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R-CHOP preceded by blood-brain barrier permeabilization with engineered tumor necrosis factor-α in primary CNS lymphoma

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Abstract:
Patients with primary CNS lymphoma (PCNSL) are treated with high-dose-methotrexate-based chemotherapy, which requires hospitalization and extensive expertise to manage related toxicity. The use of R-CHOP could overcome these difficulties, but blood-brain barrier (BBB) penetration of related drugs is poor. Tumor Necrosis Factor-a coupled with NGR (NGR-hTNF), a peptide targeting $CD13^{+}$ vessels, induces endothelial permeabilization and improves tumor access of cytostatics. We tested the hypothesis that NGR-hTNF can break the BBB, thereby improving penetration and activity of R-CHOP in patients with relapsed/refractory PCNSL (NCT03536039). Patients received six R-CHOP21 courses, alone at the first course and preceded by NGR-hTNF (0.8 μg/m²) afterwards. This trial included two phases: an "explorative phase" addressing the effect of NGR-hTNF on drugs pharmacokinetics and on vessel permeability, assessed by DCE-MRI and ^{99m}Tc-DTPA-SPECT, and the expression of CD13 on tumor tissue; and an "expansion phase" with ORR as primary endpoint, where the two-stage Simon Minimax design was used. At the first stage, if ≥4 responses were observed among 12 patients, the study accrual would have continued (sample size: 28). Herein, we report results of the explorative phase and the first-stage analysis (n=12).

CD13 was expressed in tumor vessels of all cases. NGR-hTNF selectively increased vascular permeability in tumoral/peritumoral areas, without interfering with drug plasma/CSF concentrations. NGR-hTNF/R-CHOP combination was well tolerated: there were only two SAEs and grade-4 toxicity was almost exclusively hematological, which were resolved without dose reductions or interruptions. NGR-hTNF/R-CHOP was active, with nine confirmed responses (75%; 95%CI=51-99%), eight of which were complete. In conclusion, NGR-hTNF/R-CHOP was safe in these heavily-pretreated patients. NGR-hTNF enhanced vascular permeability specifically in tumoral/peritumoral areas, which resulted in fast and sustained responses.

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R-CHOP PRECEDED BY BLOOD-BRAIN BARRIER PERMEABILIZATION WITH ENGINEERED TUMOR NECROSIS FACTOR-α IN PRIMARY CNS LYMPHOMA

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Running title: R-CHOP preceded by NGR-hTNF in PCNSL

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Preliminary results of this trial have been presented IN PART at ASCO 2018 and ASH 2018.

KEY POINT SUMMARIES

- TNF-α coupled with NGR peptide targets $CD13^+$ tumor vessels and increases vascular permeability selectively in tumor/peritumoral areas of PCNSL.
- R-CHOP preceded by low-dose NGR-hTNF was not associated with unexpected toxicity, and resulted in tumor regression in 9 of 12 PCNSL patients.

ABSTRACT

Patients with primary CNS lymphoma (PCNSL) are treated with high-dose-methotrexate-based chemotherapy, which requires hospitalization and extensive expertise to manage related toxicity. The use of R-CHOP could overcome these difficulties, but blood-brain barrier (BBB) penetration of related drugs is poor. Tumor Necrosis Factor–α coupled with NGR (NGR-hTNF), a peptide targeting $CD13^+$ vessels, induces endothelial permeabilization and improves tumor access of cytostatics. We tested the hypothesis that NGR-hTNF can break the BBB, thereby improving penetration and activity of R-CHOP in patients with relapsed/refractory PCNSL (NCT03536039). Patients received six R-CHOP21 courses, alone at the first course and preceded by NGR-hTNF (0.8 μg/m²) afterwards. This trial included two phases: an "explorative phase" addressing the effect of NGR-hTNF on drugs pharmacokinetics and on vessel permeability, assessed by DCE-MRI and ^{99m}Tc-DTPA-SPECT, and the expression of CD13 on tumor tissue; and an "expansion phase" with ORR as primary endpoint, where the two-stage Simon Minimax design was used. At the first stage, if ≥4 responses were observed among 12 patients, the study accrual would have continued (sample size: 28). Herein, we report results of the explorative phase and the first-stage analysis (n=12).

CD13 was expressed in tumor vessels of all cases. NGR-hTNF selectively increased vascular permeability in tumoral/peritumoral areas, without interfering with drug plasma/CSF concentrations. NGR-hTNF/R-CHOP combination was well tolerated: there were only two SAEs and grade-4 toxicity was almost exclusively hematological, which were resolved without dose reductions or interruptions. NGR-hTNF/R-CHOP was active, with nine confirmed responses (75%; 95%CI=51-99%), eight of which were complete. In conclusion, NGR-hTNF/R-CHOP was safe in these heavily-pretreated patients. NGR-hTNF enhanced vascular permeability specifically in tumoral/peritumoral areas, which resulted in fast and sustained responses.

KEY WORDS: Blood-brain barrier, tumor necrosis factor, CNS, diffuse large B-cell lymphoma, vascular permeability.

INTRODUCTION

Primary CNS lymphoma (PCNSL) is an aggressive malignancy with the peculiar clinical behavior to remain confined to the CNS, with rare cases of extra-CNS dissemination[1]. Accordingly, PCNSL is a stage-IE disease with a diffuse large B-cell lymphoma (DLBCL) morphology in more than 95% of cases[2]; this represents a new entity called “primary diffuse large B-cell lymphoma of the CNS” in the 2017 WHO classification of haematopoietic and lymphoid tumors[3]. In comparison with limited-stage extra-CNS DLBCL, PCNSL patients show poorer survival figures, which has been attributed, at least in part, to the inefficacy of drugs currently used to treat extra-CNS DLBCL (i.e., R-CHOP regimen) to cross the blood-brain barrier (BBB) and to achieve efficient tumor concentrations[4]. This pharmacokinetic limitation coupled to the negative results of a randomized trial[5] led to the exclusion of CHOP regimen as part of first-line treatment of PCNSL patients. Currently, PCNSL patients are treated with high-dose methotrexate-based combinations, often in association with cytarabine, alkylating agents and rituximab[6]. The diffuse use of these modern combinations has significantly improved survival in PCNSL patients, but these treatments require hospitalization, adequate direct experience and are often burdened by relevant toxicity. Conversely, enhanced CNS delivery of R-CHOP could result in important advantages: this is a well-tolerated therapy, widely used in onco-haematological centers, that does not require hospitalization. Therefore, the use of intravenous agents capable of inducing a reversible BBB permeabilization and to enhance tumor penetration of anticancer drugs is an attractive investigational approach in PCNSL patients.

Using animal models of brain metastasis, it has been demonstrated that intravenous administration of tumor necrosis factor (TNF), an inflammatory cytokine capable of altering endothelial cell-cell adhesion and barrier function, can induce selective BBB permeabilization and, consequently, can enhance tumor penetration of chemotherapeutic agents[7]. Unfortunately, the use of TNF in cancer patients is limited by prohibitive systemic toxicity, mainly due to vascular leakage syndrome resulting in haemodynamic instability, hypotension and pulmonar oedema[8]. A growing body of evidence suggests that the therapeutic index of this cytokine can be enhanced by a vascular targeting approach[8]. This can be achieved, for example, by fusing the N-terminus of human TNF with CNGRCG, a tumor vasculature-homing peptide capable of recognizing an isoform of aminopeptidase N (CD13), a membrane-bound metalloproteinase, up-regulated in angiogenic tumor blood vessels[9, 10] and barely or not at all expressed by normal blood vessels[11]. The CNGRCG-

TNF fusion protein made with human TNF (developed at the San Raffaele Scientific Institute of Milan, Italy, and called NGR-hTNF) allows the delivery of extremely low, yet pharmacologically active, doses of cytokine to the tumor vasculature, thereby avoiding systemic toxic reaction and counter-regulatory mechanisms[12]. MRI measurements with blood pool contrast agent performed in lymphoma-bearing mice have shown that low-dose murine NGR-TNF can increase the leakage of the contrast agent from the vasculature to the tumor tissue, suggesting an increase of local vascular permeability[13]. Furthermore, studies performed in melanoma and lymphoma animal models have shown that low-dose NGR-hTNF can locally enhance vascular permeability and increase the penetration of chemotherapeutic drugs in tumor tissues[8, 10, 12, 14]. The NGR-hTNF has been tested in various phase II and III trials on different types of tumors, alone and in combination with various chemotherapeutic agents, with an excellent safety profile and evidence of activity[8]. In a randomized trial on 400 patients with relapsed or refractory mesothelioma enrolled in 12 countries, although the primary endpoint was not reached, the addition of NGR-hTNF to the best investigator choice was associated with significant improved survival in the subgroup of patients with refractory or early relapsed mesothelioma without increased toxicity[15].

