	DL3	4	6,09
GD2CNS-OPBG-005	M	21	HGG with ZMIZ1:RET fusion	B	Multiple lesions: Supratentorial and infratentorial leptomeningeal involvement, ventricular and perimedullary lesions	Independent, ataxic gait. Right-sided hemiparesis.	DL3	3	2,88
GD2CNS-OPBG-006	F	6	NBL FOXR2	A	One site: Infundibular recess of the third ventricle	Left pyramidal syndrome. Nystagmus. Anisocoria (left > right). Left-sided hemianopsia and temporal field loss. Left-sided hemiparesis, hyperactive tendon reflexes on the left side. Dysarthria.	DL4	2	2,41
GD2CNS-OPBG-007	M	16	MB classic type G4	A	Two lesion site: peri/intraventricular	Convergent strabismus in the left eye and mild deficit of the left facial nerve.	DL4	3	3,95
GD2CNS-OPBG-009	F	15	HGG H3G34	B	One site: Left frontal and temporal site	Mild right hemiparesis	DL3	3	2,64
GD2CNS-OPBG-010	M	6	MB classic type G4	A	Two lesions: Linear and nodular leptomeningeal involvement in supratentorial and perimedullary regions	Negative	DL4	3	3,76

3.2 Manufacturing of GD2-CART01

GD2-CART01 was manufactured successfully in all the patients. The median total number of cell yield was 4.6 10^9 cells (range, 2.63-5.03) with a median viability of 92.15% (range, 90-95.1) and a median transduction efficiency of 62.1% (range, 47.7-71.8). In addition, the median cytotoxicity was 95.65% (range 81.5-99.3) and median vector copy number 1.9 copies per cells (range 1.1-2.6), significantly lower than the limit of 10 allowed for release.

Details regarding the drug products are provided in figure 9.

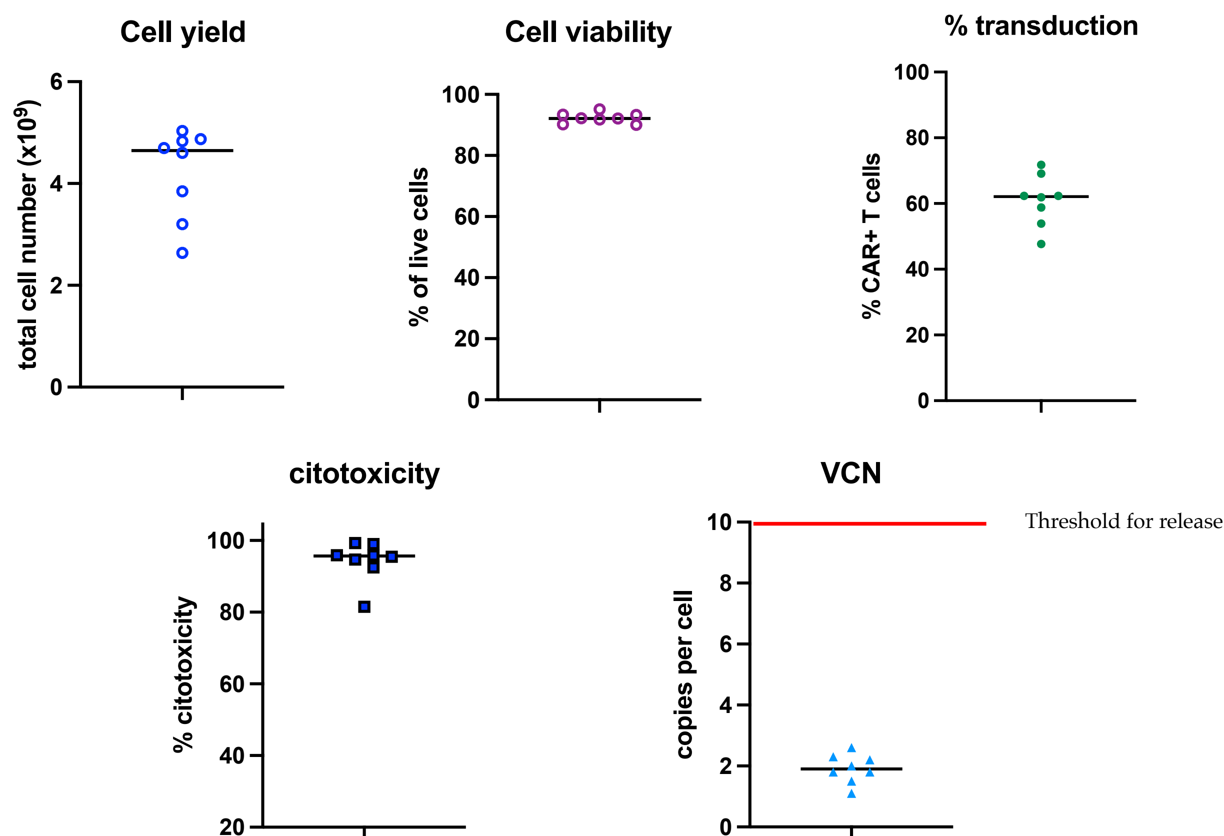


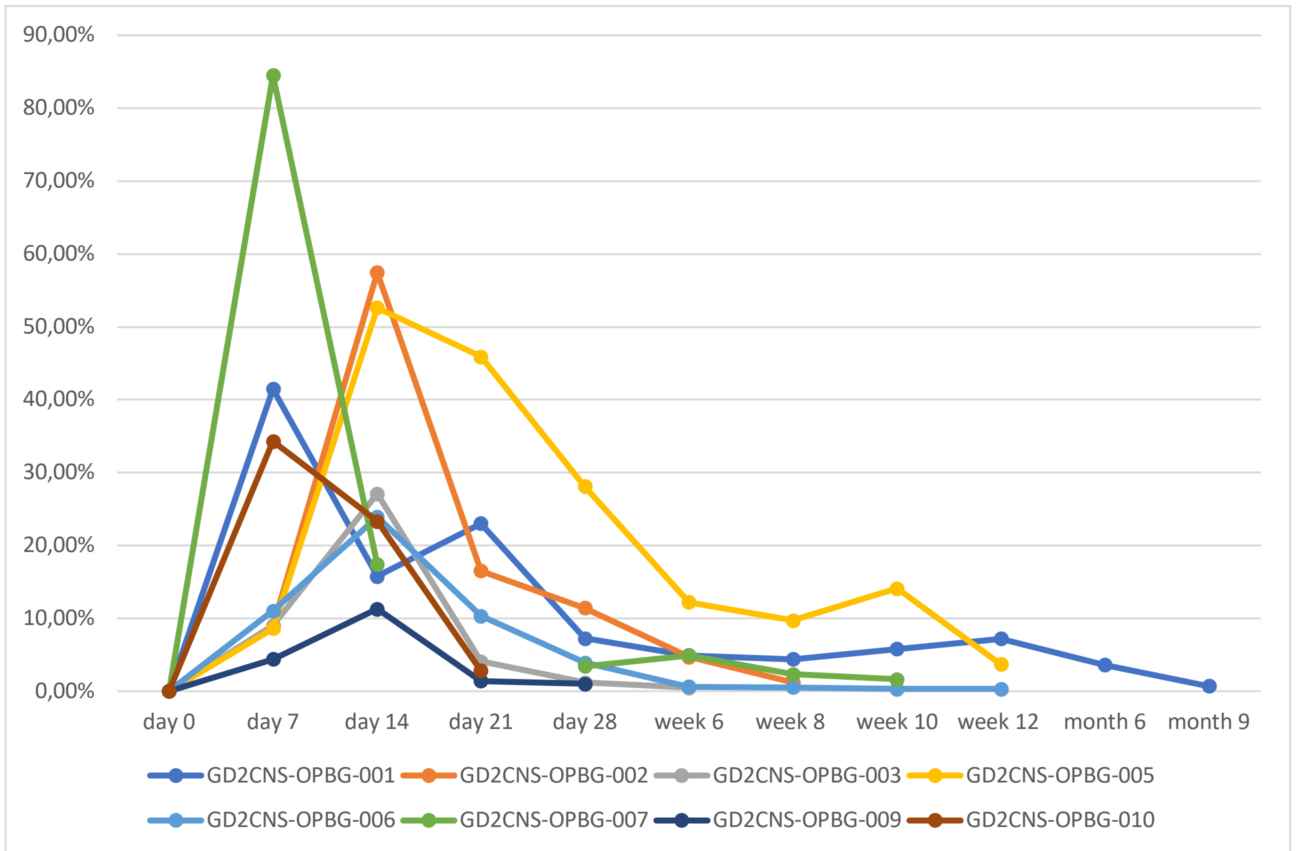
Figure 9. Cell product characteristics

3.3 GD2-CART01 In Vivo Expansion and Persistence, Cytokine Profiling

GD2-CART01 cells were detected in PB and CSF by means of flow cytometry in all patients.

The trend of CAR T-cell expansion at different time-points for each patient on PB (A) and CSF (B) is show in figure 10.

A.



B.

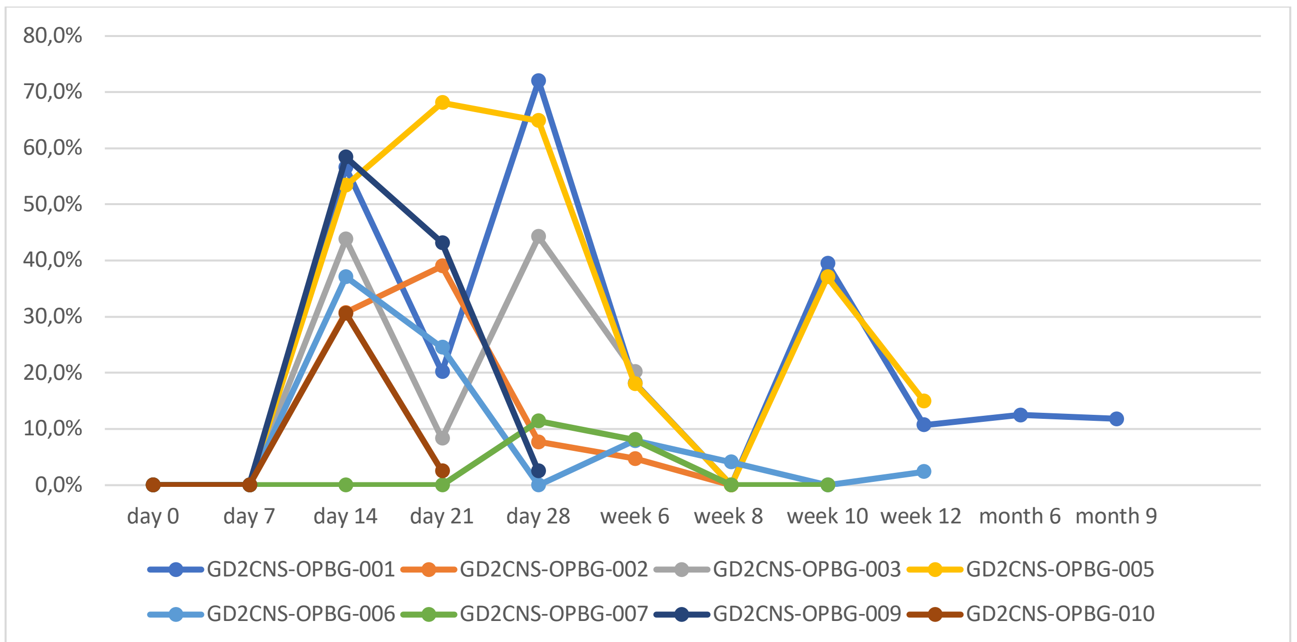
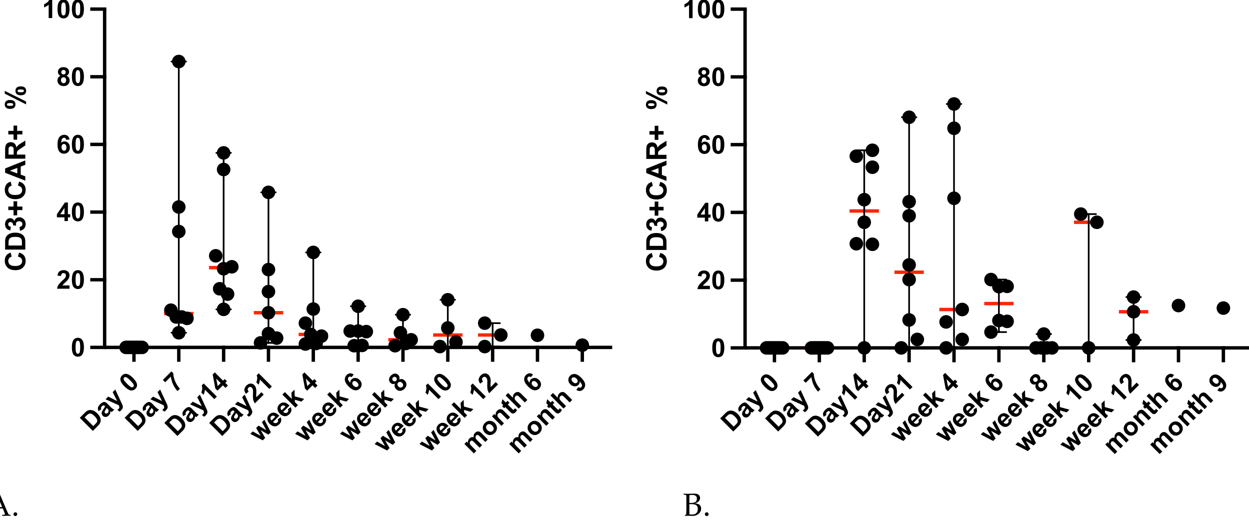


