Impact of the induction phase chemotherapy on cytokines and oxidative markers in peripheral and bone marrow plasma of children with acute lymphocytic leukemia

G.E. Broto [a], P.R.B. Silva [a], F.C. Trigo [b], V.J. Victorino [c], K.L. Bonifácio [a], W.R. Pavanelli [f], F. Tomiotto-Pelissier [f,g], M.R. Garbim [d], S.T. Oliveira [d], J.J. Jumes [d,e], C. Panis [d,e,f,1,*], D. S. Barbosa [a,1]

[a] Health Sciences Postgraduate Program, State University of Londrina (UEL), Londrina, PR, Brazil
[b] Londrina Cancer Institute, Londrina, Paraná, Brazil
[c] Federal Institute of Rio de Janeiro, Rio de Janeiro, Brazil
[d] Laboratory of Tumor Biology, State University of Western Paraná (UNIOESTE), Francisco Beltrão, PR, Brazil
[e] Health Sciences Postgraduate Program, State University of Western Paraná (UNIOESTE), Francisco Beltrão, PR, Brazil
[f] Experimental Pathology Postgraduate Program, State University of Londrina (UEL), Londrina, PR, Brazil
[g] Carlos Chagas Institute, Curitiba, Paraná, Brazil

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ABSTRACT

B-cell acute lymphocytic leukemia (B-ALL) is the main neoplasia affecting children worldwide, in which cytotoxic chemotherapy remains the main treatment modality. In this study, we analyzed the profile of inflammatory markers concerning oxidative stress and cytokines in 17 B-ALL patients. Peripheral blood (PB) and bone marrow (BM) samples were collected and evaluated for the pro-oxidative status (nitric oxide products-NOx and hydroperoxides), antioxidants (sulfhydryl groups-SH and total radical-trapping antioxidant parameter-TRAP), and cytokines (TNF-α, IFN-γ), at diagnosis (D0) to and the end of the induction phase (D28). At D28, hydroperoxides were higher in PB, concomitant to TNF-α levels. INF-γ was increased in the BM at D28. Hydroperoxides were higher in patients presenting malignant cells in BM and/or PB after treatment, a condition named minimal residual disease (MRD) when compared to those without MRD at D28. These findings suggest that oxidative stress and cytokines vary across the B-ALL induction phase, and lipid peroxidation is a potential marker associated with MRD status.

1. Introduction

The occurrence of pro-inflammatory events, as oxidative stress, is known as a pivotal event in physiological processes occurring during hematopoiesis (Gaman et al., 2014), especially during stem cell differentiation (Hole et al., 2011). The participation of oxidative stress has been documented in some aspects of hematological neoplasia, including leukemia (Er et al., 2007; Zhou et al., 2007). Cancer cells are usually under higher oxidative stress than normal cells (Cramer et al., 2017), which requires adaptation to counter the continuous impairment in the pro-oxidant environment. Since oxidative stress is necessary for the

normal functioning of the bone marrow in hematopoiesis, this environment might exhibit altered redox balance in cancer patients and impaired hematopoiesis in certain instances.

B-cell acute lymphoblastic leukemia (B-ALL) is the most common malignant neoplasia among children worldwide. Notwithstanding its relatively good prognosis, a small percentage of patients present poor clinical outcomes (Hockenberry et al., 2014), and the underlying reasons are partly reflected through the clinical aspects of the disease. ALL chemoresistance occurs when circulating cancer cells are detected, a condition known as minimal residual disease (MRD). In this context, certain cellular and molecular factors may contribute to disease progression in both the tumor microenvironment and the systemic level.

Abbreviations list

B-ALL	B-cell acute lymphocytic leukemia
PB	Peripheral blood
BM	Bone marrow
NOx	nitric oxide products
SH	sulfhydryl groups
TRAP	total radical-trapping antioxidant parameter
D0	At diagnosis
D8	Day 8 after treatment starting
D15	Day 15 after treatment starting
D22	Day 22 after treatment starting
D28	Day 28 after treatment starting
GBTLI	Brazilian Childhood Leukemia Treatment Group
LOOH	Lipid hydroperoxides
ANOVA	One-way analysis of variance
MRD	minimal residual disease
ROS	reactive oxygen species

Therefore, it is essential to understand the molecular mechanisms underlying the response to the initial phases of treatment.