Based on these notions, we hypothesized that low-dose NGR-hTNF can alter the BBB and enhance tumor penetration and activity of R-CHOP in patients with PCNSL. As a part of a translational research program of NGR-hTNF, we designed a prospective phase II trial aimed to assess the feasibility and activity of R-CHOP chemoimmunotherapy preceded by BBB permeabilization with NGR-hTNF in patients with relapsed or refractory PCNSL. In a per-protocol planned “proof-of-principle” part of the trial, we investigated changes in the BBB permeability in the lymphomatous lesions and in the normal-appearing brain parenchyma by Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI) and single photon emission computed tomography (SPECT) in enrolled patients. Changes in the concentrations of R-CHOP drugs in plasma and cerebrospinal fluid (CSF) samples and expression of CD13, the target of NGR-hTNF, in vascular cells of diagnostic biopsies were also investigated as indicators of the specificity of the effect of NGR-hTNF on the tumor vasculature. Herein, we report the results of this “proof-of-concept” study as well as the per-protocol first-step analysis as an initial effort towards the development of a simple, manageable and active treatment for PCNSL patients, analogous to the worldwide used treatment of systemic DLBCL.

PATIENTS AND METHODS

Study population and selection criteria

The "INGRID" study is a single-arm phase II trial focused on an experimental treatment consisting of six courses of conventional doses R-CHOP21 preceded by NGR-hTNF infusion in HIV-negative patients with relapsed or refractory PCNSL (EUDRACT: 2014-001532-11 – clinicaltrials.gov NCT03536039). The trial has two distinct parts: a) an exploratory phase focused on the feasibility of NGR-hTNF/R-CHOP and "proof of principle" of the effects of NGR-hTNF on vascular permeability in the first 10 enrolled patients, and b) an expansion phase focused on activity and tolerability of the experimental treatment in the whole enrolled series. Selection criteria were: 1) histologically-proven diagnosis of DLBCL according to the WHO criteria[16]; 2) disease exclusively localized in the CNS, cranial nerves, meninges, and/or eyes both at first diagnosis and trial registration; 3) lymphoma relapsed after or refractory to prior chemotherapy containing high-dose methotrexate; 4) measurable disease; 5) age 18-80 years; 6) ECOG performance status score ≤3. Patients with prior organ transplant or other forms of immunosuppression, with HBV, HCV and/or HIV infections, or other malignancies were excluded. Any kind of consolidation therapy (i.e., whole-brain radiotherapy -WBRT-, autologous stem cell transplantation -ASCT-, oral drug maintenance) during prior lines was admitted. Before trial registration, histopathological diagnostic specimens and neuroimaging exams performed at diagnosis and relapse were centrally reviewed (M.P. and N.A., respectively), and patients were assessed by physical & neurological examination, haemogram and biochemical serum profiles, echocardiography, enhanced total-body CT scan, bone marrow biopsy, contrast-enhanced brain MRI, CSF examination, ophthalmologic evaluation, and ^{18}FDG-PET. Risk was defined according to the IELSG score[17]. Written informed consent was obtained from each patient. This trial conformed to the Declaration of Helsinki and was approved by the IRBs of the San Raffaele Scientific Institute of Milano, Italy.

Trial Design and Experimental Treatment

The design of the explorative phase is summarized in figure 1. The first ten enrolled patients received a first course of R-CHOP (schedule in Supplemental Data) that was not preceded by NGR-hTNF; this was used to explore the response to R-CHOP alone and as comparator to establish the effect of NGR-hTNF on vascular permeability (see below). The

other five courses of R-CHOP were preceded by NGR-hTNF (0.8 μg/m^2 delivered 2 hours before CHOP by a 1-hour infusion). The rationale for the timing and administration schedule of NGR-hTNF is summarized in Supplemental Data. Patients enrolled in the expansion phase (after the first 10) received the six courses of R-CHOP preceded by NGR-hTNF. Oral or intravenous acetaminophen/paracetamol at a dose of 1.000 mg were delivered as prophylaxis of infusion-related reactions, 30 to 60 minutes prior starting each infusion of NGR-hTNF. No concomitant hydration was allowed during the NGR-hTNF infusion. Steroids, other than the five days of prednisone, were avoided, and, when clinically indicated, they were interrupted the day of NGR-hTNF infusion. Therapy with proton pump inhibitors was avoided as these drugs can increase chromogranin levels, which can interfere with NGR-hTNF activity. H2-blockers (i.e. ranitidine) were allowed as gastro-protective therapy.

Patients who completed the six planned courses and achieved a complete (CR) or partial (PR) response during explorative or expansion phases were evaluated for consolidative therapy. Per protocol, and accordingly to prior treatments, WBRT 30-36 Gy, carmustine-thiotepa-conditioned ASCT or oral lenalidomide maintenance were allowed.

Toxicity and response assessments

Treatment side effects were assessed separately for each chemotherapy course and graded according to the NCI-NCIC CTC version 3·0[18]. The worst toxicity per organ, per patient was considered. Periodic specialist controls, ECG, troponin levels determination, and echocardiography were performed before every treatment course to exclude cardiac toxicity. The impact of treatment on cognitive functions was not assessed by *ad hoc* tests.

All eligible patients were considered for response evaluation. Response was assessed by gadolinium-enhanced MRI of the brain performed on a 1.5 Tesla scanner after the 1st, 2nd, 4th, and 6th course of treatment (Fig. 1). In cases with concomitant positive CSF and/or vitreous, examination was performed after the 2nd, 4th and 6th courses. Response was defined according to the IPCG criteria[19]: briefly, CR consisted of disappearance of all evidence of lymphoma; PR was a >50% decrease in tumour size; progressive disease (PD) was a >25% increase in tumour size or detection of any new lesion; all other situations were considered as stable disease (SD). As an important change in the IPCG criteria, a "response" was considered only whenever tumor regression was confirmed in two consecutive MRIs; accordingly, every "response" required a minimum duration of 6-8 weeks. Response after the first course of R-CHOP did not drive therapeutic decision,

whereas patients with PD at any of the following MRIs were considered "off study" and treated according to institutional guidelines. The maximum response recorded from treatment start was considered for analyses. The duration of response was measured from the date of maximum response to the date of objective progression, death for any cause or last visit of follow-up. After end of treatment, the disease was assessed every three months.

BBB permeability assessed by neuroimaging

Variations induced by NGR-hTNF in the BBB permeability at the level of the lymphomatous lesions, areas surrounding the tumor (perilesional area) and in the normal-appearing brain parenchyma were assessed by DCE-MRI. DCE acquisition followed a standard protocol[20] that included also conventional T1, T2, Flair, DWI and Dynamic Susceptibility Contrast Perfusion (DSC) sequences. As represented in Fig. 1, DCE-MRI was performed within the conventional MRI study in day 0 (pre-treatment – baseline data) and day 1 (post-treatment) of the 1st (R-CHOP alone), 2nd (first course of NGR-hTNF/R-CHOP) and 6th (last course of NGR-hTNF/R-CHOP) treatment courses. In cases of multiple lesions, the largest one was considered. Data were suitable for analysis only in patients with responsive disease and measurable residual lesions. Post-processing of DCE-MRI was performed using Olea software (La Ciotat, France); all dynamic images were corrected for motion artifacts and co-registered to a volumetric post-contrast T1 sequences. Results were expressed as Ktrans values, which were estimated as a pre-/post-treatment ratio and normalized using contralateral white matter. Ktrans obtained after the 2nd course (after NGR-hTNF infusion) were compared with those obtained after the 1st course (without NGR-hTNF) to establish the effect of TNF on vascular permeability. Statistically significance was assessed by Wilcoxon matched pairs test.