Figure 10. Trend of CAR T-cell expansion for each patient on PB (A) and CSF (B)

The median expansion of CAR T-cells on PB (A) and CSF (B) for each time-point is show in figure 11.



A. B.

Figure 11. Median expansion of CAR T-cells on PB (A) and CSF (B) for each time-point

Overall, PB and CSF CAR T peak expansion was observed in the second week after infusion on flow cytometry.

Despite the study being ongoing for a relatively short time, in all treated patients there were still detectable CAR T-cells on both blood and CSF at last follow-up.

The correlation between the dose levels tested and GD2-CART01 level in PB (A) and LCR (B), evaluated by flow cytometry, is show in figure 12.

A. B.

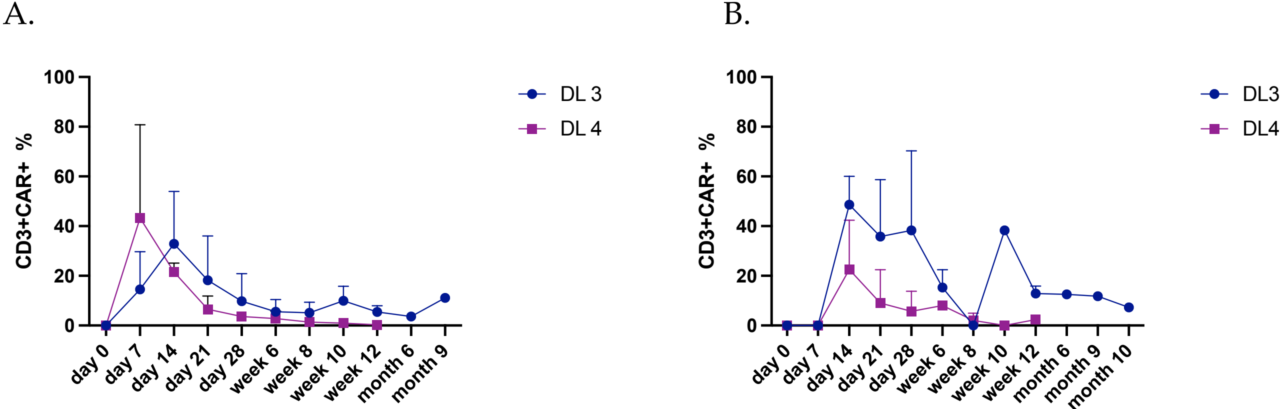


Figure 12. CAR T-cell expansion in relation to dose level

The correlation between the arm of enrollment and the GD2-CART01 level in PB (A) and LCR (B), evaluated by flow cytometry, is show in figure 13.

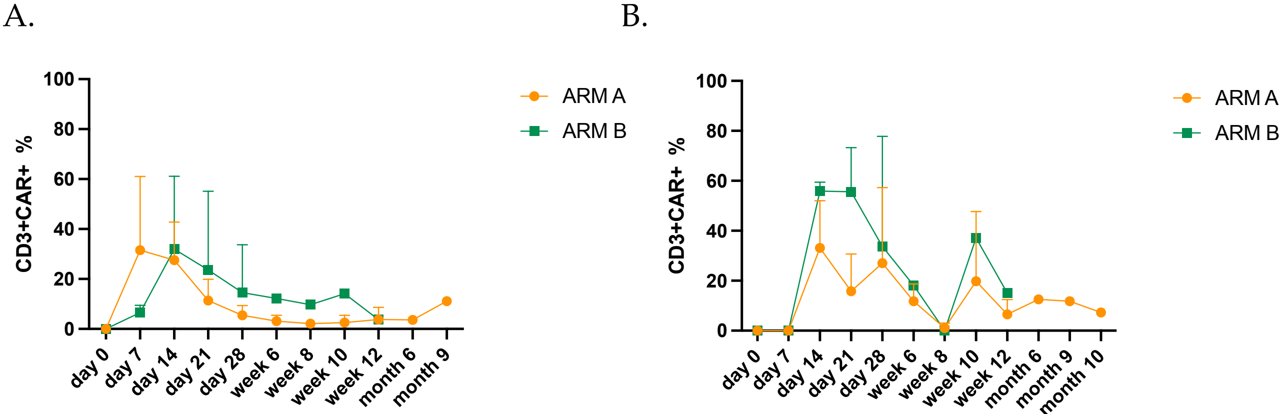


Figure 13. CAR T-cell expansion in relation to arm of treatment.

There was no significant difference in expansion between the two different dose levels tested (DL3: 1 million CAR+ T-cell/kg and DL4: 3 million CAR+ T-cell /kg), nor between arm A (MB or other embryonal tumors) and B (hemispheric HGG). However, it appears that the patients in arm B had greater expansion.

After expansion, a predominance of CD8-positive CAR-positive cells was detected in peripheral blood and was maintained at all time points, although CD4-positive CAR-positive cells expanded and were detectable as well with maximum peak at week 4 post infusion (figure 14).

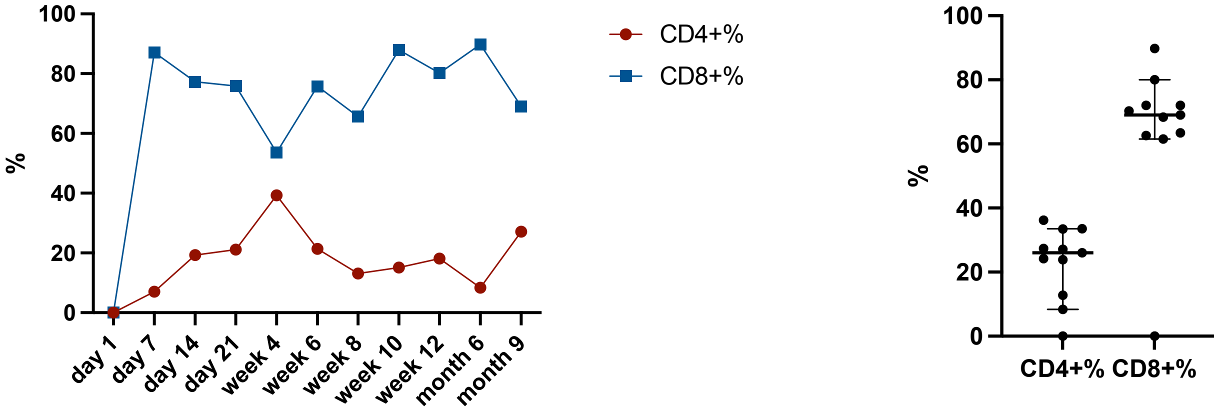


Figure 14. Distribution of CD4+CAR+ cells and CD8+CAR+ cells

Finally, we evaluated the kinetics of GD2-CART01 over time in one patient who received Rimiducid. We observed a reduction of half circulating GD2-CART01 3 hours after infusion of the dimerizing agent, with maximum reduction observed at 24 hours. Since then, circulating CAR T-cell levels have remained stable up to week 12 post-infusion, within a range of 10-15% CD3+CAR+ (figure 15).

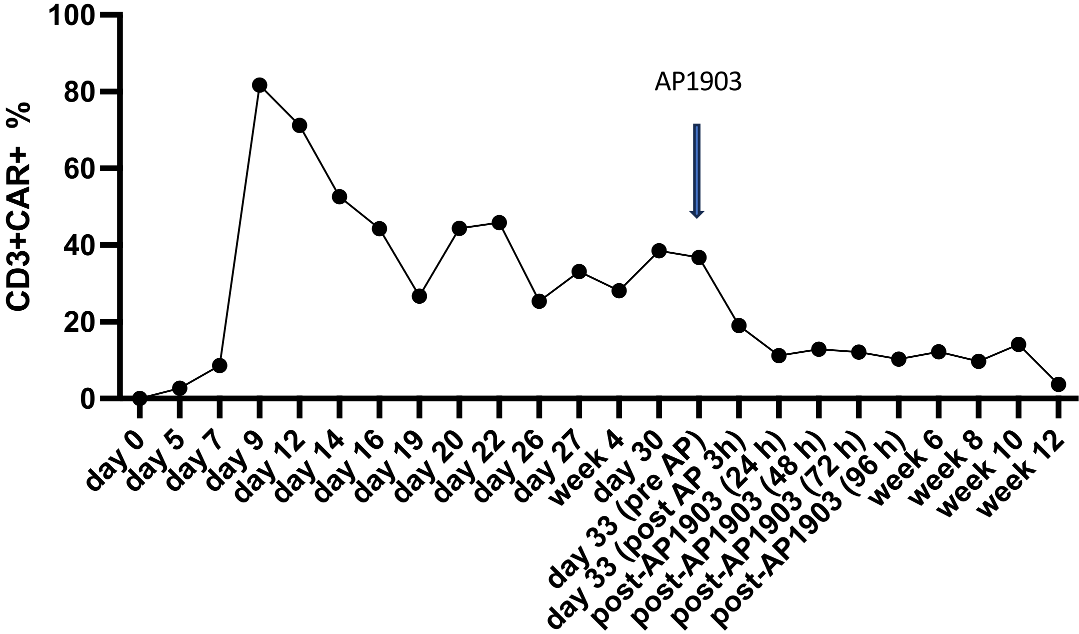


Figure 15. Circulating levels of CAR T-cells after Rimiducid

Serum levels of interferon-γ, interleukin-6, tumor necrosis factor α, and interleukin-10 were monitored in all the patients. The trend of serum cytokine levels for each patient is show in figure 16 and the trend of CSF cytokine levels for each patient is show in figure 17.

