# HYPOXIA MODULATION VS. CHEMOTHERAPY AND TUMOR SHRINKAGE ON EARLY RESPONSE ASSESSMENT IN DIFFUSE LARGE B-CELL LYMPHOMA

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## ABSTRACT

**Background:** *Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) chemotherapy remains the standard of first-line treatment for diffuse large B-cell lymphoma (DLBCL). Up to 40% of DLBCL is characterized by relapse and refractory after treatment. Preliminary study reported Hypoxia-inducible factor-1α (HIF-1α) overexpression in 88.5% of DLBCL tumors in the Dr. Kariadi Hospital. Moreover, the role of hypoxia and HIF-1α has previously never been explored in DLBCL.*

**Objectives:** *To evaluate the effect of hypoxia modulation to increased chemotherapeutic response in DLBCL.*

**Methods:** *Single blind randomized control study was performed, with pre-test and post-test control group design. Research sampling consisted of DLBCL patients. The inclusion criteria include newly diagnosed DLBCL with HIF-1α overexpression and randomized to receive hypoxia modulation consisting of carbogen inhalation and nicotinamide administration, before R-CHOP chemotherapy. The tissue biopsy, histopathology and immunohistochemical studies were done. Chemotherapeutic responses were evaluated after 10-14 days following the first cycle of R-CHOP chemotherapy.*

**Results:** *Out of twenty-six DLBCL participants with HIF-1α overexpression, there were 20 participants who completed the research protocol: 10 participants each in the intervention and control group. Demographic, clinicopathological, laboratory and disease characteristics were not statistically different between the two research groups ($p>0.05$). Baseline tumor volume to be evaluated was also considered equal (172.3 $cm^3$ vs. 152.8 $cm^3$, $p=0.597$). Following the carbogen inhalation and nicotinamide administration, serum HIF-1α and lactate reduction can be observed. There was also a significant tumor volume shrinkage in both the intervention and control (mean –85.7 $cm^3$ vs. –118.27 $cm^3$) group, though the reduction was not statistically different (Delta 58.85% vs. 65.63%, $p=0.474$).*

**Conclusion:** *The addition of hypoxia modulation to R-CHOP chemotherapy for DLBCL has shown beneficial effects on both serum HIF-1α and lactate concentration. However, the benefits did not correlate to increase a better tumor response compared to the control group.*

**Key words:** *Hypoxia modulation, serum lactate concentration, tumor volume, diffuse large B-cell lymphoma*

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## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most frequent classification of non-Hodgkin lymphoma (1). Though the majority of lymphoma are present with visible tumor or mass, they should not be mistakenly considered as solid cancer but definitely a hematological cancer. It is a cancer of lymphocytic origin with morphologic, biologic, immunology and genetic heterogeneity (2,3). DLBCL will be treated "empirically" with R-CHOP in the majority of cases through its diversity and thus bring various therapeutic responses from person-to-person (4-6). Many attempts and modifications have been made to improve the response to therapy, but currently, none can replace the R-CHOP regimen as first-line standard therapy for DLBCL (7).

In general, R-CHOP as a protocol is a combination of anti CD-20 antibody and multiple chemotherapy drugs (described later in the methods section) (5). As an established regimen, each component of R-CHOP should be directly incorporated to proliferated DLBCL cells. It binds to DNA to ultimately induced cellular apoptosis that depends on ATP, p53, and ribosomal protein (pRb) and expectedly leads to eventual tumor shrinkage (8). Hypoxia milieu and drug availability to the tumor core is also different intra-tumoral and moreover from tumor-to-tumor. It has been extensively demonstrated that tumor hypoxia is a major contributor for the failure of chemotherapy and immunotherapy (9, 10). They also need a neutral pH microenvironment to make R-CHOP work appropriately, as they are delivered to the target tissue (11).

Our preliminary study found that DLBCL is a hypoxic tumor as shown by Hypoxia-inducible Factor-1 alpha (HIF-1α) expression from the resected sample (12). Eventually, the study by Liu and Liu revealed that tumor-related tumor microenvironment correlated with therapeutic sensitivity (13). As so, Teicher et al. have shown that chemotherapy was only able to induce cell-kill in a well oxygenated tumor and a recent study by Wei et al reported that chemoresistance is regulated by tumor HIF-1α expression (14,15). Any attempt to reverse hypoxia will theoretically produce some expected benefit at least in a very simple way, which is to evaluate the effect on early tumor shrinkage in chemo-sensitive tumors like DLBCL. It would also be reasonable to combine the hypoxia-reversal agent or methods to standard chemotherapy.

## METHODS

### Study background and research design

This is a single blind, randomized controlled study with a pre- and post-test control group design (Figure 1). The population sample consisted of DLBCL patients who were treated in the Dr. Kariadi Hospital, Semarang, Indonesia. The Dr. Kariadi Hospital is a Ministry of Health vertical hospital, a central referral hospital in Central Java Province and also the main teaching hospital of the Medical Faculty of Diponegoro University. Malignant lymphoma and DLBCL cases were referred to this hospital and treated by hematologists and medical oncologists. For this study, the sampling technique was non-probability with purposive sampling i.e. to meet the predetermined inclusion and exclusion criteria as described below. Assuming the proportion of hypoxic tumor in DLBCL to be 88.5%, (12) a sample size of at least 22 in each group was needed to detect an odds ratio (OR) of 2.0 at a 95% level of confidence interval (CI) with a power of 90% (two tails).

The informed consent for this study has been approved by the committee ethics where this study was conducted according to The Declaration of Helsinki (Ethical clearance: No.736/EC/KEPK/-RSDK/2021) in 7 February 2021 and it is granted by the dr. Kariadi Hospital authority (Study licence: No.DP.02.01/I.II/2366/2021). All studies were performed at the Dr. Kariadi Hospital, Semarang, Indonesia. The trial was registered in ISRCTN77237304, protocol EAP-HYP-1.