BBB permeability assessed by SPECT

Changes in BBB permeability induced by NGR-hTNF were assessed also by brain scintigraphy. Because of its hydrophilic property, ^{99m}Tc-diethylene-triamine-pentacetic acid (^{99m}Tc-DTPA) penetrates only the disrupted BBB, and spreads into the altered tissues. The amount of tracer's uptake at the level of the brain lesions increases proportionally to the degree of vascular permeabilization. Brain scintigraphy was acquired twice (Fig. 1), in basal condition, some days before the 3rd course of treatment (median 4 days, range 1-6), and after the end of the 3rd course; details of this procedure are summarized in

Supplemental Data. A volume of interest of 30% of maximum uptake was drawn around the ^{99m}Tc-DTPA positive area(s) by an automatic isocontour method. Statistically significance of changes in volume of ^{99m}Tc-DTPA uptake between SPECTs performed in basal conditions (before NGR-hTNF) and after NGR-hTNF delivery was assessed by Wilcoxon matched pairs test.

Expression of the target receptor of the CNGRCG peptide (CD13)

The CNGRCG moiety of NGR-hTNF can recognize a CD13 form expressed by tumor vessels, resulting in targeted delivery of TNF to the tumor vessels. The presence of this CD13 form in tumors was assessed by immunohistochemical and immunofluorescence techniques on paraffin-embedded specimens of diagnostic tissue samples of enrolled patients; pericytes were detected with an anti-α–smooth muscle actin (αSMA) antibody (Methodological details in Supplemental Data). CD13 expression did not condition patient registration in the trial or experimental treatment.

Drug concentrations in CSF and plasma samples

Changes in plasmatic and CSF concentrations of rituximab, cyclophosphamide and doxorubicin, as well as in the CSF-to-plasma ratio potentially related to the treatment were considered as surrogate parameters to establish the specificity of vascular permeabilization effect of NGR-hTNF. Concentrations of these drugs were assessed on matched CSF and plasma samples collected on day 0 (baseline) and day 1 (one hour after treatment) of the 1st (R-CHOP alone), 2nd (first course of NGR-hTNF/R-CHOP) and 6th (last course of NGR-hTNF/R-CHOP) courses of treatment (Fig. 1). CSF and plasma concentrations of rituximab were determined using a validated ELISA method (lower limit of quantification, LOQ: 1.0 ng/mL). CSF and plasma concentrations of cyclophosphamide and doxorubicin were determined using liquid-chromatography-tandem mass spectrometry methods chromatographic methods (LOQ: 0.01 mg/L and 2.5 ng/mL, respectively)[21]. Statistical significance of differences between post-treatment drug concentrations achieved after the first course and after the second course was assessed by Wilcoxon matched pairs test.

Statistical considerations

Feasibility of NGR-hTNF/R-CHOP combination was the primary endpoint of the exploratory part of the trial, which required 10 enrolled patients. We considered feasibility

as an indicator of toxicity and tolerability and regarded the proportion between delivered and planned treatment courses, treatment delays, interruptions and dose reductions due to toxicity, severe adverse events, and unexpected side effects. Treatment interruptions due to lacking of tumor response were not considered to define feasibility. Secondary endpoints included overall response rate, changes in BBB permeability, expression of CD13, and changes in R-CHOP drugs pharmacokinetics. In the case the experimental treatment would be safe and some tumor responses would be recorded, the chairman, after due multidisciplinary discussion, could propose to proceed with an open, non-comparative phase II trial, with overall response rate (ORR: CR and PR) as the primary endpoint. Accordingly, the two-stage Simon Minimax design was used. The maximum ORR considered of low interest was 30% (rate reported in prior prospective trials focused on salvage treatment in PCNSL patients performed at our institution[22,23]), and the minimum ORR considered of interest was 50%; to demonstrate that difference, a total of 28 patients was needed (one-sided test; type I error .10; power .9). At the first step, 12 patients (including the 10 patients of the exploratory phase) would be registered and, if at least four responses were observed, the study would have continued up to a total of 28 patients. Herein, we report results of both the explorative phase and the first step of the Simon minimax model.

RESULTS

Study population

Median age of the 12 assessed patients was 61 years old (range 41-68); eight were males (Table 1). At trial registration, 11 patients had intermediate-high IELSG risk score, with an ECOG-PS ≥2 in seven patients, increased LDH serum level in six, high CSF protein concentration in six, involvement of deep areas of the brain in five. All patients had brain parenchymal lesions, with concomitant intraocular disease in one; no patient had meningeal disease. Patients were heavily pretreated; seven received two or more prior treatment lines, and ten received also ASCT, WBRT or both. Seven patients had refractory disease.

Feasibility and Toxicity

Experimental treatment was well tolerated (Table 2); 62 (86%) of the 72 planned courses were delivered: nine patients received the six planned courses; treatment was interrupted

due to PD in the other three patients. There were no cases of unexpected toxicity and no patient required dose reductions. Treatment delay was recorded only in three (5%) courses due to cytopenia. Two severe adverse events were recorded (Table 2), and both severe adverse events and grade-4 toxicities were solved without particular complications. There was a single case of reaction to NGR-hTNF infusion: a 65-year-old gentleman affected by arterial hypertension in treatment from ten years experienced transient grade-2 arterial hypertension during NGR-hTNF infusion at the first course; infusion was interrupted by 15 minutes, patient received symptomatic medication and, as per-protocol, completed the infusion one hour later. The patient received other 5 courses of NGR-hTNF/RCHOP with per-protocol prophylaxis without experiencing infusion reactions. Five patients required blood/platelets transfusions (three of them had received prior ASCT).

Activity

Per protocol, the first ten enrolled patients received a course of R-CHOP without NGR/hTNF; after this course, one patient had PR, seven had SD and two experienced PD (details in Supplementary Table 1). The best response to NGR-hTNF/R-CHOP combination was complete in eight patients (examples in Fig. 2) and partial in one, with an ORR of 75% (95%CI= 51-99%); three patients experienced PD (Fig. 3). Interestingly, one of the patients who experienced PD after the first course of R-CHOP (pt #8) achieved PR after NGR-hTNF/R-CHOP (after the second course of the experimental treatment), which was confirmed after the 4^{th} and 6^{th} courses. The predetermined activity threshold of the first-step analysis of at least four responses in the first 12 registered patients was largely achieved. The best response was achieved after the second course in six patients and after the fourth course in three. All the best responses were confirmed by a second MRI performed six weeks later. Consolidation in responding patients was WBRT in two patients, ASCT in three, lenalidomide maintenance in two, and combinations of these therapies in two. Response lasted more than 6 months in all responders (median 10 months; range 7-14) (Fig. 3). Six responders experienced relapse at 7-12 months; two responders (pt #2 and 10 in Fig. 3) died of complications related to progressive neurological impairment, without evidence of relapsing lymphoma. Four patients are alive at a median follow-up of 19 months (14-28).

BBB permeability assessed by neuroimaging

Per-protocol, vascular permeability changes were assessed in the seven patients with responsive and measurable disease (see Methods). The seven patients were suitable for analysis after the first and second courses, whereas only one patient who experienced partial tumor regression as the best response was assessable after the sixth course as the other six patients had CR at that time. DCE-MRI analysis showed that vascular permeability was increased after the first NGR-hTNF infusion (Fig. 4). This effect was more evident in perilesional areas. The median (range) Ktrans of contrast-enhanced areas after the first course of R-CHOP (without NGR-hTNF) was 23.5 (6.8 - 98.8) and raised to 35.3 (23.9 - 887.7; p=0.39) after the second course (NGR-hTNF/R-CHOP); increase of Ktrans values after NGR-hTNF/R-CHOP was observed in five of the seven analyzed patients. In perilesional areas, baseline values (R-CHOP alone) were lower (median 2.5; range 0.4 - 3.9), but significantly raised to 4.7 (2.2 - 37.7; p= 0.01) after NGR-hTNF infusion in the second course; this increasing effect was confirmed in all the seven assessed patients.