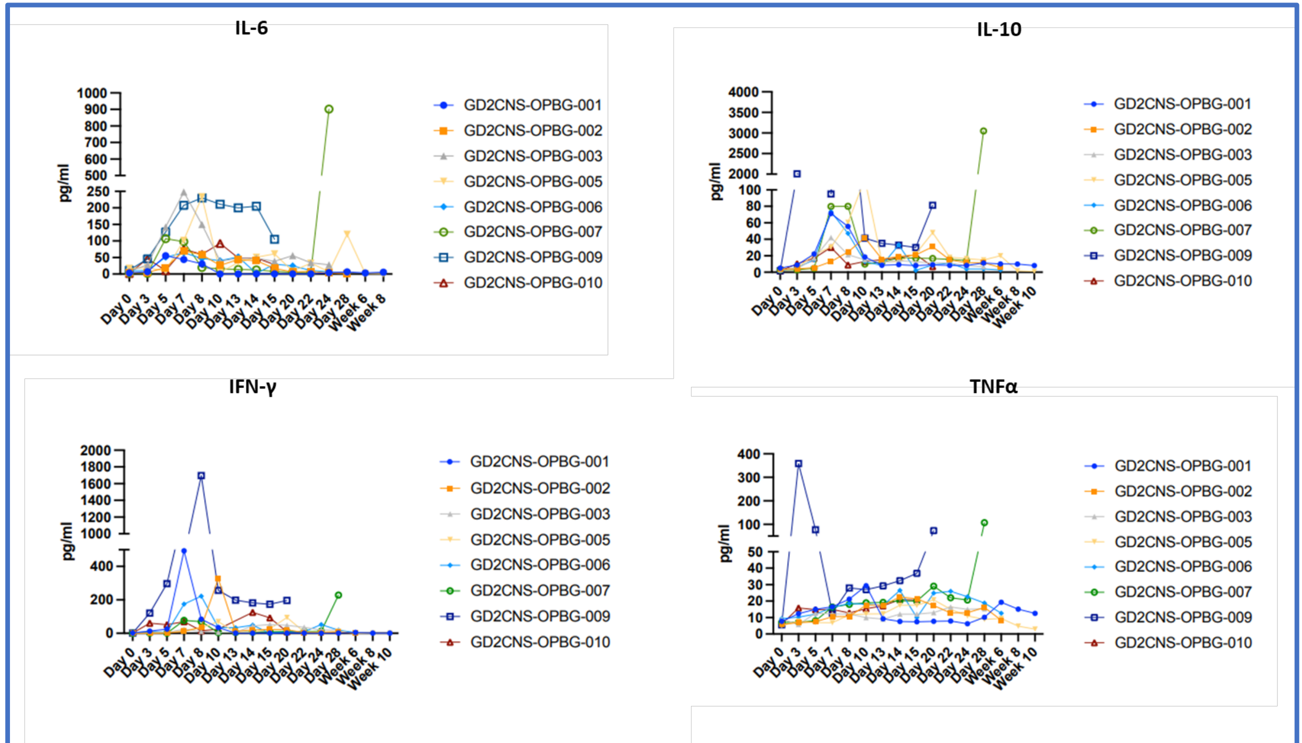


Figure 16. Trend of serum cytokine levels at different time points for each patient

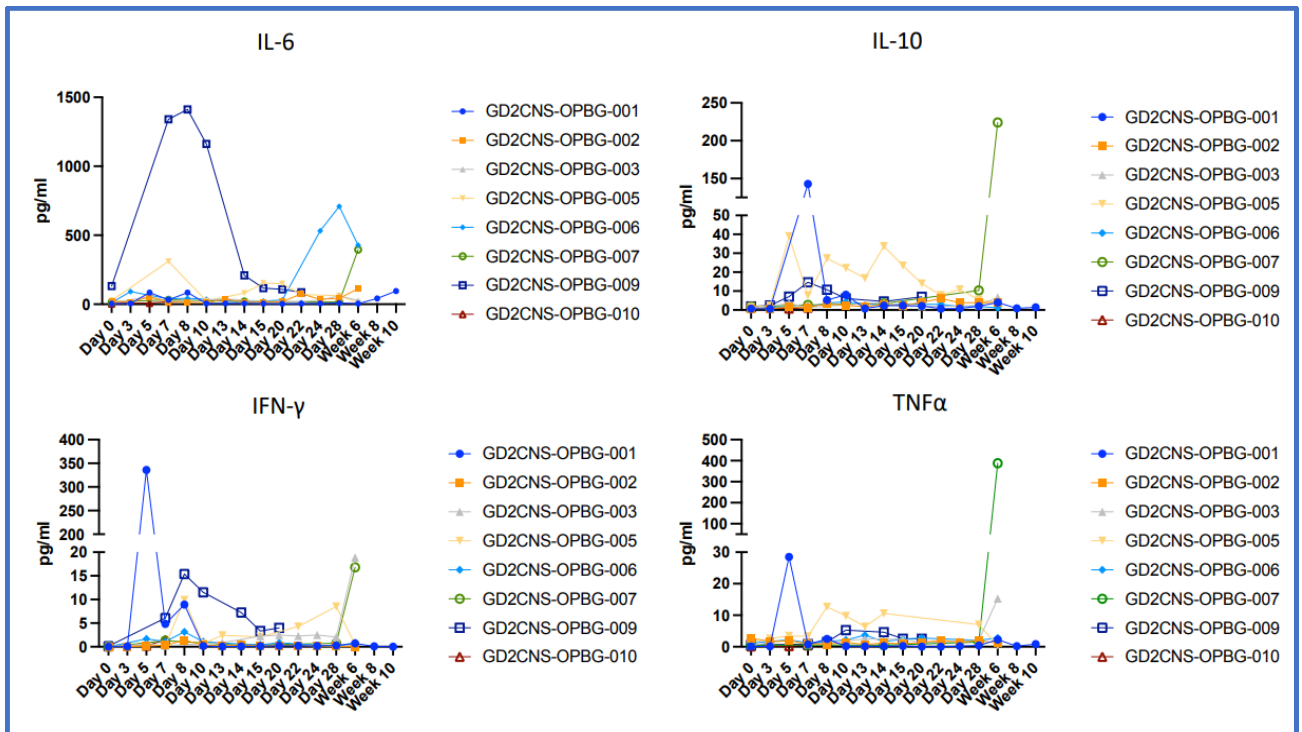
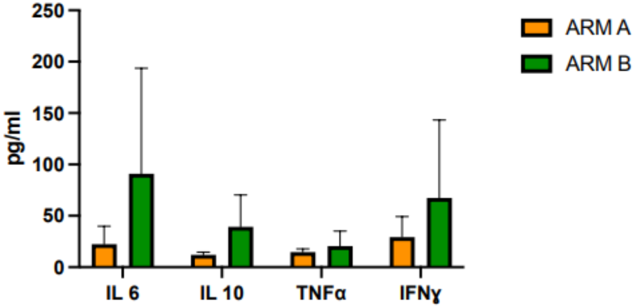


Figure 17. Trend of CSF cytokine levels at different time points for each patient

We also investigated whether there were differences in serum cytokine levels between the different arms and dose levels (figure 18). We did not find any statistically significant

differences; however, as shown in the graphs, it appears that patients in arm B (hemispheric high-grade gliomas) have higher cytokine expression levels in PB.

A.



B.

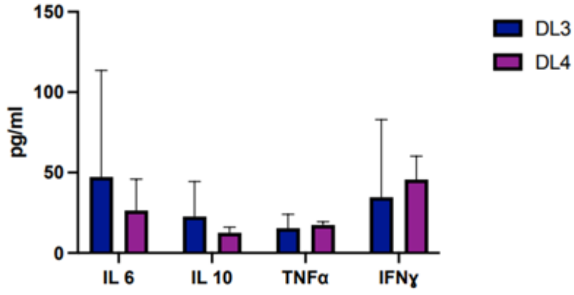


Figure 18. Difference in serum cytokine levels between the different arms (A) and dose levels (B)

Heat map of median serum cytokine concentrations (pg/ml) per each patient is illustrated in figure 19.

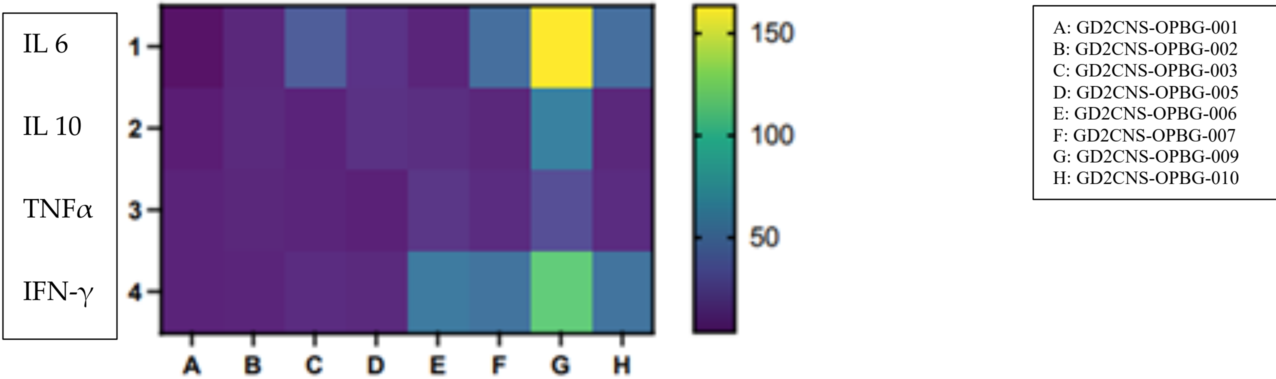


Figure 19. Heat map of median serum cytokine concentrations

We also evaluated the trends of inflammatory markers, CRP and ferritin, at different time points for individual patients (figure 20 and 21).

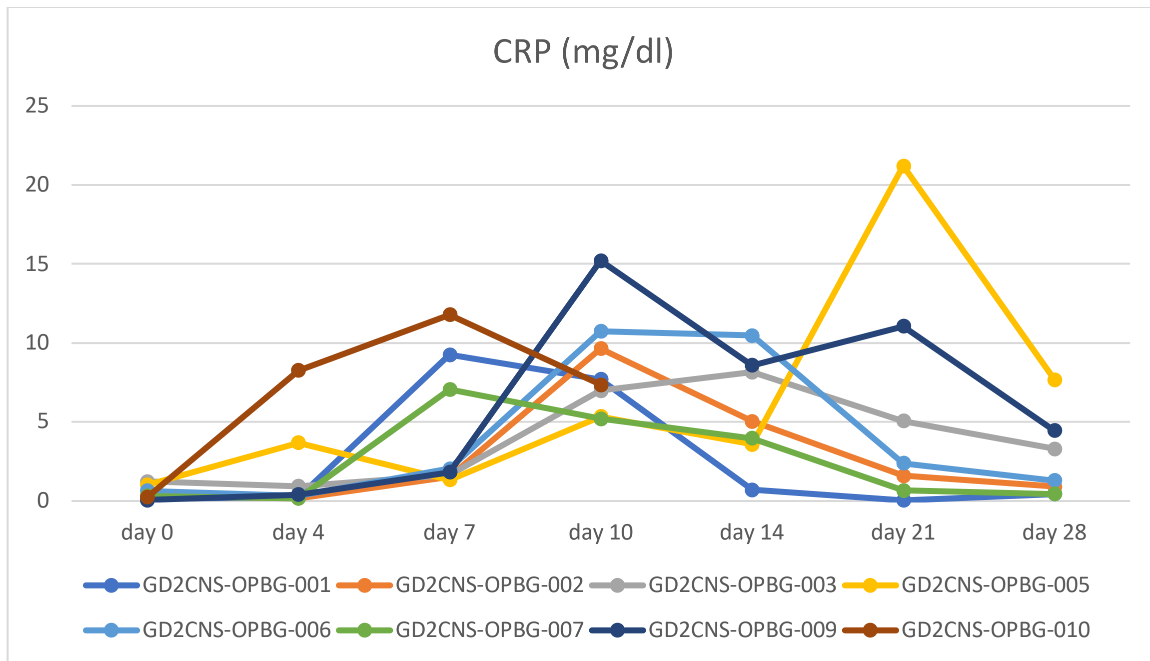


Figure 20. Trend of CRP levels for each patient.