### Diagnosis and staging of diffuse large B-cell lymphoma

Each patient diagnosis was based on excisional biopsy examination from the tumor and reviewed by two independent pathologists. Pathological diagnosis was established according to the World Health Organization classification (2). The staging work-up included history and physical examination, blood cell counts and serum chemistry, bone marrow aspiration or biopsy, thorax X-Ray, endoscopy of the gastrointestinal tract, abdominal ultrasound, and Computed Tomography. Each patient's disease was staged according to the Ann Arbor classification, stratified by the NCCN-IPI risk score.

## PATIENT POPULATION AND TREATMENT POLICY

### Patient selection

The patients that were clinically well enough to undergo MR examination were selected from those attending the clinics at the hospital, and did not receive any prior treatment. All histological groups were considered, but lesions had to be over 2 cm in size for inclusion.

## INCLUSION, EXCLUSION AND DROP-OUT CRITERIA

### Inclusion criteria

The inclusion criteria for this study were patients aged 18-65 years with newly diagnosed DLCBL, confirmed by the tissue biopsy-based sample for morphology-histopathology and immunohistochemistry, HIF-1$\alpha$ tumor expression >10%, (16,17), status performance from 0 to 2, and agreed to participate in this research by signing a written informed consent.

### Exclusion criteria

The exclusion criteria were anemia (hemoglobin concentration < 10.0 g/dL), obstructive lung disease (based on X-ray and/or spirometry), heart failure (either clinically or based on echocardiography showing significantly reduced left ventricular ejection fraction), coronary arterial disease, cerebrovascular disease, severe liver dysfunction (total bilirubin ≥2 mg/dl), severe kidney dysfunction (glomerular filtration rate ≤30 ml/min), diabetes mellitus (HbA1c >9%) pregnancy or breast feeding at screening, and history of previous chemotherapy for cancer.

### Drop out criteria

The drop out criteria are when patients decide to resign from the trial at any given time, more than 14 days delayed or overdue, or even die before post-test evaluation, allergic reactions to chemotherapy treatment or intolerant to any intervention.

## DATA COLLECTION AND PROCEDURE

### Tumor assessment and DLBCL examination

Standard methods were used for tissue fixation (10% buffered formalin) and tissue processing, described elsewhere in details (12). Sections were deparaffinized, dehydrated, and stained with monoclonal antibodies for HIF-1$\alpha$ expression (Santa Cruz Biotechnology, USA, 1:100); only samples with >10% positive staining were considered to state its overexpression state. Other immunohistochemistry examination was also performed on 5 $\mu$m-paraffin sections with an indirect method (EnVison) using the primary antibody against C-myc (Cell Marque, USA, 1:100), LDH-A (Santa Cruz Biotechnology, USA, 1:100), GLUT-1 (Santa Cruz Biotechnology, USA, 1:100), p53 (Cell Marque, USA, 1:100), Ki-67 (Santa Cruz Biotechnology, USA, 1:100), (Santa Cruz Biotechnology, USA), and the anti-rabbit/rat-IgG antibody from Dako (Carpinteria, CA, USA) as the second antibody. The other immunostains (BCL-6, CD10, MUM-1) were determined by the methods by Hans et al (18) to classify its cell-of-origin subtype.

### Serum sample and arterial blood collection

Serum samples were collected from DLBCL patients at the time of diagnosis. Blood was drawn into serum separator tubes and allowed to clot for 30 minutes at room temperature before centrifugation at 1000 × g for 10 min. The resulting serum samples were stored at −80°C until analysis. Serum levels of HIF-1$\alpha$ were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions. All other analyses such as haemoglobin, white blood cell, platelet, lactate dehydrogenase (LDH) and lactate serum were

performed according to the manual instructions provided with each assay kit.

### Hypoxic modulation using carbogen gas inhalation and nicotinamide administration

Carbogen gas (admixture of 95% oxygen and 5% carbondioxide) was delivered with a breathing circuit design, providing a flow rate of at least 10-15 liters/minute, and the gas flow 3-4 liters/minute was continually throughout the chemotherapy session to ensure it was sufficient. The mask should have an inflow/outflow valve to prevent rebreathing, and to make breathing in and out equally comfortable.

Patients were positioned sitting erectly in the most comfortable manner, with a face mask which was securely strapped. The patients' blood oxygen saturation and pulse rates were continually monitored by a pulse oximeter attached to a finger, and the respiration rate was recorded every 15 minutes by a nurse. A previous report on colorectal cancer has demonstrated an increase in intra-tumoral blood flow and chemotherapy drug delivery (19).

As the effects of breathing carbogen are at a maximum after 5-10 minutes (20), the initial assessment to provide the successful hypoxia modulation intervention should be within this time period. We did not calculate or measure intra-tumoral oxygen pressure or concentration; however, nicotinamide will provide a vasoactive agent that will accommodate better oxygenation if we deliver higher arterial oxygen tension as expected from the inhalation of carbogen gas.

The final protocol was 60 minutes after nicotinamide, 5 minutes air, 10 minutes carbogen and a further 5-10 minutes air while taking a blood sampling (Figure 2).

### Chemotherapy

All study participants will be treated with curative-intent chemotherapy R-CHOP: Rituximab, Cyclophosphamide, Hydroxy-doxorubicin, Oncovin/vincristine, and Prednisone (8). This protocol is classified as a high-emetogenic potential regimen. For tumor lysis syndrome prophylaxis, allopurinol 300 mg/24 hours for 21 days is given in the first cycle. Premedication consisted of paracetamol, ondancentron, diphenhydramine and dexamethasone intravenously. The overall plan would be six cycles of R-CHOP chemotherapy, but this study will assess the treatment response during the participant's first cycle.