In the single patient assessed after the sixth course, Ktrans were similar to those recorded after the second course, both in enhanced and perilesional areas, which suggests a sustained effect of NGR-hTNF.

BBB permeability assessed by SPECT

The capability of NGR-hTNF to increase vascular permeability in tumor and perilesional areas was confirmed by SPECT studies. Quantitative analysis showed an increase in the extent of the ^{99m}Tc-DTPA positive region(s) in all the investigated cases (an example in Fig. 5). The median volume of ≥30% ^{99m}Tc-DTPA uptake (volume of interest) measured before and after the infusion of NGR-hTNF/R-CHOP was 26 cm^3 (range 5 – 67) and 40 cm^3 (range 10 – 92), respectively (p= 0.028). There was a median volume increase of 45%, with a range of 14% - 87%.

Expression of the target receptor of the CNGRCG peptide (CD13)

Immunohistochemical staining demonstrated the presence of CD13 in diagnostic brain biopsies of the 12 registered patients; stained vessels in most instances showed narrowed lumina with irregular outlines (Fig. 6A). Immunohistochemical and confocal immunofluorescence analysis of tissue sections stained with an anti-CD13 polyclonal antibody and with anti-αSMA (a marker of pericytes) antibody showed that most stained vessels lacked pericyte layer (Fig. 6B), likely corresponding to immature vessels. 3D

projections of more mature vessels showed that CD13 was expressed on the luminal side of tumor vessels (Fig. 6C-D), which is accessible to NGR-hTNF delivered by intravenous route.

Drugs concentrations in CSF and plasma samples

The effect of NGR-hTNF was specific for the tumor area as suggested by the fact that this drug did not change plasmatic and CSF levels of R-CHOP drugs. Indeed, a significant effect of NGR-hTNF on plasma concentrations of doxorubicin and cyclophosphamide was not observed, as drug concentrations in the baseline samples collected before each course were invariably undetectable (data not shown), and concentrations in samples collected after each course were stable from the 1st to the 6th course (Table 3). In line with the well-known prolonged terminal half-life of rituximab (up to 80 days)[24], a progressive increase in the basal (from 16.6±14.1 to 73.6±43.8 ng/mL) and peak (from 45.4±17.0 to 110.2±93.4 ng/mL) plasma concentrations of this antibody was found from the 1st to the 6th course. As expected, doxorubicin and rituximab were not detected in CSF samples, while detected CSF levels and CSF/plasma ratio of cyclophosphamide did not change after NGR-hTNF delivery (Table 3).

DISCUSSION

At the best of our knowledge, this is the first prospective trial focused on the feasibility and activity of R-CHOP in patients with relapsed or refractory PCNSL. Importantly, this trial develops an innovative strategy for increasing the BBB permeability and drugs penetration in tumor and perilesional areas. This strategy exploited the use of NGR-hTNF, a TNFα derivative capable of targeting tumor blood vessels and increasing endothelial permeability. Our results indicate that the NGR-hTNF/R-CHOP combination is feasible and well tolerated, even in heavily pretreated patients. DCE-MRI and SPECT studies confirmed that NGR-hTNF selectively enhances the vascular permeability in the tumor and peritumoral areas. The tumor specificity of NGR-hTNF effects is supported also by the lack of changes in concentrations of R-CHOP drugs in plasma and CSF samples, which *bona fide* excludes nonspecific effects of this cytokine on CSF filtration in choroidal plexus and drugs pharmacokinetics. Importantly, neuroimaging and histopathological data were consistent with activity of this experimental strategy. In fact, nine out of 12 assessed patients achieved fast and prominent tumor regression after NGR-hTNF/R-CHOP

treatment, which was complete in eight and allowed the use of consolidation therapies in all responsive patients. Efficacy of NGR-hTNF/R-CHOP on tumor cells growing in compartments such as the eye or leptomeninges remains to be defined because none of the 12 treated patients had meningeal disease at trial registration, whereas only one of them had subretinal disease; interestingly, the latter patient did not experience ocular relapse after 27 months from trial registration.

This trial exhibits a few limitations. In particular, sample size of this part of the trial (n=12) seems to be small. However, this study group is appropriate to demonstrate as “proof-of-principle” the effects of NGR-hTNF on tumor BBB. This proof of concept is substantiated by well-standardized, modern diagnostic techniques, and consistency of radiological, pharmacological, histological and clinical data, which enable to draw reliable inferences about BBB changes induced by NGR-hTNF. While this manuscript is mostly focused on the biological implications of this innovative approach, the achievement of the predetermined activity threshold of the first-step Simon’s minimax design (at least four responses in the first 12 registered patients) suggests a promising activity of this strategy, and prompt us to complete the planned accrual (n=28).

Previous studies focused on NGR-hTNF and its synergistic effects with chemotherapeutic agents both in animal models and patients with solid tumors have shown that the selectivity of NGR-hTNF for tumor vessels requires the interaction with specific receptors[12, 14, 25]. In particular, NGR-hTNF, at low doses, may potentially engage high avidity interactions with CD13, TNF-R1 and TNF-R2 on endothelial cells that express these receptors, i.e. angiogenic endothelial cells[26], but not with endothelial cells lacking CD13, as in normal tissues. Importantly, findings of the present trial are in line with these knowledges: the effects of NGR-hTNF were more evident in tumor and peritumoral areas where expression of CD13 by the tumor vessels was confirmed by immunohistochemistry and immunofluorescence techniques.

The NGR-hTNF/R-CHOP combination was safe: unexpected toxicities were not recorded and, importantly, dose intensity was maintained in all cases. Hematological toxicity was well controlled with G-CSF and antibiotics and only a few patients previously treated with ASCT needed for blood/platelets transfusions. These figures are in line with good tolerability of NGR-hTNF reported in previous clinical trials, both when used alone[27, 28] or in combination with chemotherapeutic agents[29]. When doses up to 60 μg/m^2 have been used, chills and fever were the most frequently observed toxicities. In the present trial, a single case of transient infusion reaction (i.e., grade-2 arterial hypertension) was recorded.

Importantly, in line with previous trials[29], the combination of NGR-hTNF with doxorubicin was not associated with severe cardiovascular events.

Published experience with R-CHOP in PCNSL patients is anecdotal, mostly due to the diffuse belief that CNS bioavailability of related drugs is poor. A few, available pre-rituximab clinical studies including more than 20 patients support this notion, which is in line with undetectable concentrations of assessed drugs in CSF samples collected after the first R-CHOP course (without NGR-hTNF) in the present series (Table 3). When used as upfront treatment, CHOP chemotherapy was associated with low response rate and did not contribute to improve disease control in combination with high-dose-methotrexate-based chemotherapy or with WBRT, with a 2-year overall survival after CHOP-WBRT of only 20-40%[5, 30-32]. Studies focused on CHOP ± rituximab in patients with relapsed or refractory PCNSL do not exist; however, disappointing results reported as first-line treatment[5, 30-32] suggest that CHOP ± rituximab should be inactive as salvage therapy. In line with these reports, response achieved after R-CHOP alone in the first 10 enrolled patients was insignificant; most patients had stable or progressive disease, which excludes *bona fide* that responses reached later on by the same patients were exclusively due to R-CHOP activity. Conversely, fast and consistent tumor regression recorded in nine of the 12 assessed patients suggests that the addition of NGR-hTNF results in improved activity of R-CHOP combination. However, these results should be taken into account with caution because PCNSL exhibits important molecular and biological differences with respect to systemic DLBCL, and some of them are related *per se* to a poorer efficacy of R-CHOP. In particular, most cases of PCNSL have activated B-cell-like phenotype[2], a subtype of DLBCL less sensitive to R-CHOP, and exhibit frequent genetic alterations of NF-κB and components of the Toll-like receptor signaling pathway (*MYD88*, *CARD11*, *CD79B),* which is a component of the proximal B-cell receptor signaling pathway[33,34]. These genetic alterations activate the related pathways, increase NF-κB activity and, importantly, are more commonly associated with the less sensitive activated B-cell-like phenotype. Conversely, genetic signature of PCNSL closely resembles that of primary testicular lymphoma[35], an entity that usually exhibits excellent outcome when treated with R-CHOP and suitable CNS and testicular prophylaxes[36]. Accordingly, and as planned by the protocol, the encouraging activity and excellent safety profile of NGR-hTNF/RCHOP, recorded in a group of heavily pretreated patients, deserve to be addressed on a larger number of patients and completion of the estimated accrual of the INGRID trial is warranted.