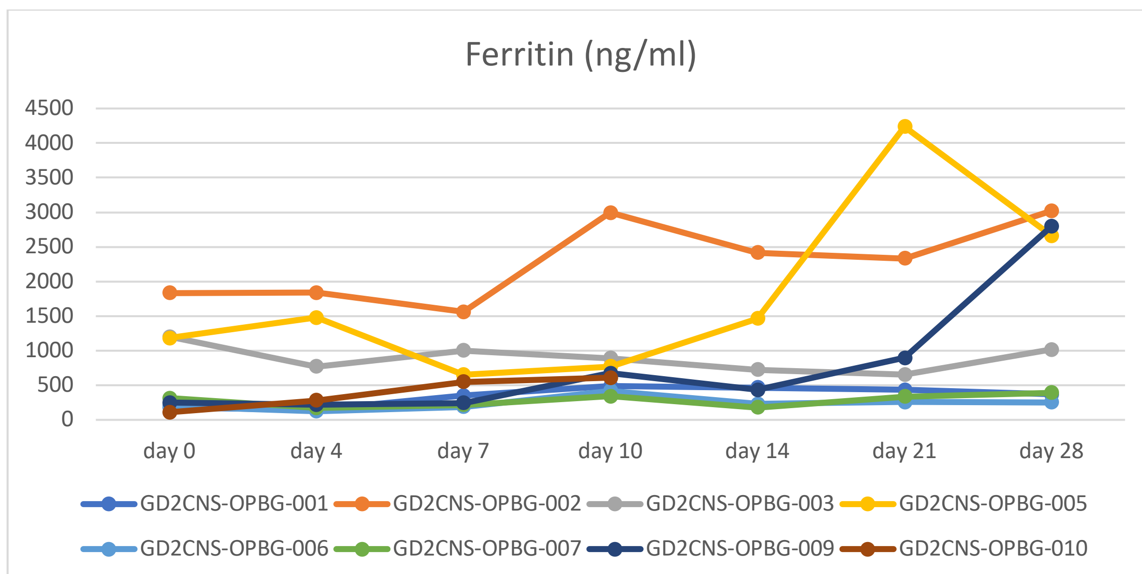


Figure 21. Trend of ferritin levels for each patient.

The median levels of CRP and ferritin at the different time points are described in figure 22 and 23.

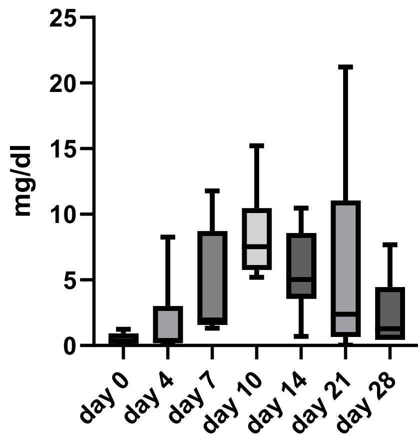


Figure 22. Median CRP levels

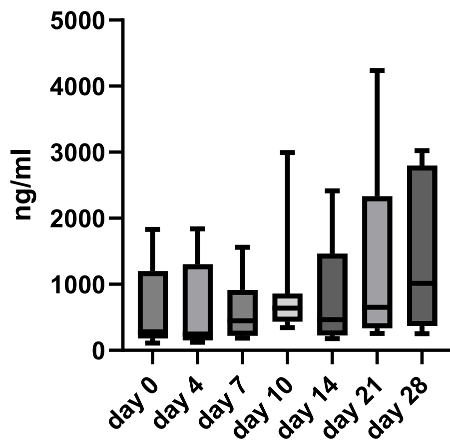


Figure 23. Median ferritin levels

3.4 Safety of infusion

No dose-limiting toxic effects were reported in patients treated to date.

Cytokine release syndrome was one of the most common drug-related adverse events, observed in 7 of 8 patients (87,5%) after infusion. In all cases CRS was classify as grade 1 according to Lee et al. (57). The CRS began between day +6 and day +8 post-infusion and median duration was 4 days (range 3-15).

Regarding neurotoxicity, two patients (25%) experienced ICANS. Patient GD2CNS-OPBG-001 developed grade 2 ICANS, characterized by aphasia, drowsiness, and psychomotor slowing, for which low-dose steroids and anakinra were administered. The symptoms resolved completely within 12 days. In patient GD2CNS-OPBG-009, grade 1

ICANS (drowsiness) resolved completely without intervention in 7 days. The diagnosis of ICANS was supported by the EEG recording and imaging in both cases.

Type 2, grade 1 TIAN was reported in two cases (25%). These patients experienced a temporary worsening of their baseline disease-related symptoms. In one case (GD2CNS-OPBG-005), the patient experienced a recurrence of seizure activity and the symptoms completely resolved following an adjustment of the antiepileptic therapy. In the second case (GD2CNS-OPBG-009), after an initial presentation of ICANS grade 1 characterized by drowsiness and slowing on the EEG tracing, there was a worsening of the left hemiparesis due to a right temporal lesion, which resolved spontaneously in one week.

It is important to emphasize that all patients underwent ICP telemetric monitoring during hospitalization. In case of intracranial pressure values above 20 mmHg, CSF aspirations were performed and no patients presented complications from intracranial hypertension.

Although the condition was not attributable to CAR T toxicity, due to the severity of the event and to avoid any interference related to a potential toxicity of CAR T cells, one patient (GD2CNS-OPBG-005) received a single infusion of rimiducid, along with steroids and anakinra, following the development of a brain hemorrhage due to tumor bleeding. The event occurred 4 weeks after the infusion and was not related to the experimental product.

Hematologic toxic effects developed in all the patients, induced by lymphodepletion and sustained after GD2-CART01 infusion. In addition, grade 3, transient elevated transaminases developed in one patient after infusion ad grade 2 hyponatraemia in 2 cases.

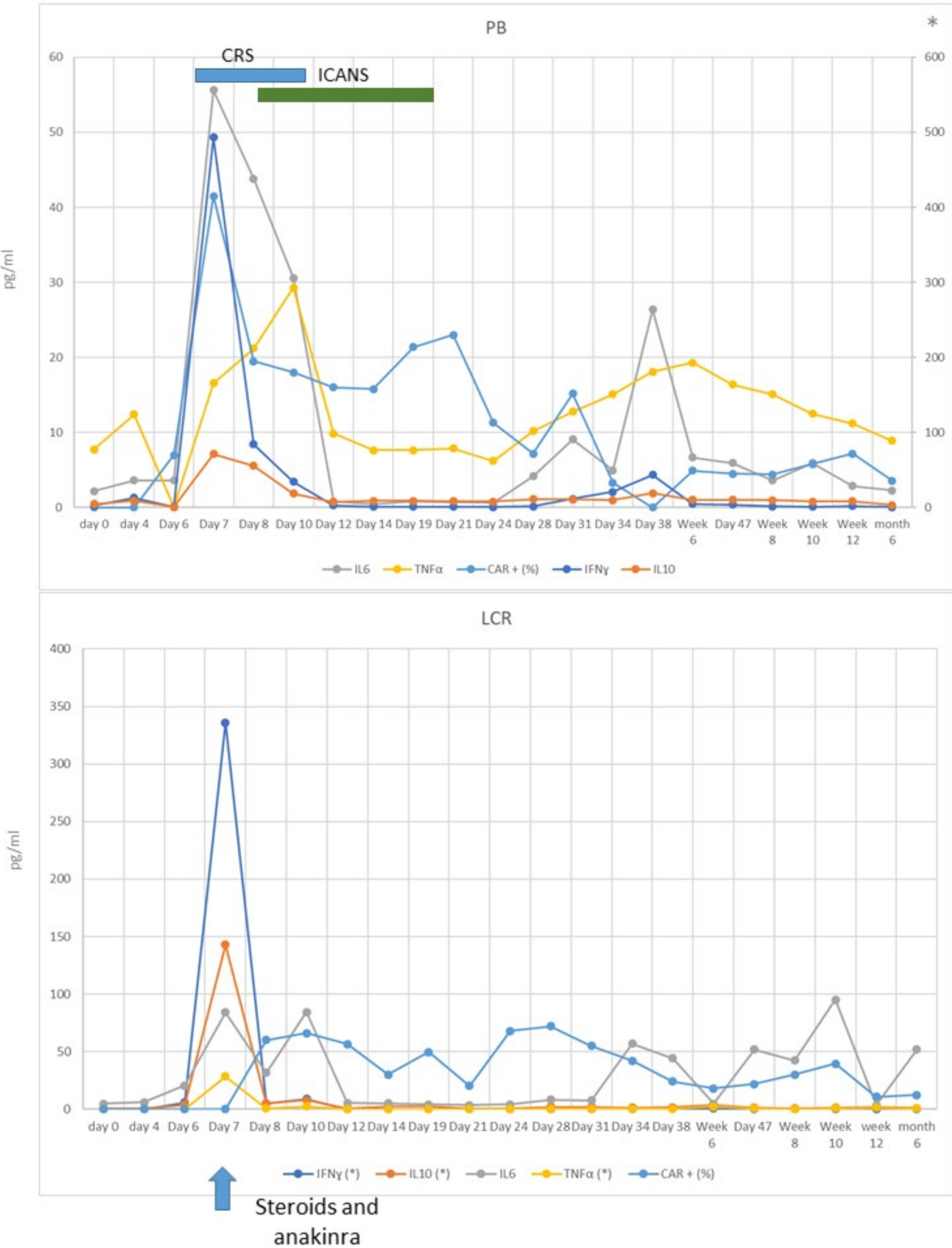
All the adverse events are listed in table 6.

Table 6. Adverse events in patients after infusion of GD2-CART01

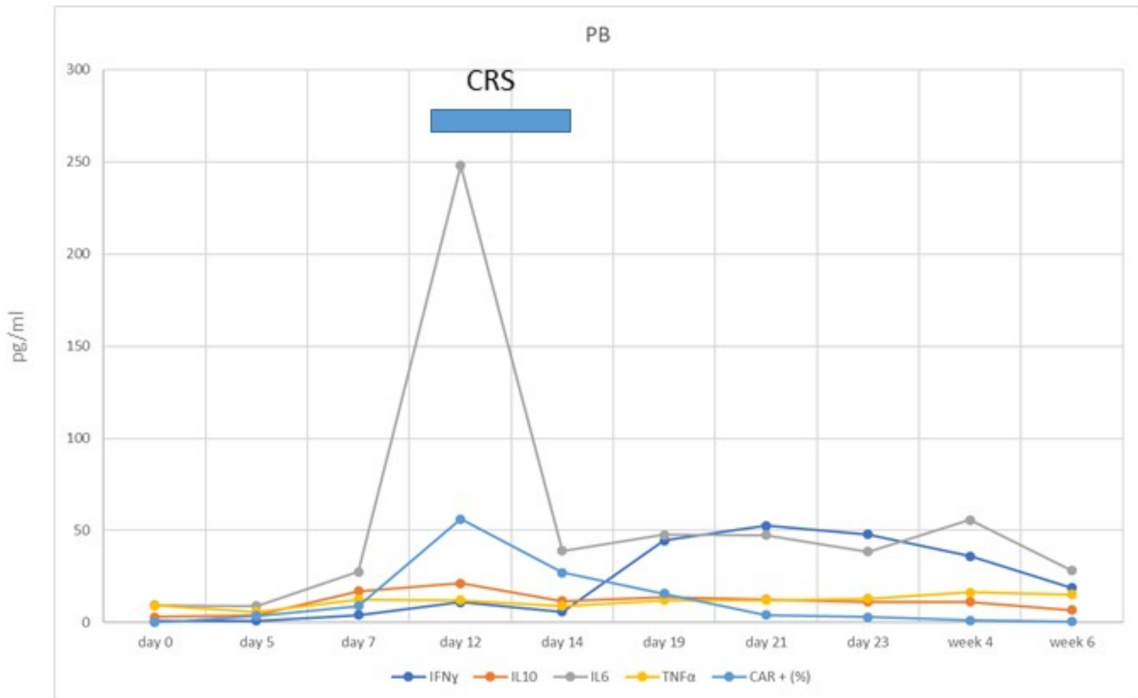
Pt ID	CRS	Neutotoxicity	Hematological toxicity	Others	treatment
GD2CNS-OPBG-001	G1	ICANS G2	Anemia (G2); thrombocytopenia (G1); neutropenia (G4)	No	Steroids Anakinra
GD2CNS-OPBG-002	G1	No	Anemia (G2); thrombocytopenia (G3); neutropenia (G4)	No	
GD2CNS-OPBG-003	G1	No	Anemia (G2); thrombocytopenia (G3); neutropenia (G4)	No	
GD2CNS-OPBG-005	G1	TIAN type 2 G1	Anemia (G3); thrombocytopenia (G4); neutropenia (G4)	Hyponatraemia (G2), Elevated transaminases (G3). Brain hemorrhage (not related to GD2-CART01)	Steroids Anakinra AP1903
GD2CNS-OPBG-006	G1	No	Anemia (G2); thrombocytopenia (G2); neutropenia (G4)	Skin rash (G1)	
GD2CNS-OPBG-007	No	No	Anemia (G2); thrombocytopenia (G3); neutropenia (G4)	No	
GD2CNS-OPBG-009	G1	TIAN type 2 G1/ICANS G1	Anemia (G2); thrombocytopenia (G1); neutropenia (G4)	Skin rash (G1), Hyponatraemia (G2),	
GD2CNS-OPBG-010	G1	No	Anemia (G3); thrombocytopenia (G3); neutropenia (G4)	No	