### MRI measurement

Immediately before the start of chemotherapy, MRI was performed on all patients. MRI examinations were obtained on a 1.5 T scanner (Gyroscan; Philips Medical Systems, Eindhoven, Netherlands) using the system's body coil and reviewed by experienced radiologists (GS).

The tumor volume was assessed in each MRI examination independently by ROI-based volumetry and diameter-based measurements. For the ROI-based measurement, the entire tumor region identified and traced on the MR workstation on all T2-weighted sagittal imaging slices throughout the tumor. The volume of each outlined tumor was measured using the ellipsoid formula ($V = d_{CC}^{\circ} \times d_{AP}^{\circ} \times d_{LL}^{\circ} \times \Pi / 6$ in $cm^3$) to calculate the diameter-based volume (V).

### Follow-up

The patient received a follow-up within 10-14 days after chemotherapy. Evaluation included a history and physical examination, complete blood count, serum chemistries, HIF-1$\alpha$, lactate, and tumor MRI at the patient's most convenient time.

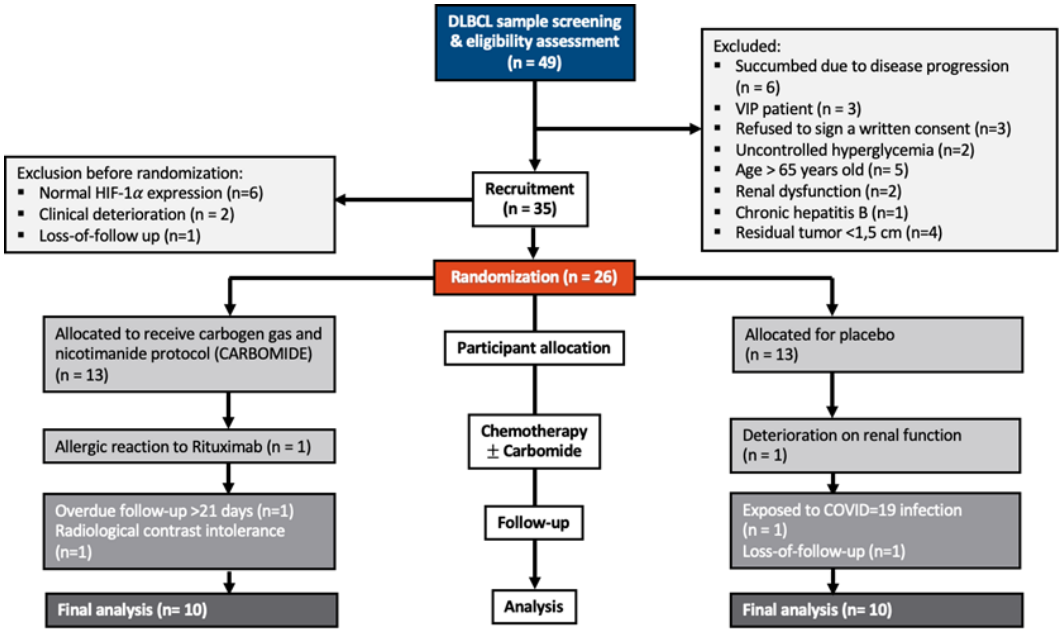


**Figure 1.** Consort diagram of clinical trial.

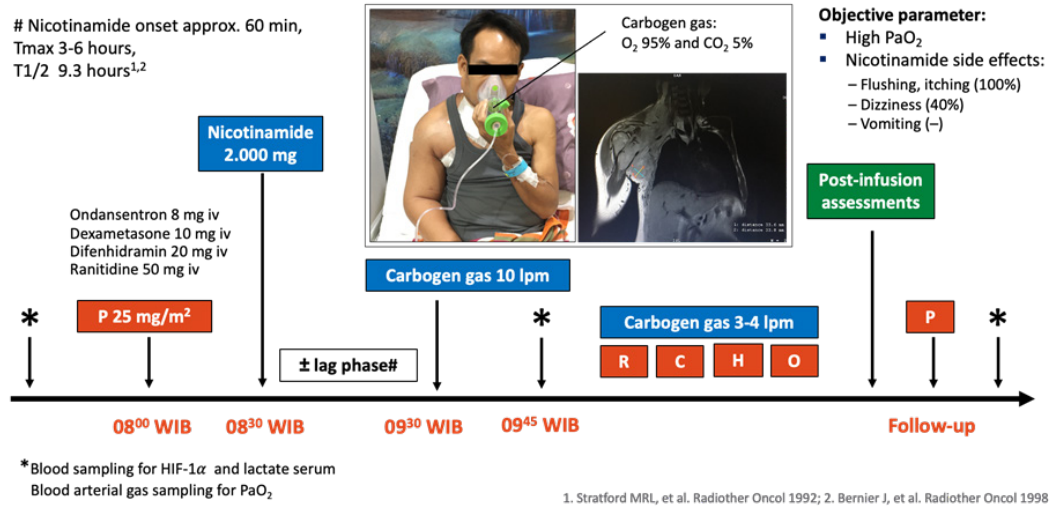


**Figure 2.** Experimental design and protocol of carbogen breathing, nicotinamide administration and the delivery of standard R-CHOP chemotherapy. The sitting position, face mask application and coronal image of DLBCL nodal tumor in the axillary area from patient #7 were also shown. R, rituximab; C, cyclophosphamide; H, hydroxy-doxorubicin; O, oncovin (vincristine); P, prednisone.

## STATISTICAL ANALYSIS AND OUTCOME

For statistical data processing, the SPSS statistic (v. 21) software package was used. The distributions of data were checked for normality using the Shapiro–Wilk test. The level of HIF-1α, lactate and tumor volumes at different time points were compared with paired t-test after checking the normality. The variable that can predict the treatment response >50% tumor shrinkage was assessed with descriptive statistical and multivariate analysis. To detect the differences in the subgroups, the Student's t-test was used. For each test, the respective p value level of significance was quoted, with p values less than 0.05 considered significant.