Bone marrow stromal cells can mediate redox adaptation in ALL by increasing its mitochondrial oxidative stress, which has been associated with drug resistance and disease recurrence (Liu et al., 2015). Furthermore, enhanced oxidative stress has been reported as a possible cause of systemic injury during disease treatment (Ki Moore et al., 2015; Cole et al., 2015). ALL treatment is based on 3-step chemotherapy targeting the billion continuously proliferating cells. One-month induction eliminates approximately 99.9% of cancer cells, achieving ALL remission in more than 90% of patients, and the responsiveness to this phase of treatment will determine the long-term prognosis of ALL. To attain this goal, this phase comprises the administration of combinatorial chemotherapy comprising vincristine, L-asparaginase, anthracyclins, methotrexate, and steroids, all known to generate oxidative stress (Hockenberry et al., 2014). Induction alters the antioxidant capacity and malondialdehyde levels in serum of children with ALL, causing redox imbalance when comparing patients at diagnosis and those at the end of treatment (Al-Tonbary et al., 2011).

Few studies have focused on the clinical relevance of oxidative stress profile, as well as its relationship with other inflammatory mediators as cytokines, especially considering the bone marrow environment. Hence, it is essential to characterize the inflammatory profile of both the systemic and bone marrow microenvironments in B-ALL patients to determine whether oxidative mediators and cytokines could help explain disease behavior during chemotherapy.

Although the recent overall survival rates for B-ALL are very high in developed countries, access to promising therapies as the CD19 CAR-T is not a reality in developing ones. Therefore, B-ALL treatment imposes a great challenge in poor countries due to the high cost of such personalized therapies, becoming cytotoxic chemotherapy the main modality of treatment. The response to chemotherapy in the induction phase is a determinant of treatment success, but the mechanisms underlying MRD are not clear in the context of inflammation.

In this context, the present study aimed to characterize the inflammatory scenario of B-ALL patients by evaluating their peripheral blood/bone marrow oxidative profiles and cytokine levels, comparing them at the start and the end of the induction phase of treatment.

2. Methods

2.1. Study design

This study has a longitudinal observational design and was approved by the Institutional Ethics Board (24498213.0.0000.5231). Pediatric patients diagnosed with B-ALL with ages between 0 and 18 years were recruited from January 2015 to January 2016, after signed consent forms. All patients were from the Institute of Cancer in Londrina, Paraná-Brazil, and were ongoing the chemotherapeutic schedules recommended by the Brazilian Childhood Leukemia Treatment Group (GBTLI, 2009). Since we aimed to compare the oxidative stress and cytokine profiles of patients through induction phase treatment, whole blood samples were obtained from patients at diagnosis (D0, no treatment) and the end of the treatment, at the twenty-eighth day (D28). Following the routine sample collection employed by the GBLTI protocol, D0 started with corticoid (prednisone), then the following drugs were introduced: vincristine, prednisone, daunorubicin and asparaginase. The drug schedule adopted was the following (Fig. 1): Prednisone 60 mg/m^2 orally from D1 to D7, then 40 mg/m^2/day orally, divided into 2–3 doses for 3 weeks (D8-29), suspending regressively in 3–4 days. If necessary, Prednisolone can be administered intravenously, divided into 3 doses. Vincristine: 1.5 mg/m2/week intravenously, maximum dose of 2 mg) administered in days 8, 15, 22 and 29. Daunorubicin: 40 mg/m^2/week intravenously, administered in days 8, 15 and 22. L-Asparaginase: 10,000 IU/m^2 intramuscular or intravenous (if thrombocytopenia <75000/mm^3) every 3 days, from day 8 of treatment, for a total of 9 doses. Cyclophosphamide: 500 mg/m2 intravenously on days 22 and 23 of induction for patients in the slow response subgroup. Intrathecal medication (ITM): triple therapy with Methotrexate, Ara-C and Dexamethasone will be administered at age-adjusted doses, on days 15 and 29 of induction (>1 < 3 years: 10 mg/m^2 and 20 mg/m^2, for Methotrexate and Ara-C respectively; > 3 < 9 years: 12 mg/m^2 and 24 mg/m^2, respectively; > 9 years: 15 mg/m^2 and 30 mg/m^2, respectively. The dose of Dexamethasone is uniform (2 mg/m^2. Max. 2 mg dose). Medical records were assessed to obtain clinicopathological data. For risk categorization were considered as low-risk children between 1 and 9 years, white cells <50000/mm$^{3.}$ The others were high risk. MRD assessment was determined in PB and BM samples by flow cytometry following the Recommendations from the MRD Working Group of the Brazilian Society of Bone Marrow Transplantation (Ikoma-Colturato et al., 2020). Patients were considered MRD positive when this analysis detected cells bearing leukemia-associated immunophenotypes, such as asynchronous antigen expressions or cross-lineage markers considering a multiparametric panel composed by CD20, CD45, CD38, CD10, CD66c/CD123, CD19, CD34, CD73, CD304, and CD81 antibodies (see Fig. 2).