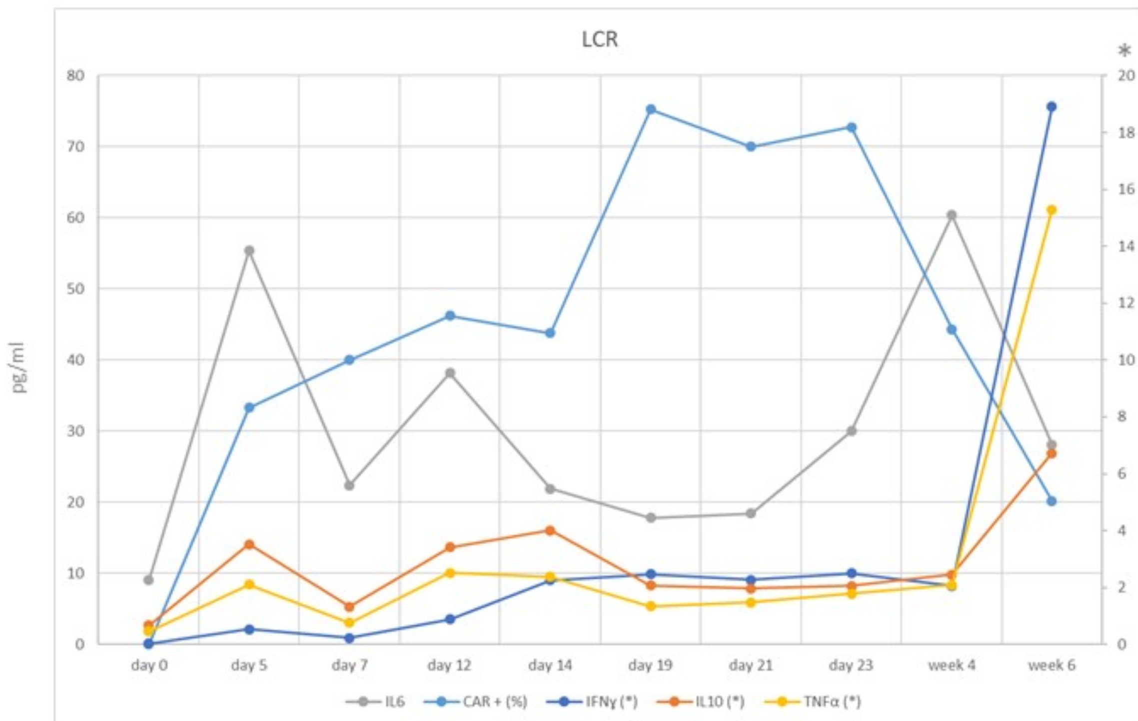
Detailed cytokine profile, CAR-T expansion and toxicity for each patient are illustrated in the following graphs.

 # GD2CNS-OPBG-001 (ARM A DL3)

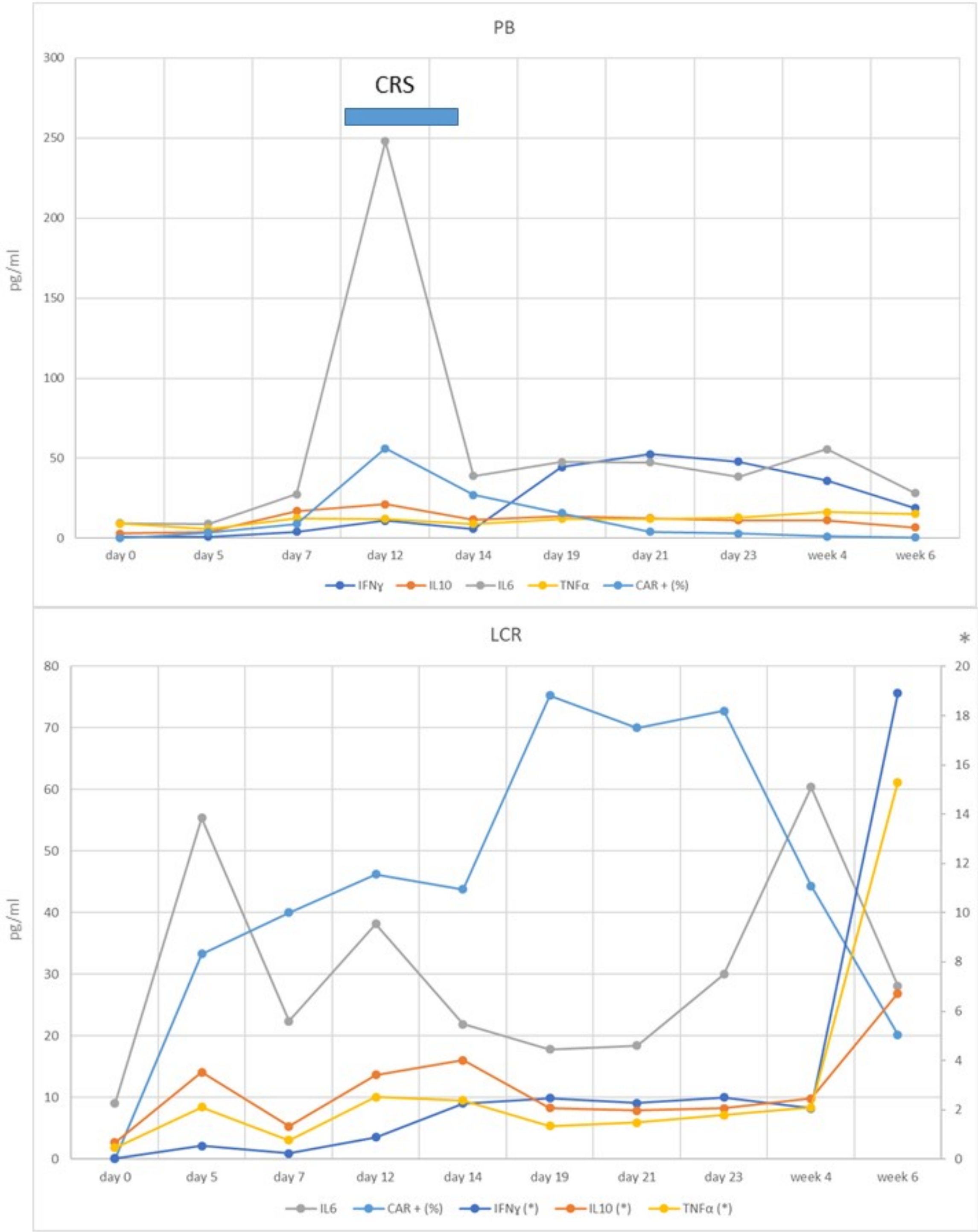


GD2CNS-OPBG-002 (ARM A DL3)