## RESULTS

### Baseline characteristics and clinical data

Twenty patients were investigated for the analysis. The mean follow-up time for patients was 17.6 days (range, 9 to 31 days). This clinical trial lasted for an average of 18.2 ± 5.47 days per participant, from day screening to serum sampling and imaging evaluation. In general, there was no difference between the intervention and the placebo group that would receive R-CHOP ($p>0.05$).

It can be observed from Table 1 that the tumor ROI size in the treatment group was larger (median 172.3 cm3 vs. 152.8 $cm^3$, $p=0.597$) at inclusion and more with bulky mass ($n= 7$ vs. 4, $p=0.597$) compared with the control group although the differences were not statistically different. The largest tumor dimension (23.14 cm) and volume (2,349.7 $cm^3$) were found in the treatment group. There was also no significant difference in the DLBCL stage according to the Ann-Arbor classification and NCCN-IPI prognostic score ($p>0.05$).

### The effect of Carbomide protocol upon hypoxia parameters

During the intervention protocol, there was a rise in pulse rate in 5, a decrease in 2 and no change in 3 participants. The respiration rate increased in 2 and remained static in 10. Facial flushing appeared in 8 participants, and temporary tingling and hand numbness in 4 participants of the intervention group. In the remaining 6 patients, no side effects were recorded after oral nicotinamide, but there were two complaints about the tightness of the mask during carbogen inhalation. All participants could tolerate the experimental treatment without technical error to be considered.

### The effect on oxygen arterial partial pressure

Measurements of arterial blood gases were carried out with carbogen breathing after nicotinamide administration. There was a significant increase in PaO2 (mean 186.22 vs. 93.20, $p<0.001$) and a significant change in the anion gap (10.33 vs. 8.11) compared to the control group that was only breathing in room air. The results are given in Table 2.

**Table 1.** Comparison of general characteristic, baseline data and DLBCL tumor profile.

| General characteristics | Intervention group (n=10) | Control group (n=10) | P |
|---|---|---|---|
| Age (years), median (min-max) | 55.0 (24-61) | 58.5 (21-65) | 0.307† |
| Sex | | | |
| • Male | 7 (70%) | 7 (70%) | 1.000§ |
| • Female | 3 (30%) | 3 (30%) | |
| Body surface area ($m^2$), mean SD | 1.56 0.12 | 1.55 0.10 | 0.07‡ |
| Status performance | | | |
| • ECOG 0–1 | 8 (80%) | 7 (70%) | 0.606§ |
| • ECOG 2 | 2 (20%) | 3 (30%) | |
| Hb concentration (g/dL), median (min-max) | 13.0 (10.1-13.9) | 11.7 (10.3-13.9) | 0.384† |
| Glomerular filtration rate (mL/min), median (min-max) | 57.5 (31.7-92.5) | 67.05 (56.8-127.5) | 0.096† |
| Serum LDH concentration (U/L), mean SD | 914.4 389.65 | 885.40 286.55 | 0.190‡ |
| Serum lactate concentration (mMol/L), mean SD | 3.83 1.31 | 3.65 0.75 | 0.384‡ |
| Serum HIF-1α concentration (pg/mL), median (min-max) | 45.81 (33.71-255.30) | 43.60 (30.0-57.02) | 0.676† |
| Comorbidities | | | |
| Diabetes mellitus | 1 (10%) | 2 (20%) | 0.531§ |
| HbA1c (%), median (min-max) | 5.65 (5.0-8.2) | 6.05 (5.1-8.9) | |
| HbA1c >7% | 1 (10%) | 2 (20%) | |
| Overweight/obesity | 2 (20%) | 2 (20%) | 1.000§ |
| DLBCL stage and tumor profile | | | |
| Ann-Arbor stage | | | |
| • Stage I-II, n (%) | 9 (90%) | 7 (70%) | 0.264§ |
| • Stage III-IV, n (%) | 1 (10%) | 3 (30%) | |
| Presence of B-symptoms, n (%) | 10 (100%) | 9 (90%) | 0.305§ |
| Cell-of-origin subtype | | | |
| • Germinal center B-cell DLBCL | 3 (30%) | 2 (20%) | 0.606§ |
| • Non-Germinal Center B-cell DLBCL | 7 (70%) | 8 (80%) | |
| Primary extranodal, n (%) | 3 (30%) | 4 (40%) | 0.390§ |
| Primary nodal, with extranodal in-volvement, n (%) | 4 (40%) | 5 (50%) | 0.653§ |
| NCCN-IPI score | | | |
| • Low and low-intermediate risk, n (%) | 5 (50%) | 3 (30%) | 0.361§ |
| • High-intermediate and high risk, n (%) | 5 (50%) | 7 (70%) | |
| Tumor highest dimension (cm), mean SD | 11.05 7.73 | 9.2 6.36 | 0.582‡ |
| • Min-max | 2.70-23.14 | 2.70-20.10 | |
| Bulky tumor, n (%) | 7 (70%) | 4 (40%) | 0.178§ |
| Tumor volume as ROI ($cm^3$), median (min-max) | 172.3 (6.5-2,349.7) | 4.3-1,542.6) | 0.597† |

GCB, Germinal center B-cell; ROI, region of interest; SD, standard deviations; NCCN-IPI, International score for Prognostic Index from NCCN
* Primary extranodal in gastrointestinal 5, testicular 1, liver 1
** Largest tumor dimension > 7,5 cm in either nodal or extranodal, measured according to imaging investigation at the baseline
† Mann-Whitney U-test;
‡ Independent t-test;
§ Chi-square test