In conclusion, low-dose NGR-hTNF exerts relevant effects on vascular permeability in patients with relapsed or refractory PCNSL. These effects were specific in tumor and peritumoral areas, and were consistently demonstrated by standardized DCE-MRI, SPECT and plasma/CSF pharmacokinetics studies. NGR-hTNF/R-CHOP combination was well tolerated and followed by fast and prominent tumor regression in nine of the 12 assessed patients. Accrual completion of this trial is warranted, and, in the case of positive results, this innovative approach deserves to be addressed in first-line treatment prospective trials.

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Part of these results have been presented at the 2018 Annual Meeting of the American Society of Hematology and published as abstract meeting: “R-CHOP Preceded by Engineered Tumor Necrosis Factor (TNF) in Patients with Relapsed or Refractory (r/r) Primary CNS Lymphoma (PCNSL): Results of Antitumor Activity, Safety and Blood-Brain Barrier (BBB) Permeabilization in the "Ingrid" Phase II Trial“. Ferreri AJM, Calimeri T, Conte GM, Cattaneo D, Fallanca F, Ponzoni M, Scarano E, Sassone M, Perrone S, Cecchetti C, Lopedote P, Ilariucci F, Rudà R, Angelucci E, Visco C, Pisani F, Fabbri A, Petrucci L, Bregni M, Marino D, Gini G, Ciceri F, Corti A, Anzalone N. Blood 132 (Suppl. 1): abstract 1687, 2018.

AUTHORSHIP CONTRIBUTIONS

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Development of methodology: AJMF, GC, LP, AC, DC, FC, and CB.

Administrative support and data management: ES

Statistics: AN

Treatment of patients: TC, MF, SG, MS, SP, and CC

Neuroimaging assessment and analysis: GMC and NA

SPECT assessment and analysis: FF

Pharmacology assessments and analysis: DC

Histopathology, immunohistochemistry and immunofluorescence: MP, AC and FCu

Acquisition of clinical data: TC and PL

Analysis and interpretation of data: AJMF, TC, AC, NA, and AN

Writing manuscript: AJMF, AC, NA, and MP

Revision and approval of the manuscript: all the authors

DISCLOSURE OF CONFLICTS OF INTEREST

No potential conflicts of interest are disclosed by the authors, with the exception of C.B. who reports employment and equity ownership from MolMed SpA, and A.C. who reports consultancy to MolMed SpA, during the conduct of the study. A.C and F.C. are inventors of a patent on NGR-hTNF.

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Table 1. Patient characteristics

Median Age	61 (range 41-68)
Male:Female	2:1
ECOG – Performance Status >1	7
High lactic dehydrogenase serum level	6
High cerebrospinal-fluid protein concentration*	6
Involvement of deep areas	5
IELSG risk score	
Low	1
Intermediate	9
High	2
Intraocular disease	1
Meningeal dissemination	0
Prior lines	
Prior lines ≥2	7
Prior Autologous Stem Cell transplantation (ASCT)	3
Prior Whole-Brain Irradiation (WBRT)	3
Both ASCT + WBRT	4
Refractory to prior lines	7

*Lumbar puncture was contraindicated in three patients; CSF protein concentration was considered as unfavorable feature in IELSG risk score in these patients.

Table 2. Tolerability and toxicity

	Grade 1-2	Grade 3	Grade 4	Grade 5
Neutropenia	6 (10%)	6 (10%)	35 (56%)	-
Thrombocytopenia	21 (34%)	15 (24%)	11 (18%)	-
Anaemia	47 (76%)	6 (10%)	1 (2%)	-
Febrile Neutropenia	-	3 (5%)	1 (2%)^	-
Hepatotoxicity	7 (11%)	1 (2%)	1 (2%)	-
Urinary tract infection	-	1 (2%)	-	-
Constipation	1 (2%)	-	-	-
Cardiotoxicity[#]	1 (2%)^	-	-	-
NGR-hTNF Infusion reaction[§]	1 (2%)	-	-	-

All toxic events other than alopecia are reported.

Denominator is the total number of delivered courses (n= 62).

^ Severe adverse event.

[#] Transient grade-2 left ventricular ejection fraction reduction.

[§] Transient grade-2 arterial hypertension.

Table 3. Concentrations of doxorubicin, cyclophosphamide and rituximab in plasma and cerebrospinal fluid samples

Drugs concentrations	**Without NGR-hTNF**[a]	**After NGR-hTNF**[b]	**p-value**
Plasma			
Doxorubicin, ng/mL	29.6 ± 7.4	26.0 ± 6.7	0.43
Cyclophosphamide, mg/L	26.3 ± 7.7	27.8 ± 7.9	0.17
Rituximab, ng/mL	45.4 ± 17.0	69.1 ± 13.4	0.04
Cerebrospinal fluid (CSF)			
Doxorubicin, ng/mL	<2.5 (all samples)	<2.5 (all samples)	
Cyclophosphamide, mg/L	14.1 ± 3.5	15.5 ± 4.8	0.27
Rituximab, ng/mL	<1.0 (all samples)	<1.0 (all samples)	
CSF/plasma ratio[c]			
Cyclophosphamide, %	60 ± 20	62 ± 19	0.73

[a] Samples collected after the first course of treatment, that is after R-CHOP without NGR-hTNF.

[b] Samples collected after the second course of treatment, that is after NGR-hTNF followed by R-CHOP.

[c] The ratio was not estimated for doxorubicin and rituximab because CSF concentrations resulted below the LOQ.

FIGURE LEGENDS

Figure 1: Trial design.

Enrolled patients received a first course of R-CHOP that was not preceded by NGR-hTNF, while the other five courses were preceded by NGR-hTNF. Each of the lines corresponds to a treatment course. The first column regards Gadolinium-enhanced magnetic resonance imaging (Brain MRI) performed for response assessment; the second column represents cerebral dynamic contrast-enhanced MRI (DCE-MRI) and single positron emission computerized tomography (SPECT) performed before treatment course (day 0) and used as baseline data; the third and fourth columns regard treatment courses; the fifth column represents DCE-MRI and SPECT performed after treatment and used to assess changes in BBB permeability. Arrows represent collection of CSF and plasma samples.

Figure 2: Examples of responses to R-CHOP preceded by NGR-hTNF.

A) Gadolinium-enhanced T1 weighted scan shows a large homogeneous enhancing lesion in the left frontal lobe (arrows) in a 62-year-old woman at the second relapse after high-dose-methotrexate and after salvage high-dose-ifosfamide-based therapy; disease was refractory to prior lines. B) Tumor regression after two courses of experimental treatment. C) Gadolinium-enhanced T1 weighted scan shows a large enhancing left temporal lesion (arrows) in a 65-year-old gentleman at the second relapse after high-dose-methotrexate and after salvage whole-brain irradiation. D) Tumor regression after two courses of experimental treatment.

Figure 3: Swimmer plot of responses and duration of responses.

The best response to NGR-hTNF/R-CHOP was complete in eight patients (blue) and partial in one (green); three patients experienced progressive disease (red). Response lasted more than 6 months in all responders. Six responders experienced relapse (R) at 7-12 months; two responders (patients #2 and 10) died of complications related to progressive neurological impairment, without evidence of relapsing lymphoma. Four patients (#4, 7, 8, and 12) are alive at 27, 19, 19, and 13 months from trial registration. Bars were cut at 16 months for clarity.

Figure 4: Changes in BBB permeability assessed by DCE-MRI in responders.