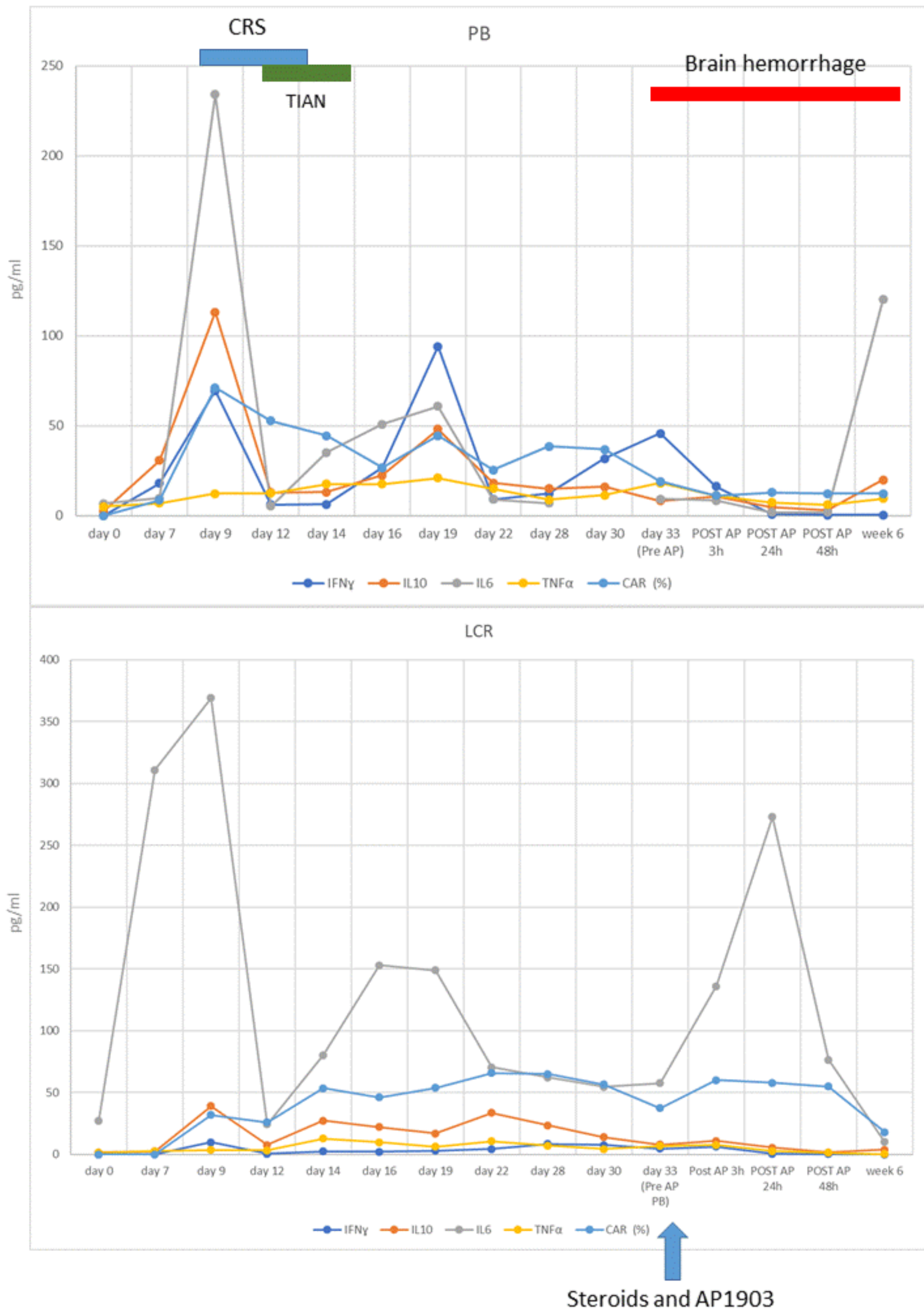




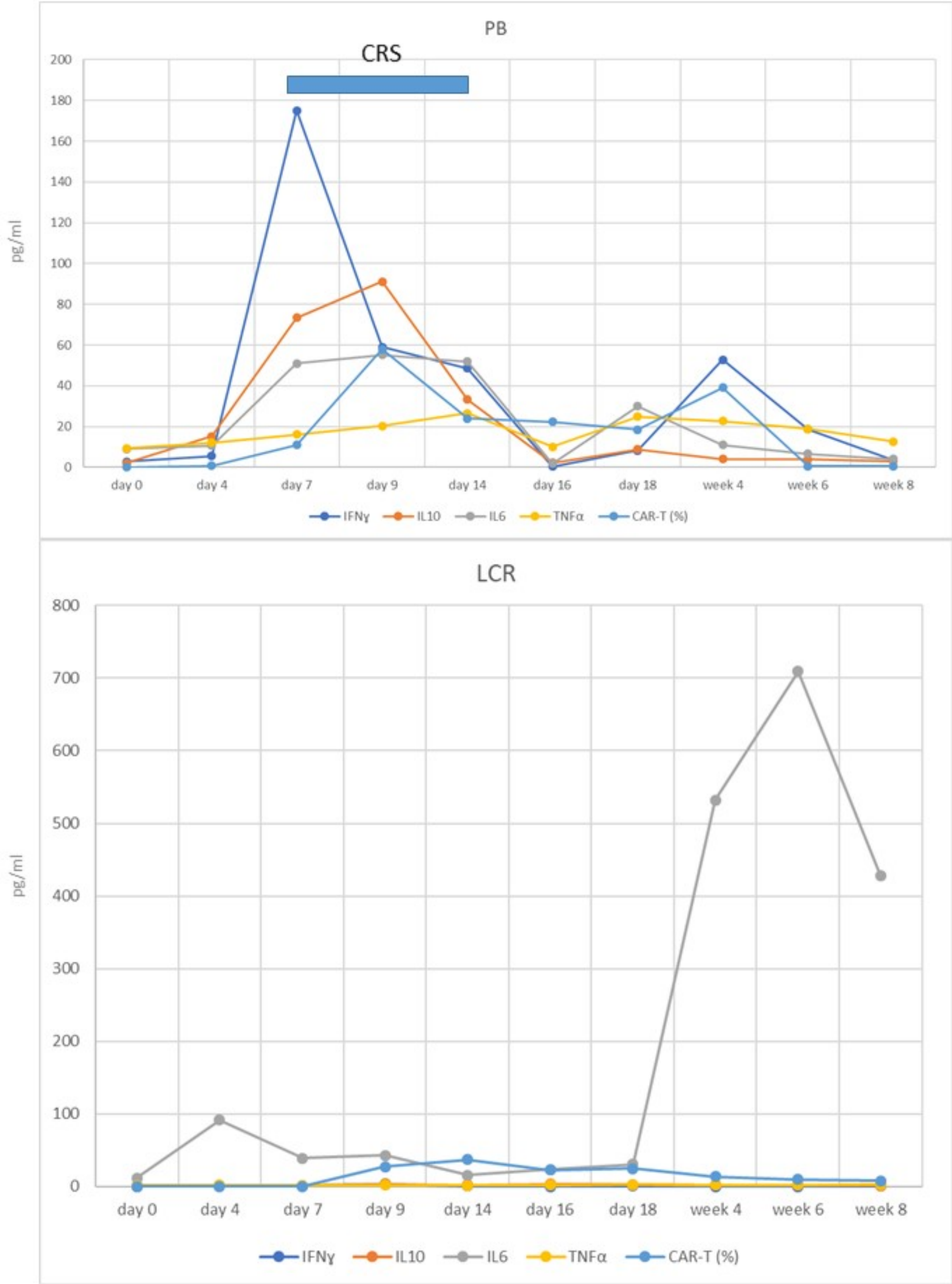
GD2CNS-OPBG-003 (ARM A DL3)



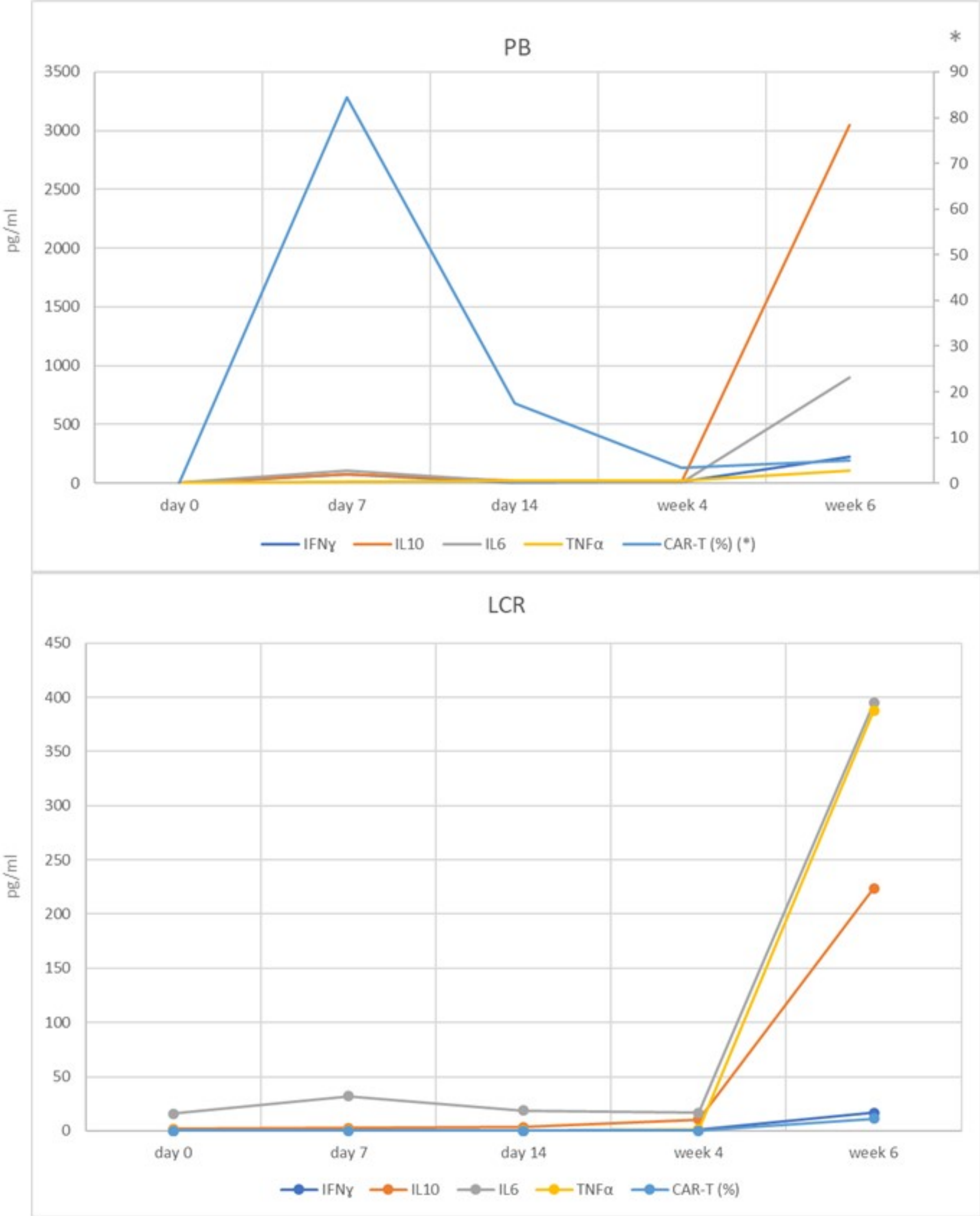
GD2CNS-OPBG-005 (ARM B DL3)



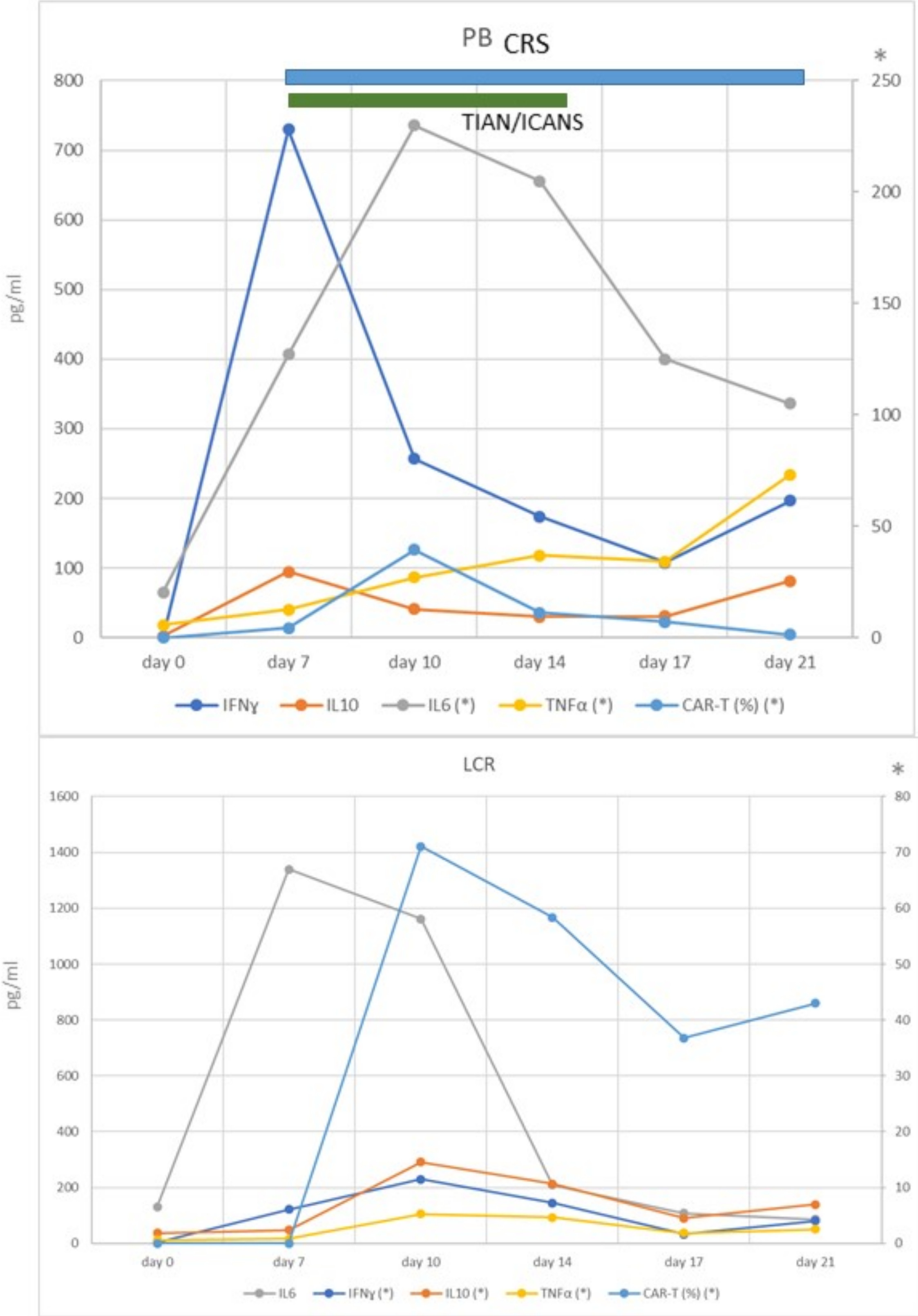
GD2CNS-OPBG-006 (ARM A DL4)