**Table 2.** Oxygenation status of all participants before chemotherapy.

| Oxygenation and acid base status | Intervention group | Control group (n=10) | P |
|---|---|---|---|
| pH | 7.43 ± 0.03 | 7.44 ± 0.04 | 0.550† |
| $PaO_2$ (mmHg) | 186.22 ± 18.36 | 93.20 ±14.37 | <0.001† |
| Anion gap | 10.33 ± 5.50 | 8.11 ± 3.37 | 0.010† |

†Unpaired t-test

### The effect on HIF-1α serum

Please be informed that this study was only performed in hypoxic tumors so that more than 10% of HIF-1α expression should be detected in the DLBCL sample. The relationship of HIF-1α protein expression with its serum level was also examined in this study and showed a significant positive relationship (Kruskal-Wallis' test with Mann-Whitney post-hoc analysis, p=0.015). The serum concentration was increased progressively as the tumor expressed more HIF-1α, and the highest level was shown in the more overexpressed tumor.

In this study, when the HIF-1α serum was analyzed as a dependent variable among all patients, there was a decreasing trend regarding its concentration after the Carbomide protocol (median 5.81 to 37.29 pg/mL) as depicted in Figure 3A. However, in the R-CHOP cohort patients (Figure 3B), the HIF-1α also decreased after chemotherapy, interestingly in the same final results compared to the intervention arm (35.81 vs. 38.03 pg/mL). From the serological point of view, hypoxia modulation using carbogen inhalation and nicotinamide administration was able to reduce the degree of tumor hypoxia irrespectively of tumor volume (Figure 4).

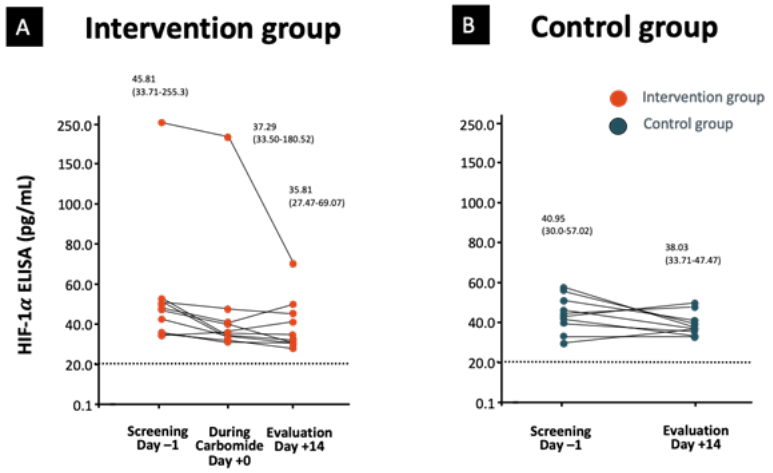


**Figure 3.** HIF-1α dynamics in A) intervention vs. B) control group. Individual serum concentration is indicated in different color and different timeline during the study: at screening, during carbogen breathing after nicotinamide administration (only for intervention group), and day +14 after chemotherapy.

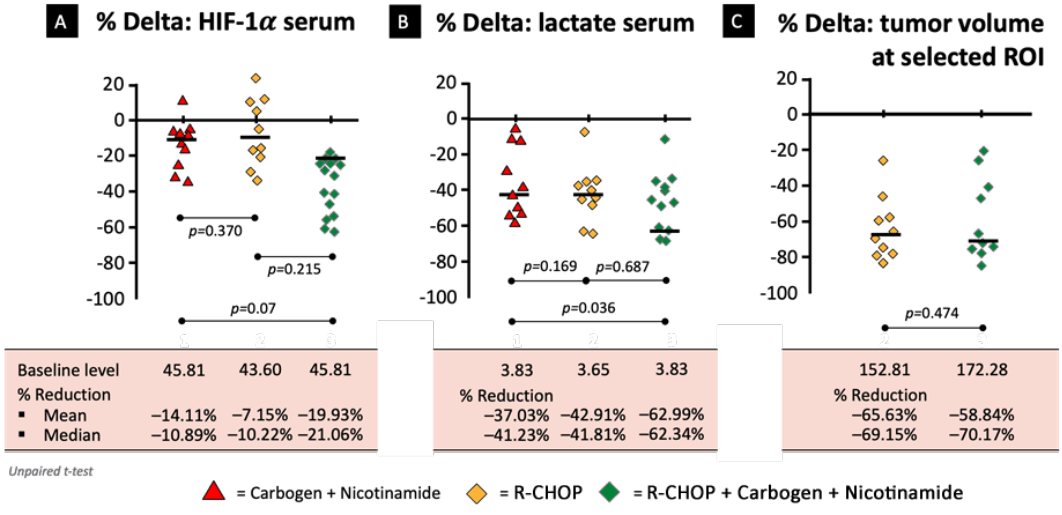


**Figure 4.** Median value, delta change on A) HIF-1α, B) Lactate serum, C) Tumor volume
The bar line (zero point) indicates the baseline value and the symbols represent their change relatively to the first measurement.

### The effect on lactate serum

Lactate is a glycolytic byproduct, sine qua non of the Warburg phenomenon in cancer. It emphasizes the shift in cancer metabolism in favor of glycolysis over oxidative phosphorylation as the preferred energetic mechanisms due to activation of the transcription factor HIF-1α and its downstream signaling. Metabolic reprogramming of tumor cells by HIF-1α modifies the metabolic fluxes as expected by high GLUT-1 expression (21).

Baseline lactate data in this study showed that the level of all DLBCL samples were above the normal limit (median 3.6 pg/mL, reference value <2 pg/mL). The acid base status corresponds to the state of anion gap lactic acidosis which increases to a mild moderate degree as the results of an overwhelming lactate production. As depicted in Figure 4B, carbogen inhalation and nicotinamide administration provided a lactate reducing effect (mean – 37,03% from baseline) when the blood samples were taken just 5-10 minutes after the intervention. The intervention group at the end of observation showed the greatest reduction compared to chemotherapy alone as the control group (62.99 vs. 42.91%, p=0.687).