All samples (peripheral blood and bone marrow) were centrifuged to obtain plasma (3500×g, 5 min) and stored at −80 °C until oxidative stress analyses. Aiming to compare the results, only plasma samples (not cells) were included.

2.2. Investigation of oxidative status

To determine the oxidative status of samples, we quantified the following parameters:

a) **Sulfhydryl groups (SH):** Sulfhydryl groups from proteins were evaluated by the method adapted by Taylan and Resmi (2010) (Taylan and Resmi, 2010). SH method is based on the reaction of 5, 5-dithiobis-2 nitrobenzoic acid (DTNB) with sulfhydryl groups. Determination was conducted in a microplate reader, Perkin Elmer®, model EnSpire (Waltham, MA, USA) at 412 nm. Results are expressed as μM of sulfhydryl.

b) **Total radical-trapping antioxidant parameter (TRAP):** TRAP was evaluated according to the method described by Repetto et al. (1996) (Repetto et al., 1996) in microplate reader Victor X-3, Perkin Elmer® (Waltham, MA, USA). Experimental conditions were: running time of 25 min, response range from 300 to 620 nm and a temperature of 30 °C. This method detects hydro and/or liposoluble antioxidants present in serum. The results were expressed as μM trolox.
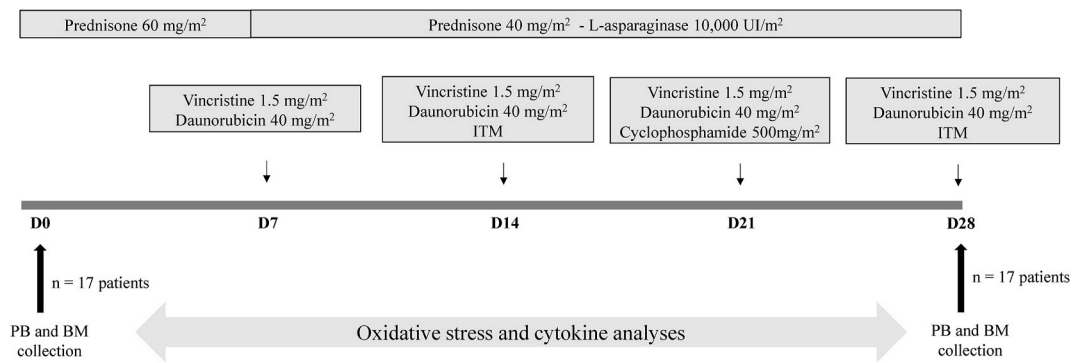
Fig. 1. Design of the study. A total of 17 patients were included in the study. Drug schedule: Prednisone 60 mg/m2 orally from D1 to D7, then 40 mg/m2/day orally, divided into 2–3 doses for 3 weeks (D8-29), suspending regressively in 3–4 days. If necessary, Prednisolone was administered intravenously, divided into 3 doses. Vincristine: 1.5 mg/m2/week intravenously, maximum dose of 2 mg) administered in days 8, 15, 22 and 29. Daunorubicin: 40 mg/m2/week intravenously, administered in days 8, 15 and 22. L-Asparaginase: 10,000 IU/m2 intramuscular or intravenous (if thrombocytopenia <75000/mm3) every 3 days, from day 8 of treatment, for a total of 9 doses. Cyclophosphamide: 500 mg/m2 intravenously on days 22 and 23 of induction for patients in the slow response subgroup. Intrathecal medication (ITM): triple therapy with Methotrexate, Ara-C and Dexamethasone will be administered at age-adjusted doses, on days 15 and 29 of induction (>1 < 3 years: 10mg/m2 and 20mg/m2, for Methotrexate and Ara-C respectively; > 3 < 9 years: 12 mg/m2 and 24 mg/m2,respectively; > 9 years: 15mg/m2 and 30mg/m2, respectively. The dose of Dexamethasone is uniform (2 mg/m2. Max. 2 mg dose). PB = peripheral blood, BM = bone marrow aspirate, ITM = intrathecal medication.
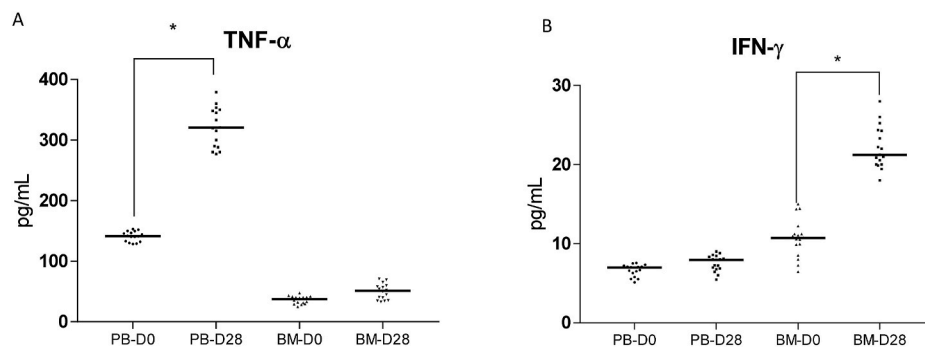
Fig. 2. Tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) levels in peripheral blood (PB) and bone marrow (BM) plasma at diagnosis (D0) and in the end (D28) of the induction phase chemotherapy. * indicates statistical significance (p < 0.05). Dots represents cytokine level for each individual.