Changes in the enhanced areas are represented on the left and in the perilesional areas on the right; results are expressed in Ktrans. Values at the first course (without NGR-

hTNF) and second and sixth course (with NGR-hTNF) for each patient are linked with a line. Data were suitable for analysis only in patients with responsive disease and measurable residual lesions. Median and range values per subgroup are reported at the bottom of each graphic.

Figure 5: An example of increase of ^{99m}Tc-DTPA uptake after the infusion of NGR-hTNF followed by R-CHOP at the 3rd course of treatment.

The volume of ≥30% ^{99m}Tc-DTPA uptake is contoured in two SPECT studies performed before (left imagine – blue line) and after (middle imagine – green line) administration of NGR-hTNF and R-CHOP. Comparison of contoured volumes are represented in the gadolinium-enhanced T1-weigthed MRI showing the tumor (right imagine). The volume of interest before and after NGR-hTNF/R-CHOP delivery was 22 cm^3 and 40 cm^3, respectively.

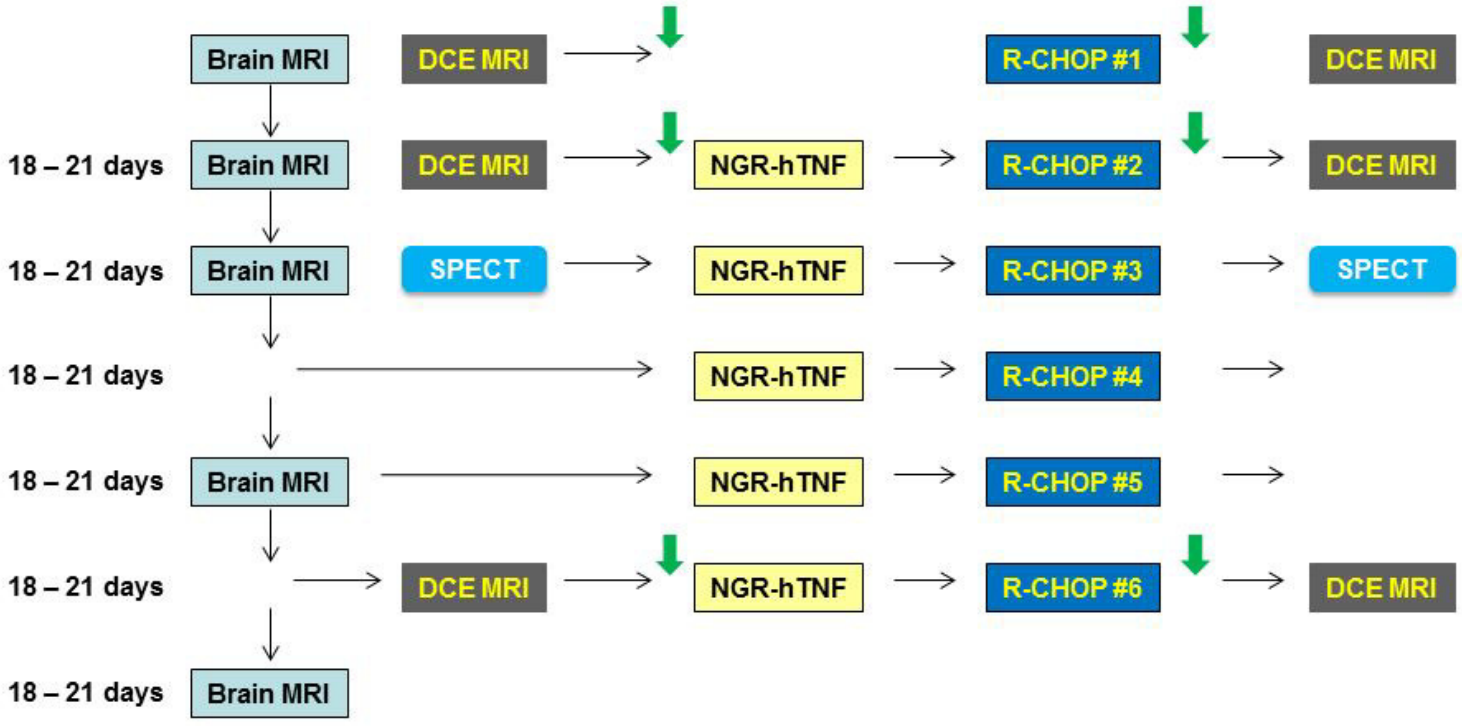
Figure 6: Expression of CD13 by tumor vasculature.

A) Immunohistochemical analysis of CD13 expression within lymphomatous component of diagnostic brain biopsy of an enrolled patient. Staining was performed using the anti-CD13 monoclonal antibody SP187 alone (brown signal, 400x).

B) Immunohistochemical analysis of CD13 and αSMA (a marker of pericytes). The co-staining was performed with the anti-CD13 monoclonal antibody SP187 (brown) and the anti-αSMA monoclonal antibody 1A4 (red). Black arrows indicate CD13-positive vessels; red arrows indicate αSMA-positive perivascular cells (bar 20 μm; 630X). Left panels: representative photograph of areas with large vessels with pericyte coverage (red) and some microvessels, showing CD13 staining (brown) also in the absence of pericytes. Right: representative photograph of an area with several CD13-positive structures, likely corresponding to microvessels (brown).

C-D) Confocal immunofluorescence analysis of a tissue section stained with a polyclonal anti-CD13 antibody (green) and with the anti-αSMA antibody 1A4 (red) (400x, bar: 50 μm). Inset: 3D projection of CD13 and αSMA staining of a mature vessel (asterisk) (400x, bar: 25 μm) showing that CD13 was expressed on the luminal side of the vessels.

Fig. 1


Brain MRI
DCE MRI
R-CHOP #1
DCE MRI
18 – 21 days
Brain MRI
DCE MRI
NGR-hTNF
R-CHOP #2
DCE MRI
18 – 21 days
Brain MRI
SPECT
NGR-hTNF
R-CHOP #3
SPECT
18 – 21 days
NGR-hTNF
R-CHOP #4
18 – 21 days
Brain MRI
NGR-hTNF
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DCE MRI
18 – 21 days
Brain MRI