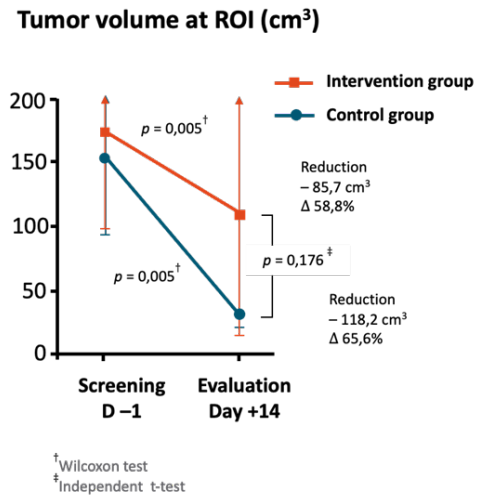


**Figure 5.** Tumor regression in response to treatment – intervention vs. control group.

### The effect of the Carbomide protocol upon tumor volume

Overall, for both treatment arms, the intervention and control group, a significant tumor volume reduction was obtained in every participant (p=0.005) (Figure 5). Please note that the median tumor volume which is designated as Regio of interest, i.e. the largest tumor mass, their baseline values were larger in the intervention group compared to the control group even though not statistically significant. However, from the oncological point of view, tumor size determines the proliferation fraction and ultimately chemosensitivity.

Seventy percent of the participants had a 50% change in tumor volume (n=14); 7 patients had at least a 75% reduction. At the end of observation, there were no differences in mean delta tumor reductions (–58.84% vs. –65.63%, p=0.176) between the intervention group and the control group. This result indicates that carbogen inhalation and nicotinamide administration upon standard therapy did not give additional benefits to standard chemotherapy R-CHOP in hypoxic DLBCL tumor.

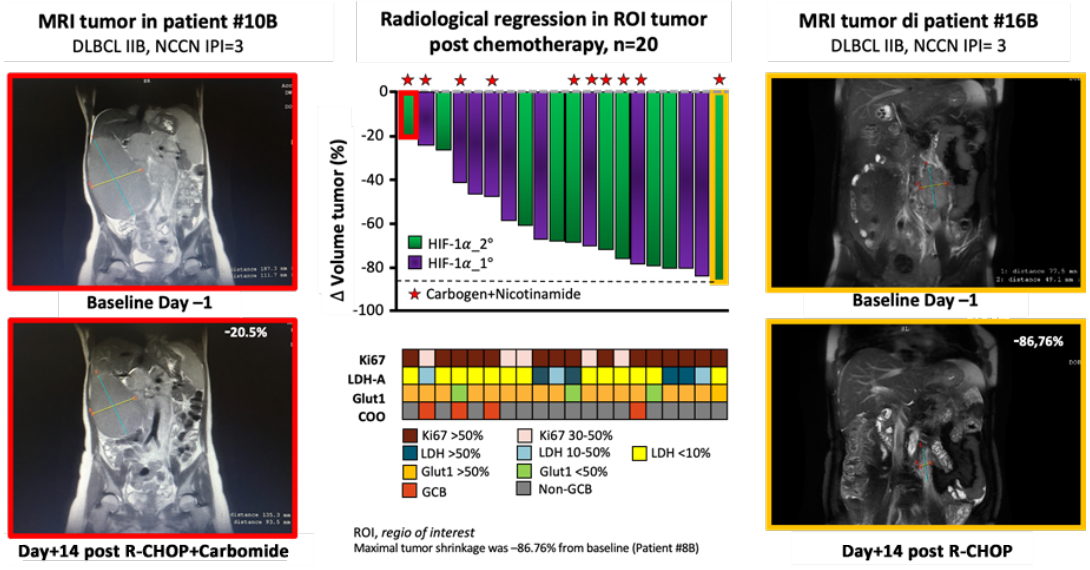


**Figure 6.** Radiological regression of the tumor volume after the 1st cycle of chemotherapy
The green bar in the middle indicates tumor with an overexpression of HIF-1α >50%, while the purple bar was 10-50%. The intervention group was marked with a star symbol. Each waterfall bar was a plot with the corresponding tumor shrinkage

Figure 6 demonstrates intrinsic tumor variability as assessed by immunohistochemistry opposed to the treatment response after the first cycle of chemotherapy. Tumor shrinkage that was displayed and calculated as a delta value did not show a relationship with a certain biological profile or even specific intervention. The greatest tumor reduction was delta –86.76%. Namely, the participant from the intervention arm showing specific characteristics of

Ki-67>50%, Glut1>50%, LDH<10% and non-GCB subtype, turned out to be an identical profile to the participant with the least tumor shrinkage (delta –20.5%). Both the good and bad response after standard R-CHOP chemotherapy occurred even though they have additional intervention with the hypoxia modulation. However, the bulky mass as shown on the left side of the figure that probably possesses more complex vasculature was taken into account.

### Correlation with the proliferation index and protein expression and cell-of-origin subtype

We performed a survey of several protein expressions in tumors that define the proliferation index (Ki-67), cell-of-origin subtype (GCB vs. Non-GCB subtype), p53, C-myc and HIF-1α expression, and its target genes/protein (LDH-A and Glut-1) in two ordinal categorical variables. As shown in Figure 7, tumor reduction with the greatest dimension was observable in tumors with high Ki-67, high Glut-1 expression, and non-GCB subtype in the intervention group. Our study did not allow identifying tumor biology that accurately differentiates its associated varying response to either hypoxia modulation or standard chemotherapy.