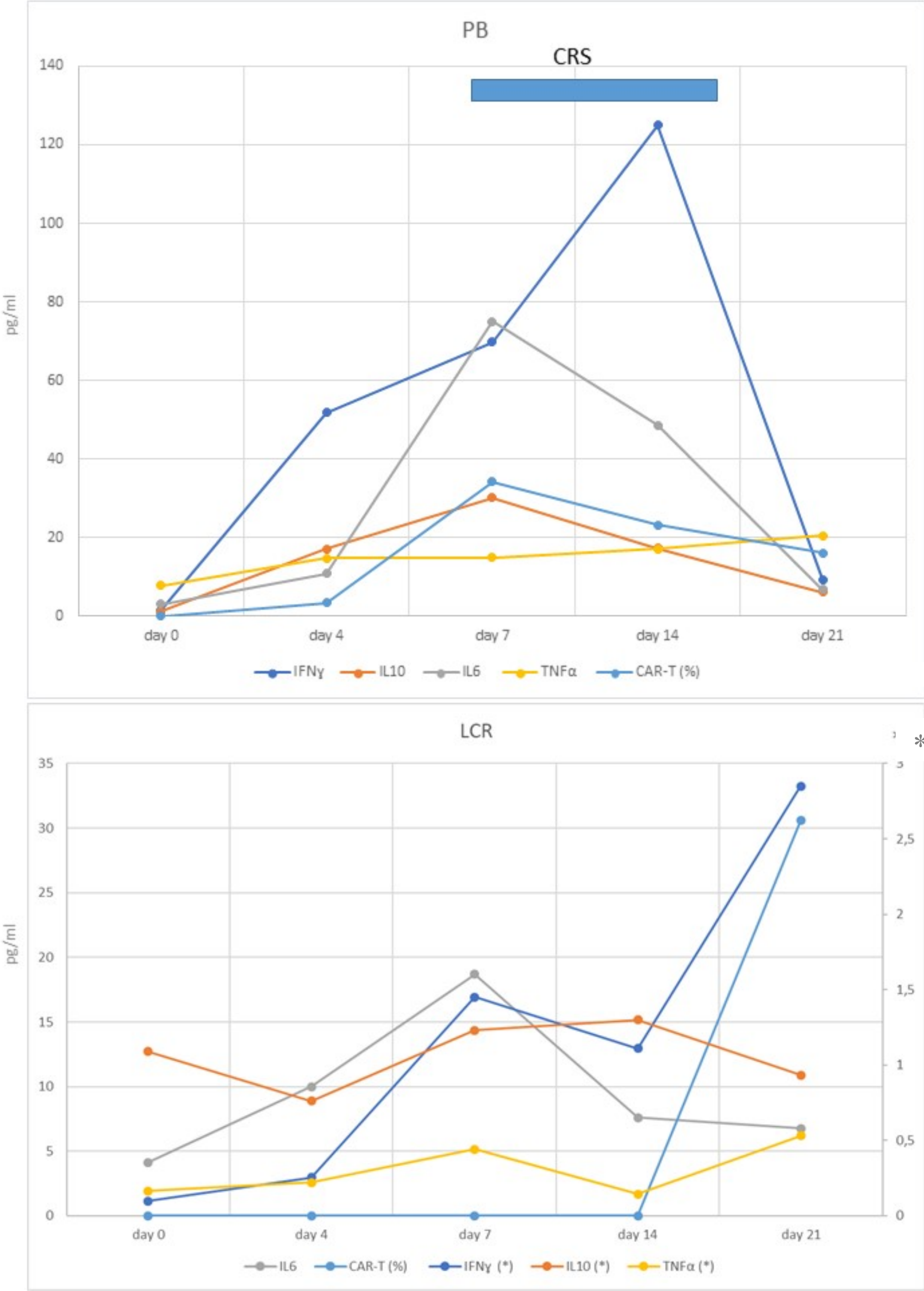
GD2CNS-OPBG-007 (ARM A DL 4)



GD2CNS-OPBG-009 (ARM B DL 3)



GD2CNS-OPBG-010 (ARM A DL 4)



3.5 Radiological responses after GD2-CART01 Infusion and clinical outcome

All patients underwent disease reassessment with brain and spine MRI and CSF cytology according to RAPNO criteria at the time-points specified by the study.

The median follow-up was 2.92 months (range 10.53 - 0.77).

One patient (GD2CNS-OPBG-001) presented PR at week 6 post infusion MRI, SD at week 12, and PD at month 6 after infusion. The patient underwent surgery to remove the lesion in the posterior cranial fossa, where high levels of GD2 CAR T-cells were detected in the tumor samples, along with sustained high GD2 expression. Currently, the patient is at a 10.5-month follow-up since CAR T-cells, with an OS of 116.7 months from diagnosis and with a negative neurological examination.

Three patients (GD2CNS-OPBG-002, 003 and 006) progressed after week 6 post infusion. GD2CNS-OPBG-002 died at 5,5 months after infusion, with an OS of 16.4 months after diagnosis. GD2CNS-OPBG-003 died at 3 months after infusion, with an OS of 73.10 months after diagnosis. Patient GD2CNS-OPBG-006 is alive, with stable neurological status compared to pre-infusion and an OS of 3.2 months from CAR T-cells and 29 months from diagnosis.

One patient (GD2CNS-OPBG-007) shows a suspected pseudoprogression at week 6 post-infusion (also supported by the fact that CAR T-cells and cytokines in cerebrospinal fluid increased at that time-point). The patient is awaiting reassessment with new imaging to confirm this finding and currently has an OS of 47.6 months from diagnosis with stable neurological condition.

Patient GD2CNS-OPBG-005 showed SD at week 6 post-infusion and PD at week 12, in line with a worsening of his neurological symptoms. He is still alive with OS of 34.7 months from diagnosis.

The patients GD2CNS-OPBG-009 and 010 have not yet been reassessed for disease status.

Figure 24 summarizes patients' survival from GD2-CART01 infusion and current disease status.

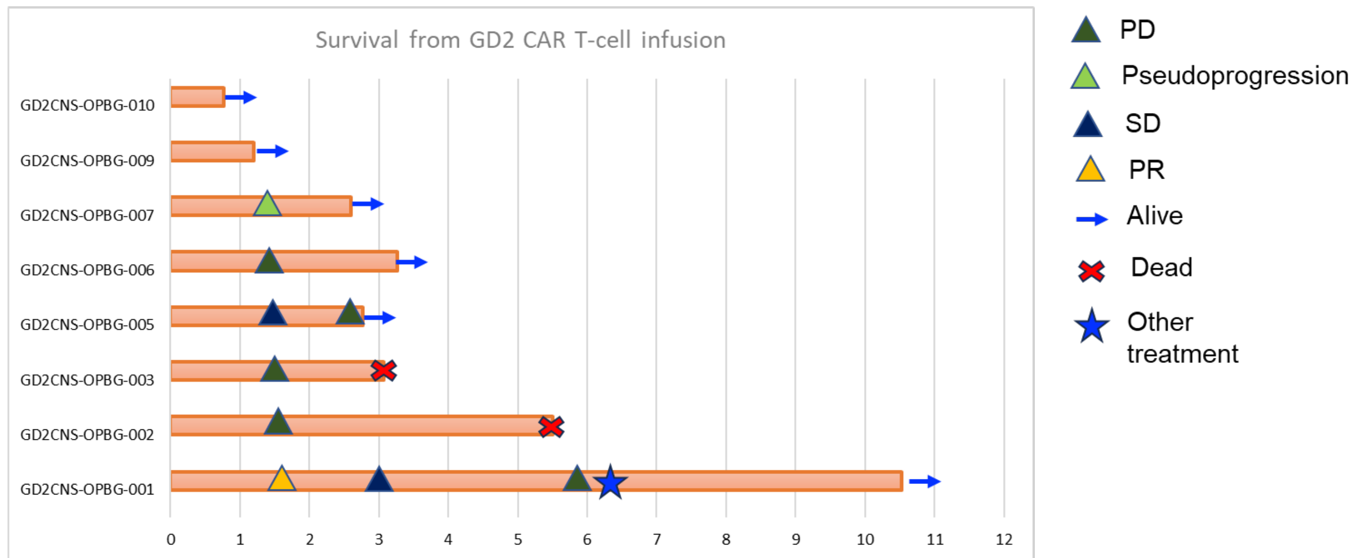


Figure 24. Outcomes after GD2-CART01 infusion.

4. Discussion

Childhood malignant brain tumors remain a significant cause of death in the pediatric population, despite the use of aggressive multimodal treatments. New therapeutic approaches are urgently needed for these patients in order to improve prognosis, while reducing side effects and long-term sequelae of the treatment. Immunotherapy is an attractive option and, in particular, the use of gene-modified T-cells expressing a chimeric antigen receptor CAR T-cells represents a promising approach. Major hurdles in the clinical application of this approach in neuro-oncology, however, exist. The peculiar location of brain tumors leads to both a difficulty of access to the tumor mass, shielded by the BBB, and to an increased risk of potentially life-threatening neurotoxicity, due to the primary location of the disease in the CNS and the low intracranial volume reserve. Additionally, the highly heterogeneous nature of brain tumors makes it difficult to identify a single, specific antigen target for CAR T-cells, raising the risk of off-target effects and neurotoxicity. Finally, the immunosuppressive microenvironment within the brain can hinder CAR T-cell function and durability, limiting therapeutic efficacy.

The GD2 disialoganglioside is an antigen expressed in various histotypes of brain tumors. Our experience on DMG and medulloblastoma have shown high expression in this category of tumors and a good response to CAR T-cell therapy in preclinical setting (55,56). These data, in addition to the good results and safety we obtained in the phase I/II trial on neuroblastomas (7), were preliminary to the launch a phase I study of anti-GD2 CAR T-cells in pediatric and young adult patients affected by relapsed/refractory central nervous system tumor (NCT05298995).

From November 2023 to October 2024, we enrolled a total of 12 patients, of whom 8 received treatment. The manufacturing of GD2-CART01 was successful for all the enrolled patients. The median transduction was 62.1%, and cell viability was 92.5%. In all cases, the cytotoxicity of the product was high and the mean copy number of the vector was significantly lower than the threshold required for release.

In our trial, all patients had expansion of GD2-CART01 in PB and LCR. The peak of expansion was observed at the second week post-infusion, and CAR T-cells are still present in the cerebrospinal fluid and peripheral blood of all patients at the last follow-up.

In order to maintain tumor control, it is essential that CAR T-cells persist over time. This aspect has also been underlined in the pioneering work by Louis and colleagues (68), who found a significant correlation between persistence longer than 6 weeks and longer time to disease progression (69). All our patients had persistence of CAR T-cell at last follow up. We detected CAR T-cells in the peripheral blood and LCR of the first infused patient even 9 months post-infusion, possibly because of the incorporation of two costimulatory domains in the construct and the use of interleukin-7 and interleukin-15 in the manufacturing of GD2-CART01 (70,71).

We did not find a correlation between the expansion peak and different treatment arms or dose levels, but it appears that patients in arm B showed a greater CAR T-cells expansion in both blood and cerebrospinal fluid.

Moreover, consistent with previously published data (7), CD8-positive CAR T-cells were predominant in peripheral blood at all time-points. CD4-positive CAR T-cells also expanded and were detectable, reaching their highest levels at week 4 post-infusion.

Another particularly interesting point relates to the use of rimiducid. Only one patient of our cohort received AP1903, although the event that triggered its use was considered by the clinical study team to be unrelated to the CAR T-cells. The use of rimiducid resulted in the rapid reduction of circulating GD2-CART01. We observed a reduction of half circulating GD2-CART01 3 hours after infusion of the dimerizing agent, with maximum reduction observed at 24 hours. Since then, circulating CAR T-cell levels have remained stable, ranging between 10-15% of total CD3 up to week 12 post-infusion. The fact that CAR T-cells were not completely eliminated with the rimiducid suggests that this approach led to an effective control of the toxic effects without completely eliminating the CAR T-cells.

Our data support the hypothesis that CAR T-cells expand in peripheral blood and cerebrospinal fluid, also confirming their ability to cross the blood-brain barrier, as has already been established in other conditions (7,40,46). The ability of CAR T-cells to reach their target was demonstrated in the first treated patient. In this patient, we performed a resection of a progressing lesion in the posterior fossa, where a high degree of CAR T-cell infiltration was observed, confirming successful targeting.

CAR T-cells induced an immune-related response that was manifested by mild cytokine release syndrome in most of the patients (87,5%). CRS began around days 6-8 in all patients with a median duration of 4 days and was reversible in all cases without intervention. As already reported with other CAR constructs and immunotherapy approaches (68), this complication correlated with the peak serum levels of the cytokines interleukin-6, interferon-γ, tumor necrosis factor α, and interleukin-10. In all patients who experienced CRS, there was a concurrent rise in cytokine levels in both serum and cerebrospinal fluid. Moreover, simultaneously with CRS, the peak expansion of CAR T cells and the increase in cytokines levels, a rise in serum inflammatory markers such as CRP and ferritin was observed.