c) **Nitric oxide metabolites (NOx):** NOx levels were assessed indirectly by determining the plasma nitrite concentration using an adaptation of the technique described by Navarro-gonzálvez et al. (1998) (Navarro-gonzálvez et al., 1998). This method is based on the reduction of the nitrate present in the sample to nitrite by oxidation-reduction reactions mediated by the system cadmium-copper reagent. Thereafter, Griess reagent was added to induce diazotization, forming a colored complex and subsequent detection at 540 nm. The quantification of NOx was made in a microplate reader Asys Expert Plus, Biochrom® (Holliston, MA, USA). The nitric oxide metabolites concentration was expressed as µM nitrite.

d) **Lipid hydroperoxides - chemiluminescence assay (LOOH):** LOOH were determined according to an adaptation of the technique described by Gonzales-Flecha et al. (1991) (Flecha et al., 1991) and Panis et al. (2012) (Panis et al., 2012). This method uses the compound tert-butyl hydroperoxide to start a lipid chain reaction that can be detected by photon emission during the formation of lipid hydroperoxides. Readings were performed in a Glomax luminometer (TD 20/20 Turner Designers, E.U.A.) over 1 h at 1 readings/s. Results were expressed as relative units of light (RLU).

2.3. Cytokine measurement

Considering that TNF-α and IFN-γ are relevant pro-inflammatory cytokines in the B-ALL context, we chose to measure its levels in the collected samples. To reach this goal, we used enzyme immunoassay kits

(e-Biosciences, USA) following the manufacturer's instructions, with internal controls. Results were calculated in pg/mL by fitting the data to a standard curve obtained using each human recombinant cytokine.

2.4. Statistical analysis

Comparisons were performed according to the normal distribution of data in the Shapiro-Wilk test, as well as the homogeneity by Levene's test. Differences were evaluated by comparing D0 x D28 from PB and D0 x D28 from BM by Student's T-test (parametric data) or Mann-Whitney's test (non-parametric data). Graphs are presented as dot plots and medians. All analyses were performed in the software GraphPad Prism 7.0 (GRAPHPAD Software, San Diego, CA). A p < 0.05 value was considered significant.

3. Results

Clinicopathological data of patients are shown in Table 1. Seventeen patients (52.95% male, 88.23% Caucasian; mean age, 7.8 years) were included in this study; most being categorized into the low-risk group.

Table 2 shows the oxidative status of PB and BM at D0 and D28. For PB, none of the evaluated parameters (NOx, SH, TRAP, and LOOH) varied significantly. For BM, a significant augment in LOOH levels was detected at the end of treatment. Specific median, minimum and maximum values are described in this Table.