### Multivariate logistic regression analysis

The overall stage, grade and biological profile are potential confounders of this unexpected response. Using multivariate logistic regression, we analyzed Ann-Arbor staging, bulky tumor, the primary extra-nodal status, NCCN-IPI, COO subtype, proliferation rate, degree of HIF-1α overexpression, as dependent variables for correlation with tumor shrinkage among DLBCL participants who received standard R-CHOP chemotherapy. All variables were dichotomized as positive vs. negative against more than 50% of tumor shrinkage as independent variables after the first cycle of chemotherapy. In the multivariate analysis as shown in Table 3, none were found to be a significant factor for an improved response other than chemotherapy itself.

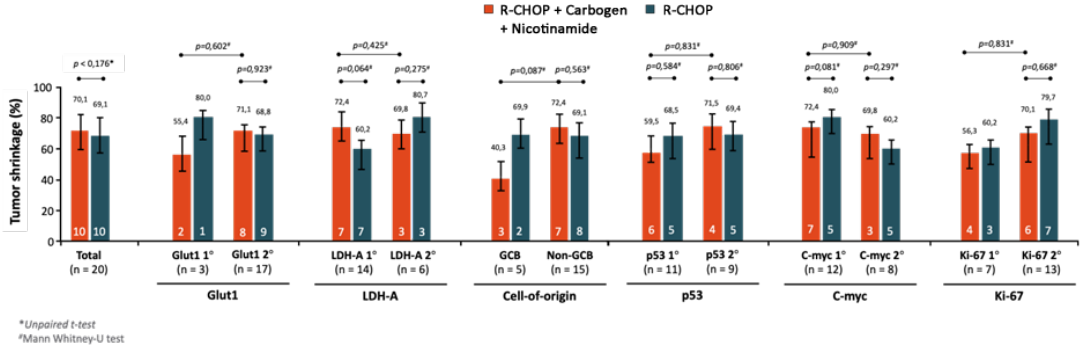


**Figure 7.** The degree of tumor shrinkage based on biological characteristics: Glut-1, LDH-A, Cell-of-origin prifile, p53, C-myc, and Ki-67 expression.

**Table 3.** Multivariate analysis of the selected variables with tumor shrinkage in patients with DLBCL after the first cycle of chemotherapy.

| Dependent variable | Total | 50% tumor shrinkage | OR | 95% CI | Unadjusted P |
|---|---|---|---|---|---|
| Ann-Arbor staging | | | | | |
| Ann-Arbor I-II | 16 (80%) | 12 (75%) | | | |
| Ann-Arbor III-IV | 4 (20%) | 2 (50%) | 0.33 | 0.35-3.20 | 0.34 |
| NCCN-IPI score | | | | | |
| Low to low-intermediate | 8 (40%) | 5 (62.5%) | | | |
| High-intermediate to high | 12 (60%) | 10 (83.3%) | 5.00 | 0.64-39.05 | 0.12 |
| Disease | | | | | |
| Primary nodal | 13 (65%) | 8 (61.5%) | | | |
| Primary extra-nodal | 7 (35%) | 6 (85.7%) | 3.75 | 0.34-41.08 | 0.27 |
| Bulky tumor | | | | | |
| Non-bulky | 9 (45%) | 7 (77.7%) | | | |
| Bulky | 11 (55%) | 7 (63.6%) | 0.28 | 0.04-1.82 | 0.18 |
| Ki-67 status | | | | | |
| 30-50% | 7 (35%) | 5 (71.4%) | | | |
| >50% | 13 (65%) | 7 (53.8%) | 1.11 | 0.14-8.37 | 0.92 |
| HIF-1α overexpression | | | | | |
| 10-50% | 10 (50%) | 6 (60%) | | | |
| >50% | 10 (50%) | 8 (80%) | 1.80 | 0.26-12.50 | 0.55 |
| Cell-of-origin subtype | | | | | |
| GCB DLBCL | 5 (25%) | 2 (40%) | | | |
| Non-GCB DLBCL | 15 (75%) | 12 (80%) | 6.00 | 0.67-53.68 | 0.11 |
| Interventional therapy | | | | | |
| R-CHOP | 10 (50%) | 8 (80%) | | | |
| R-CHOP+ Carbo-gen+Nicotinamide | 10 (50%) | 6 (60%) | 0.37 | 0.05-2.77 | 0.37 |

†Unpaired t-test

## DISCUSSION

This study examines the pathogenic pathway of hypoxia in DLBCL in the setting of clinical trials, specifically in a population with an HIF-1α overexpression for the first time. The range of tumor diameters is from 2.7 cm to 23.4 cm (being the largest), from an early to advanced stage, from low risk to high risk NCCN IPI (see Table 1).

We proposed modifying the hypoxia microenvironment and knocking the HIF down using hyperoxia therapy and a vasodilator as intended by carbogen inhalation and nicotinamide administration as an addition to standard R-CHOP chemotherapy. DLBCL is a chemosensitive cancer, which means that the clinical response can be or should be seen after the first cycle of the treatment (22-24). Chemotherapeutic drugs in R-CHOP target those that divide/proliferate and CD20+ cells, so that highly proliferative DLBCL can be a suitable cancer model to allow early evaluation.

The result of this study demonstrated a reduction of 20.5 – 86.7% in tumor volume after the first cycle of R-CHOP chemotherapy, which can be considered one reduction fraction or log-cell killing and which can be regained with a repetition dose of chemotherapy. This number represents DLBCL cells that possess chemosensitivity and an area that can be reached by the drug (s) through vascularization. All the tumors exhibit HIF-1α overexpression in a representative biopsy sample. The cut-off for HIF-1a staining was 10% to indicate overexpression, as reported previously in our preliminary study (12) and by several authors (16, 17).