Fig. 2

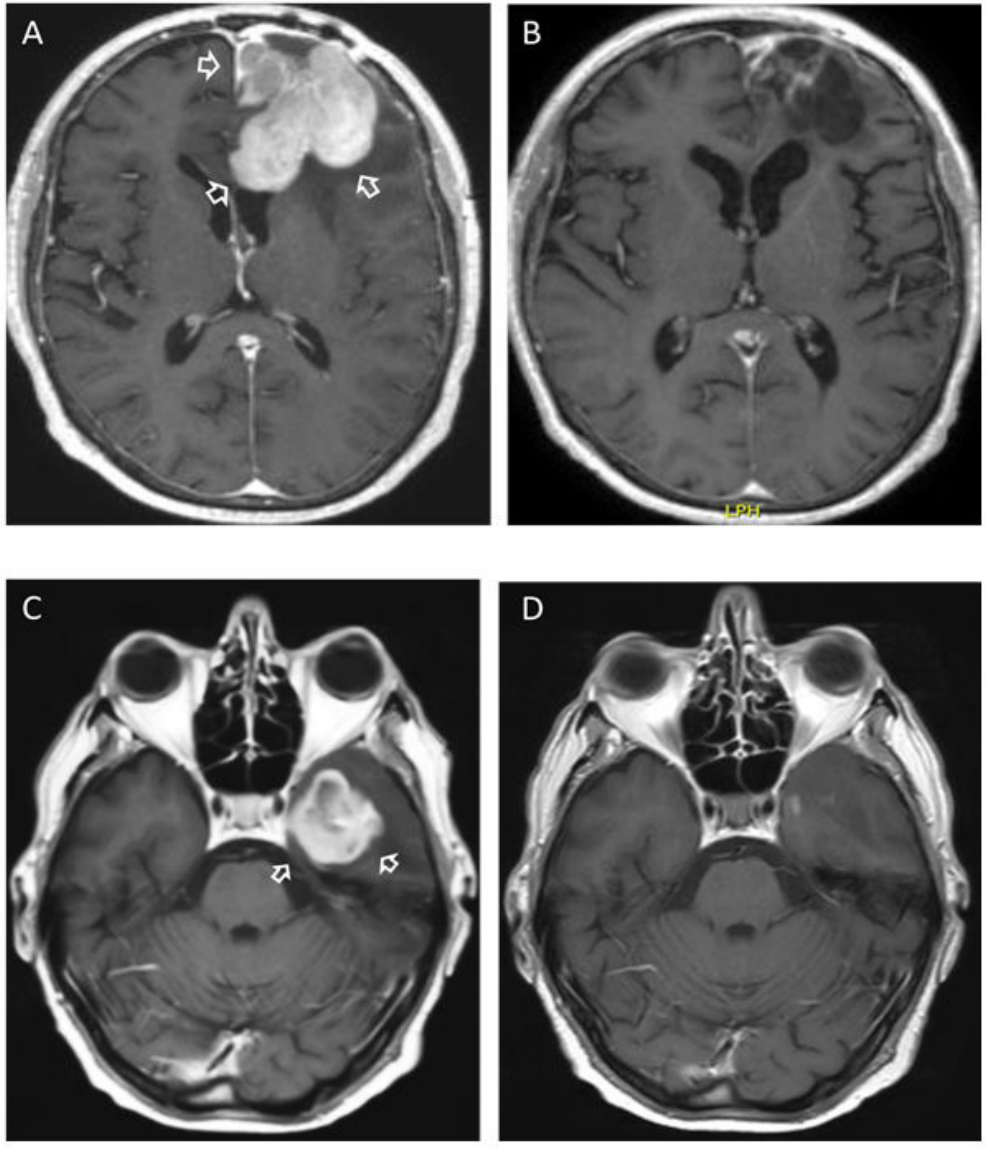
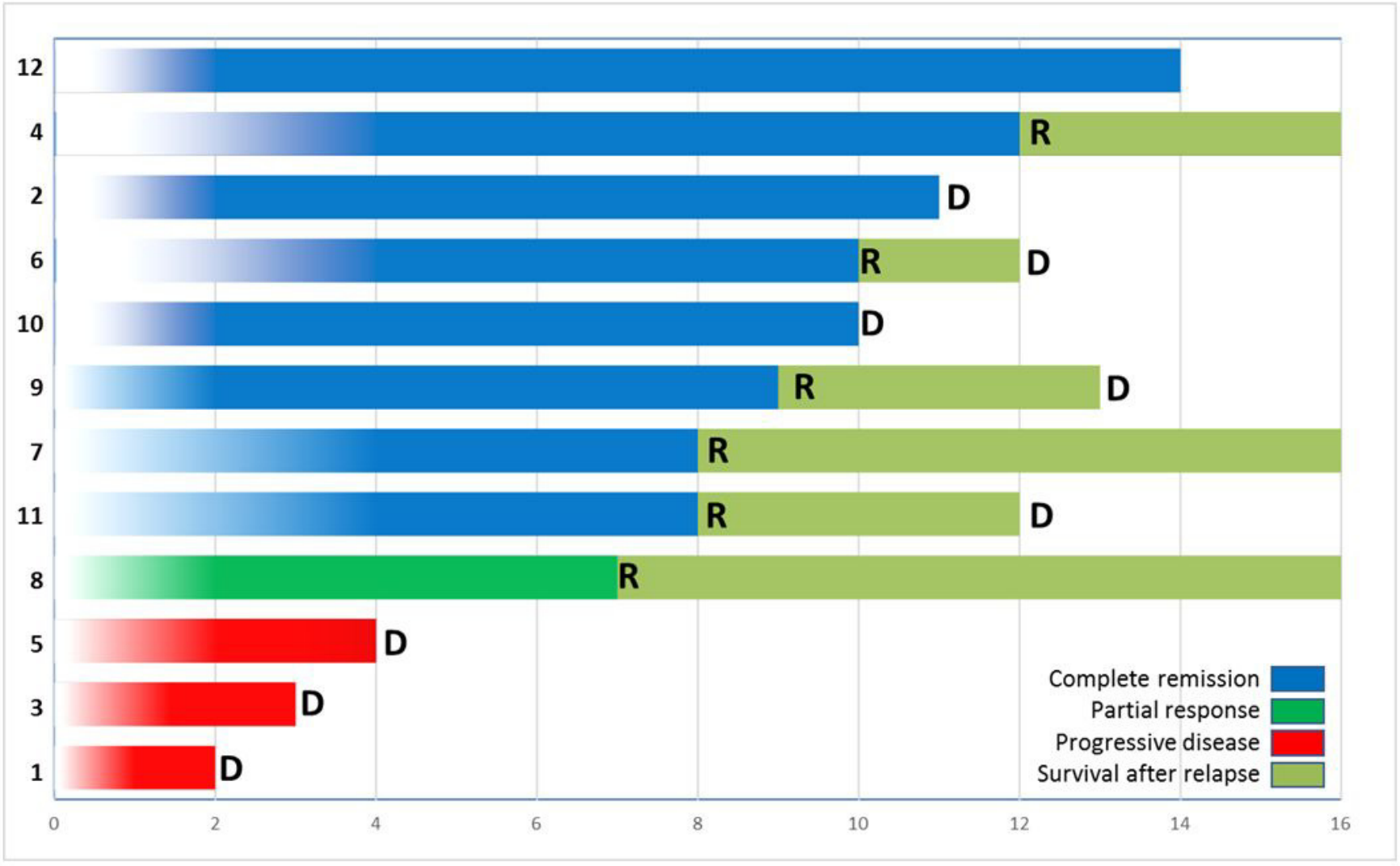


Fig. 3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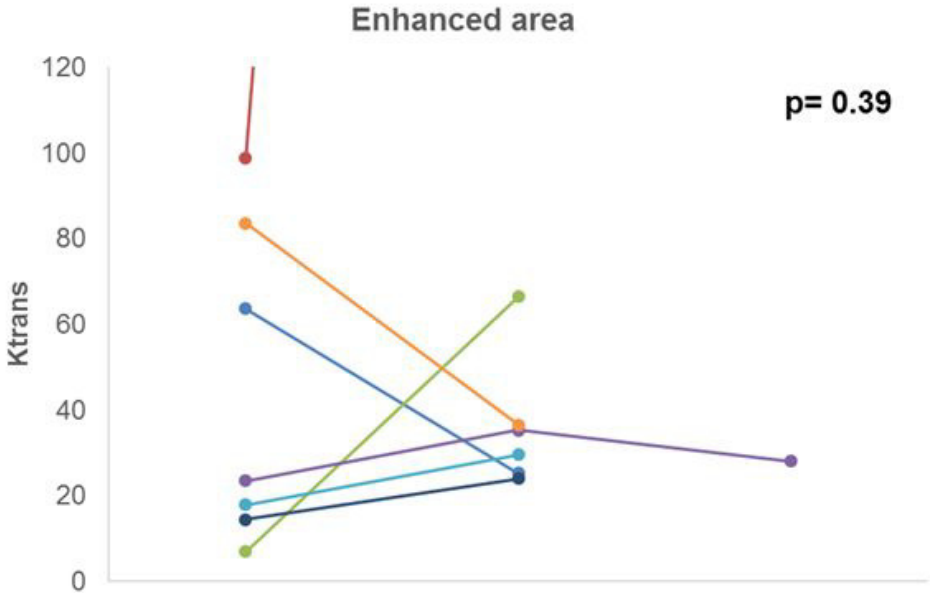
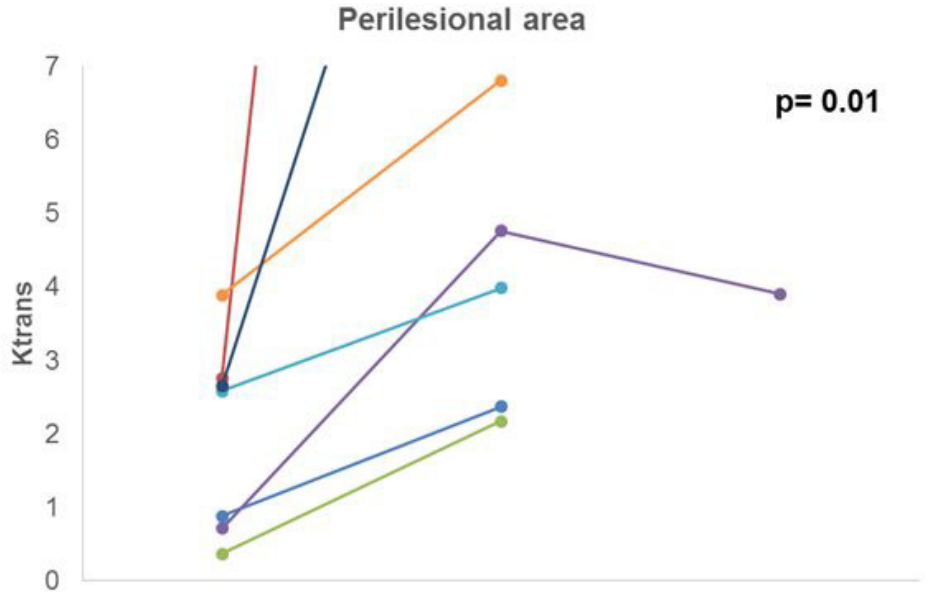
Partial response
Progressive disease
Survival after relapse