As for CAR T-cell expansion, we did not find significant differences between the entity of cytokine level increase and treatment arm or dose level. However, patients in arm B had a higher median cytokine level compared to those in arm A.

Although manageable, the side-effect profile of this type of immunotherapy is not negligible. Majzner et al. described their experience in four patients with DMG treated with GD2 CAR T-cells. They reported CRS in all cases (grade 1 in two patients, grade 2 in one patient, and grade 3 in one patient). The CRS was manageable and resolved with the use of steroids, tocilizumab, siltuximab or anakinra (9).

ICANS is a recognized neurological complication of CAR T-cell therapy, most commonly seen in hematologic malignancies but increasingly noted in solid cancers (72), including brain tumors. ICANS symptoms can range from mild cognitive changes and confusion to severe encephalopathy, seizures, and even cerebral edema. The pathophysiology of ICANS isn’t fully understood, but it's thought to involve inflammatory cytokines,

disruption of the blood-brain barrier, and direct effects of immune cell activity on the CNS.

In our study population, two cases of ICANS were documented. The first case presented with grade 2 ICANS, for which we initiated treatment with steroids and anakinra, leading to complete resolution of clinical symptoms within 12 days. The second case involved grade 1 ICANS, marked by somnolence and slowed activity on EEG tracing, which resolved spontaneously within 7 days.

Majzner et al. described one case of grade 4 ICANS associated with no MRI changes in the normal brain and diffuse slowing with triphasic waves on continuous EEG monitoring, consistent with a reversible inflammatory encephalopathy. Given the extensive tumour invasion of brain structures they were unable to distinguish between ICANS and TIAN as the primary cause for encephalopathy (9).

In our experience CRS and ICANS were similar to that described with other CAR T-cell therapies (73,74), but patients also developed signs and symptoms consistent with CAR T-cell-mediated inflammation in sites of CNS disease, consistent with TIAN. As Majzner highlights in his study, distinguishing between ICANS and TIAN in brain tumor patients treated with CAR T-cells is not straightforward (9). TIAN most often manifested as transient worsening of existing deficits but also resulted in episodes of increased ICP secondary to brainstem edema and consequent obstructive hydrocephalus, which would have been life-threatening unless immediately and appropriately managed.

In our patients, none exhibited intracranial hypertension and this is thanks to our non-invasive ICP monitoring. The intracranial pressure monitoring device enabled us to closely monitor our patients, allowing for timely interventions. In cases of elevated ICP levels (> 20 mmHg), we were able to perform CSF aspiration via the Rickham reservoir. This proactive approach ensured effective management of ICP and improved patient safety during treatment. Furthermore, this monitoring capability allows us to limit the use of steroids, which have lymphosuppressive effects.

In our population, two cases of type 2 grade 1 TIAN were identified, characterized by a transient worsening of symptoms related to the underlying disease. In one case seizure

activity recurred but fully resolved after modifying the antiepileptic regimen. The other patient presented worsening of right hemiparesis linked to a left temporal lesion, which resolved spontaneously within a week.

Although patients developed symptoms of on-tumour neurotoxicity, they did not manifest any signs or symptoms of on-target, off-tumour toxicity. GD2 is expressed on normal neural tissues, including the brain and peripheral nerves, and treatment with anti-GD2 antibody dinutuximab for neuroblastoma is associated with transient painful neuropathy in most children (75,76). None of these patients developed painful neuropathies or any other clinical or radiographic indication of on-target, off-tumour toxicity.

Hematologic toxicity is another common occurrence with CAR T therapy, even in patients with solid tumors (7). Hematologic toxic effects, which were thought by the investigators to be mainly induced by the lymphodepleting chemotherapy and sustained by GD2-CART01, were another relevant adverse event that was reported and it resolved in all patients.

One patient with a high tumor burden experienced bleeding at the tumor site 33 days after infusion of the experimental product; this event was deemed unrelated to the treatment. Considering the severity of the event, rimiducid was administered in addition with steroids and anakinra to further mitigate any additional risks. As previously mentioned, rimiducid has significantly reduced the number of circulating CAR T-cells without eliminating them completely. This is a crucial point, as it allows us to limit toxicity when it is too severe, while also preserving potential therapeutic effect of CAR T-cells.

In conclusion, to date, we have not observed any dose-limiting toxicities in our study. While still in its early stages, our initial experience suggests that the cell product is safe.

Regarding patient outcomes, our experience has documented one partial response at six weeks post-infusion, three disease progression, one case of pseudoprogression, and one stable disease. The patient who showed a partial response at six weeks post-infusion maintained stable disease at week 12, but progressed at six months. Remarkably, she still

has circulating CAR T-cells in both blood and cerebrospinal fluid nine months post-infusion. Following the surgical removal of the progressed tumor mass, the patient presents with essentially stable residual lesions and negative neurological examination. Given her long medical history of 9.7 years and the numerous treatments she has undergone, this result is considered remarkable. The patient with stable disease at six weeks experienced disease progression at twelve weeks post-infusion. This patient had a diagnosis of high-grade glioma that was both very aggressive and extensively spread at the time of enrollment. One patient was deemed to be in pseudoprogression based on radiological and clinical criteria at six weeks post-infusion. This assessment is supported by the observation that, in this case, the expansion of CAR T-cells and the increase in cytokine levels occurred precisely at six weeks post-infusion. Of the three patients who exhibited disease progression on the first MRI at six weeks, two died at 5.5 and 3 months post infusion respectively, while one is currently alive in stable clinical and neurological conditions, with only a single tumor lesion and no other signs of dissemination.

Nonetheless, it is important to recognize that the majority of these patients had a long history of disease and had already received multiple lines of treatment. Additionally, it's noteworthy that our patients with single or few lesions did not experience further disease dissemination. This leads us to speculate that patients with a lower tumor burden may be the ideal candidates for this treatment, as demonstrated in other diseases (7).

There are no published scientific data that can be compared to our experience. The only trial reporting outcomes for four patients treated with GD2 CAR T-cells in brain tumors is the one conducted by Majzner et al. (9). In their experience, only patients with DMG were included (for which we are still awaiting to open the cohort). Furthermore, in their clinical study design, intraventricular infusions were allowed following an initial intravenous infusion if the patient demonstrated a clinical benefit from the intravenous treatment. The first patient they described experienced progression one month after i.v. GD2-CAR T-cell infusion and died three months later. The second patient showed significant neurological improvement and tumor reduction by one month post-infusion. However, symptoms recurred, leading to a second intracerebroventricular (i.c.v.)

infusion, which resulted in marked improvement. He received a total of five i.c.v. infusions but died from an intratumoral hemorrhage before the planned sixth infusion, surviving 10 months overall. Patient 3 presented clinical and radiological improvement by 1 month post i.v. infusion. She was re-treated with 2 doses of GD2-CAR T-cells i.c.v. 3 months after and she died from tumour progression 7 months after her first GD2-CAR T-cell administration. The last patient, with spinal DMG, achieved over 90% tumor volume reduction by day +32 post i.v. infusion but experienced progression by day +75, necessitating a new i.c.v. dose. Subsequent improvement was noted, but both spinal and brain disease progressed further by two months post-infusion, and she survived 11 months after her initial GD2-CAR T-cell administration (9).

The other available data currently published concerning pediatric patients pertains specifically to intraventricular infusions. Vitanza et al. described three patients treated with a second-generation HER2-targeted CAR T therapy, specifically one case of anaplastic astrocytoma and two cases of ependymoma. The treatment was administered locoregionally, and notably, no dose-limiting toxic effects were observed (10). Data outcome were not described by the authors. In a second study by Vitanza et al. three patients affected by DMG were treated with a second-generation B7H3-targeted CAR T-cell. The treatment was administered locoregionally, resulting in two cases of PD and one PR observed through 12 months on study. Importantly, also in this case, no dose-limiting toxic effects were associated with the treatment (11).

Despite the documented ability of intravenous administered CAR-T cells to cross the BBB, local delivery of T-cells within the CNS presents an appealing strategy to mitigate systemic toxicity while enhancing CAR T-cell migration and homing in the tumor site. To date, there are several studies in both adults and children setting that confirm the efficacy and reduced toxicity of local infusions compared to systemic infusion (9–11,77–79). Moreover, Majzner et al found that intraventricular administration was associated high levels of cytokines and decreased immunosuppressive cell populations in CSF, compared with intravenous delivery (9).

Despite clinical and preclinical evidence demonstrating that CAR T-cells expand and reach their targets on CNS, the response outcomes are often not as expected. This discrepancy can be attributed to various other factors including optimal target antigen, TME and exhaustion.

One potential approach to optimize CAR T-cell therapy in brain tumors is to focus on target selection. Identifying and validating additional tumor-specific antigens could enhance the specificity of CAR T-cells, thereby minimizing off-tumor effects and improving overall efficacy. Multitarget CAR T-cell therapies are designed to recognize and attack multiple tumor antigens simultaneously, enhancing their ability to target heterogeneous tumors. This approach may improve treatment efficacy and reduce the risk of tumor escape due to antigen loss, potentially leading to better outcomes for patients with complex malignancies. Seattle Children's Hospital recently launched a new trial for DMG and other recurrent and refractory CNS tumors (NCT05768880) with CAR T-cells targeting four different antigens (B7-H3, EGFR806, HER2, IL13-Zetakine). Additionally, exploring combination therapies, such as pairing CAR T therapy with immune checkpoint inhibitors or other immunomodulatory agents, may boost the immune response and enhance tumor eradication (80).

The TME poses numerous challenges to CAR T therapy, including the presence of a suppressive tumor stroma consisting of tumor-associated macrophages and MDSCs, as well as hypoxic conditions that impede its effectiveness (81). Countless research studies have been conducted to counteract the antagonistic effect of the microenvironment on the efficacy of CAR T-cells against cancer.

The mechanisms underlying CAR T-cell exhaustion are incredibly intricate and warrant thorough exploration. Inadequate CAR T-cell structure may trigger ligand-independent tonic signaling, consequently predisposing CAR T-cells to exhaustion. Additionally, both the cytokine milieu and the duration of in-vitro expansion play roles in influencing CAR T-cell exhaustion. Finally, the TME harbors immunosuppressive factors, which further contribute to this phenomenon. Prolonged persistence of CAR T-cells is a feature of new-generation CAR T constructs. Numerous studies have delved into CAR T engineering

strategies, highlighting that prioritizing a central memory phenotype could be pivotal in inhibiting exhaustion and bolstering CAR T-cells proliferation and persistence (82,83). In addition to modifying the CAR costimulatory signals themselves, engineering approaches aimed at producing suitable cytokines are also essential for the full activation of CAR T-cells (84).

Given the limited number of patients included in the study thus far, we cannot draw definitive conclusions regarding the use of CAR T-cells in brain tumors. It is essential to evaluate all dose levels up to the maximum permitted for the trial and to complete patient enrollment also for the conditions included in arm C.

Continued research will be essential to optimize this therapeutic approach, aiming to improve outcomes for children and young adults with aggressive CNS tumors.

5. Conclusion

Our initial findings indicate that GD2 CAR T-cell therapy appears to be safe, as no dose-limiting toxicities have been recorded to date. The side-effect profile of this type of immunotherapy is not negligible. The use of devices to detect non-invasive ICP is crucial, as it enables timely monitoring and intervention to prevent complications associated with intracranial hypertension. In term of kinetics, we observed that the CAR T-cells expand and cytokine increase in all cases. However, a single systemic infusion may not be sufficient to control and reduce the disease, underscoring the need to explore additional intraventricular infusions. Furthermore, patient selection appears crucial, with a lower tumor burden potentially enhancing treatment efficacy. Moving forward, further exploration of CAR T-cell therapy for pediatric brain tumors is warranted, with a focus on optimizing target selection, improving the understanding of tumor microenvironment challenges, and enhancing CAR T-cell persistence.

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