The expression level of HIF-1α in tumors is unrelated to the patient's age and sex, but is positively correlated with the clinical stage (25). Hypoxia is nearly present in tumors, which is considered a hallmark of cancer (26). The mechanism may be that as the tumor continues to grow and increase in size because of its oncogenic driver, hypoxic necrosis occurs due to insufficient blood supply, which induces the expression of HIF-1α to promote the transcription of the Vascular Endothelial Growth Factor (VEGF), one of its target genes (27). VEGF increases angiogenesis, tumor growth, invading surrounding tissues, and the occurrence of distant metastasis; thus, the clinical stage increases and the survival rate decreases.

From a theoretical point of view, oncology studies have shown that HIF-1α can regulate at least 100 downstream target genes (28). If HIF-1α is used as the target, all the downstream growth factors are inhibited as well; therefore, the application range of HIF-1α inhibitors makes sense and is logical in many aspects. A large number of existing drugs are small molecule compounds that inhibit the function of HIF, while there is no specific HIF-1 inhibitor (29).

Unexpectedly, our efforts to reverse tumor hypoxia and knock the HIF down by applying carbogen inhalation and nicotinamide administration in addition to R-CHOP chemotherapy did not lead to tumor mass reduction that is not even bigger (mean –58.84%) compared to

the control group (mean–65.63% reduction, p>0.05). From the serological point of view, intervention was able to reduce the HIF and lactate serum significantly (Figure 4A & 4B), though it does not necessarily have a greater tumor reduction effect (Figure 4C). A similar study was conducted by Nawangsih et al. on rectal cancer to assess the tumor response post-chemoradiation using carbogen alone without nicotinamide. They reported positive results where tumor volume reduction was better (63.53 vs. 30.03%, p= 0.024), which is evaluated 4-8 weeks after the completion of chemoradiation (30).

This study included 25% of GCB DLBCL, while the rest was a non-GCB subtype with varying HIF-1α expression. Various clinical studies show that the ABC (or non- BCG) subtype has a worse prognosis than the GCB subtype (31). Contrary to this study, the chemotherapy response was better with HIF-1α modulation in non-GCB DLBCL based on logistic regression, though it is not statistically significant. This information contains some possible important implications - whether the cell-of-origin approach was analyzed with molecular profiling to define high-risk DLBCL that is double- and triple-hit DLBCL involving c-myc, bcl-2 and/or bcl-6 re-arrangement. This high-risk DLBCL occurs more commonly in the GCB subtype (31,32).

On the basis of consistent upregulation of HIF-1α expression in DLBCL, we found a slightly increased tumor response in those with >50% overexpression, though not statistically significant (OR 1.8, p=0.55). It is important to note that the associations and interactions between the microenvironment and expression of cellular DLBCL HIF-1α are not yet known. In a survey of HIF-1α target genes and metabolic reprogramming, one important finding is reducing a significant lactate serum with hypoxia modulation irrespectively of the chemotherapeutic effect of tumor shrinkage (Figure 4B).

Statistical tests show that there was a significant lactate reduction as an effect from carbogen inhalation and nicotinamide administration with R-CHOP chemotherapy. Lactate dynamic can be seen in Figure 4B. The reduction effect was equal to the post-chemotherapy level in the control group (p=0.169). The result of this study demonstrated that the lactate level in the intervention group showed the greatest reduction (Delta 62.99 vs. 42.91%, p=0.003). The previous study by Bhatt et al. explains that the lactate was a by-product of aerobic glycolysis that is usually seen in patients with malignant lymphoma (33). Lactate production adjusts to the rate of DLBCL cells proliferation and glucose flux (34). In this case, a decrease in lactate levels reflects an improved metabolic milieu leading to a switch from abnormal glycolysis to normal oxidative phosphorilization (Ox-Phos). Lactate normalization may also delineate the end result of tumor shrinkage as the consequence of a reduced number of cancer cells (35).

If this concept is interpreted based on the changes in lactic acid levels, hypoxia and HIF-1α modulation by applying carbogen inhalation and nicotinamide administration is expected to increase the partial pressure oxygen in the arterial induced oxidative metabolism. At the end of the reaction, this attempt reduces lactate production and intracellular acidosis. Carbon dioxide and nicotinamide both act as vasodilator agents, thus improving oxygen delivery and shifting the dissociation curve to the right so that diffusion is more effective for chemotherapy to be expected.

Our findings in this limited series study had several main limitations. 1) This study only looked at the response after the first cycle of chemotherapy. A more periodic evaluation at the end of the chemotherapy cycle is possible to achieve more concrete results. 2) Additional studies of a larger group of patients and involving other chemotherapeutic regimens are required to verify our results, and prospective long-term evaluation to determine the effect of each pre-treatment parameter on the clinical outcome. 3) Our approach does not allow capturing such major differences. However, it makes it impossible to predict the trend of tumor resistance to treatment very well. 4) The availability of clinical or biological markers would be highly useful as well as the need of an additional genetic study of tumors.

## CONCLUSION

We observed the changes in three parameters (oxygenation, HIF-1α, and the lactate serum) at an early time, after the start of the intervention as our intention for hypoxia modulation. Significant tumor reduction was achieved in every cohort, ranging from 20.5 to 86.7% in tumor shrinkage from the baseline. However, there was no difference in tumor volume reduction between the two groups after the first cycle of chemotherapy, regardless of the addition of carbogen inhalation and nicotinamide administration in the intervention group.

### Declaration of competing interest

None of the authors of this study have a financial or personal relationship with other individuals or organizations that could inappropriately influence or bias the content of this article.

### Ethic statement

The study was performed in accordance with the Declaration of Helsinki. The procedure was approved by the institutional review board of the Dr. Kariadi Hospital (study licence: No.DP.02.01/I.II/2366/2021). A written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this manuscript.

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### Data availability

The data sets used and analyzed during this study are available from the corresponding author.

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