Fig. 4





Course	First	Second	Sixth	First	Second	Sixth
NGR-hTNF	NO	YES	YES	NO	YES	YES
Median	23.5	35.3		2.5	4.7	
Range	6.8 – 98.8	23.9 – 887.7		0.4 – 3.9	2.2 – 37.7	

Fig. 5

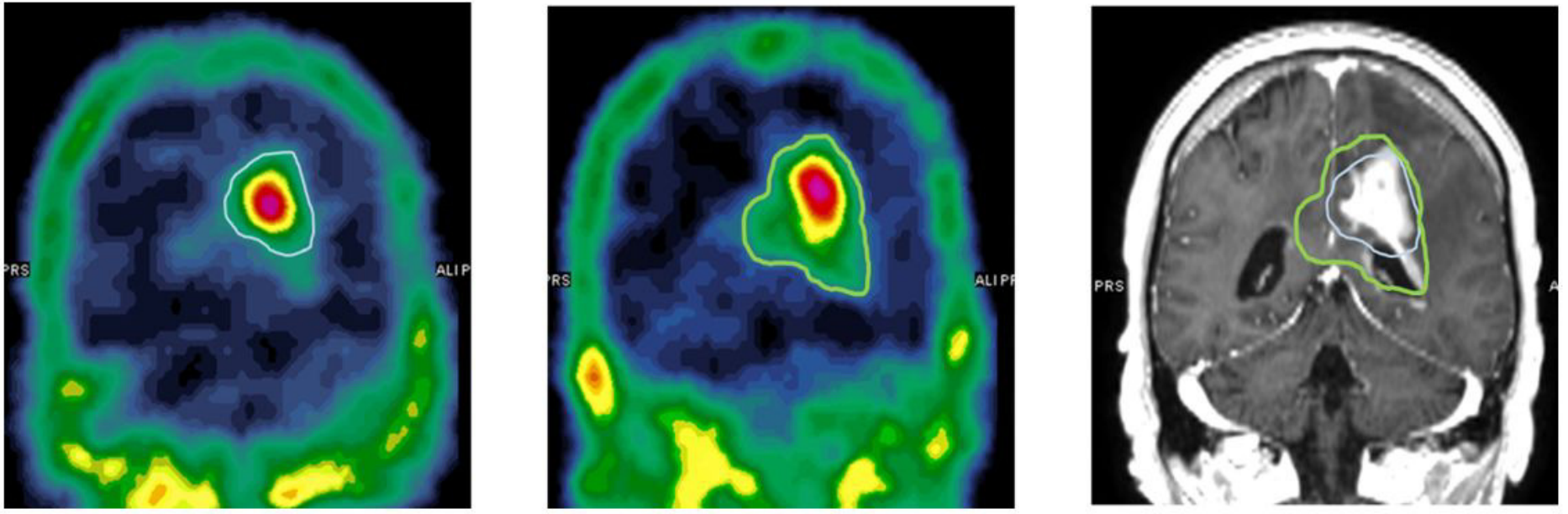
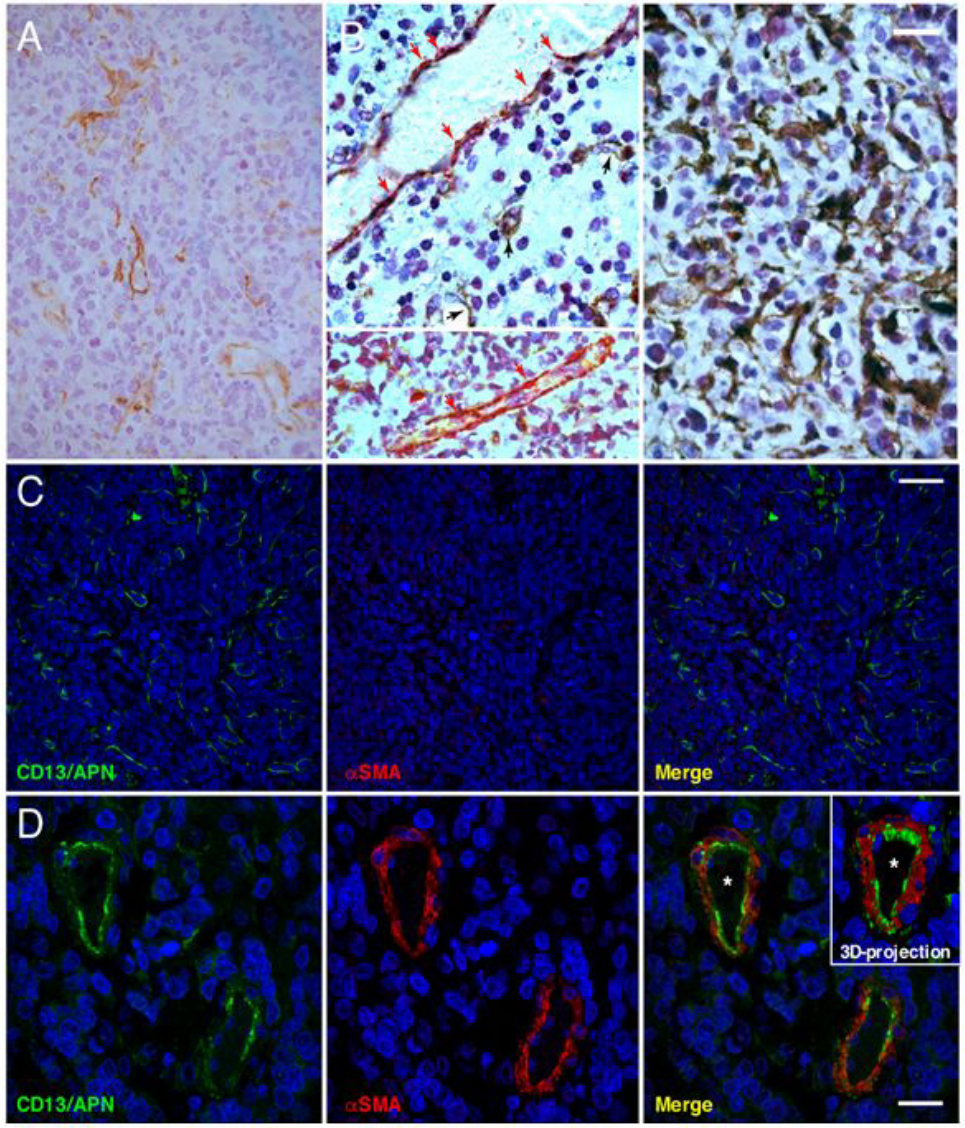


Fig. 6



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R-CHOP preceded by blood-brain barrier permeabilization with engineered tumor necrosis factor- α in primary CNS lymphoma

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