Cytokine levels for TNF-α and IFN-γ are displayed in Fig. 1. TNF-α levels showed a significant augment at D28-PB about D0-PB (p =

Table 1
Clinicopathological characterization of patients.

Number of patients	N = 17
Mean age at diagnosis (years)	7.8 ± 0.95
Mean BMI (kg/m2)	16.46 ± 0.6
Ethnicity	
Caucasian	88.23%
African	11.77%
Gender	
Female	47.05%
Male	52.95%
Risk categorization	
Low risk at D28	N = 9
High risk at D28	N = 8

Legend: BMI = body mass index, D28 = 28 days after starting the induction phase of chemotherapy.

0.0286, Fig. 1A), and did not vary in the other groups (141.7 ± 5.04 pg/mL for PB-D0, 276.6 ± 379.1 pg/mL for PB-D28, 35.97 ± 5.5 pg/mL for BM-D0, and 49.32 ± 9.23 pg/mL for BM-D28). For IFN-γ, a significant augment was observed in BM-D28 when compared to BM-D0 (p = 0.0002, Fig. 1B), without any change for the other groups (6.7 ± 0.46 pg/mL for D0-PB, 7.71 ± 0.75 pg/mL for D28-PB, 10.63 ± 1.61 pg/mL for D0-BM and 23.72 ± 4.2 pg/mL for 23.72 ± 4.27 pg/mL).

On investigating whether the evaluated parameters affected disease outcome, we compared the presence of minimal residual disease (MRD+) or not (MRD-) at D28 in both PB and BM plasma compartments (Fig. 3). Based on MRD status, patients were equally distributed (about 50% of patients for each group).

From all parameters analyzed, only LOOH was significantly altered regarding MRD status, showing augmented in the PB of MRD + patients at D28 (p = 0.0006), concerning DRM - group (min-max intervals: 2694258–3724906 RLU for MRD + PB, 1104889–6824291 RLU for MRD + BM, 799447–1861685 RLU for MRD – PB, and 1052169–7708277 RLU for MRD – BM). NOx, SH, TRAP, and cytokines did not vary statistically according to MRD status (data not shown).

4. Discussion

The present study establishes a comparative landscape between the analysis of plasma samples from two different sources, representing systemic (PB) versus local tumor (BM) microenvironme1nts in B-ALL patients at the light of inflammatory mediators. To reach this goal, we analyzed the levels of proinflammatory cytokines (TNF-α and IFN-γ) and oxidative stress parameters (NOx, anti, and pro-oxidants) in both compartments, at the start (D0) and the end (D28) of induction chemotherapy. Our findings indicate that cytokines and oxidative stress mediators are differentially produced by both compartments, depending on the moment of the chemotherapy challenge. Further, these findings indicated a relevant meaning for lipid peroxides (LOOH) in the context of the minimal residual disease, which is the main challenge to solve in

B-ALL management. Therefore, to the best of our knowledge, this information is a novelty.

Cytokine production and oxidative stress generation are intrinsically linked processes, which can regulate each other levels. Oxidative stress is the result of the imbalance generated by the pro-oxidants (as LOOH and NOx) and antioxidants (as SH and TRAP) reactions. It is well known that normal hematopoiesis depends on low oxidative stress levels (Hole et al., 2011) and is negatively affected by an increase in reactive oxygen species (ROS) levels, thereby leading to defective cell proliferation and possibly tumorigenesis (Hole et al., 2011; De Cavanagh et al., 2002). ROS production is a crucial phenomenon that can directly affect cellular components like lipids, proteins, and DNA. Therefore, characterization of the oxidative status from both systemic (PB) and tumor microenvironment (BM) in B-ALL may help to understand the dynamics of oxidative stress mediators and their behavior concerning chemotherapy-induced changes and disease behavior.

In this context, analysis of PB samples based on oxidative stress parameters revealed a significant increase in LOOH levels on D28 of the induction phase of treatment. In this phase, an intensive bone marrow hypocellularity and consequently, systemic pancytopenia, reflecting a common side effect of non-target-specific chemotherapeutic drugs (Irwin and Rivera-Del Valle, 2013). This phase mainly comprises the sequential use of corticosteroids, methotrexate, vincristine, doxorubicin, and L-asparaginase. The latter 4 drugs are known to generate oxidative stress and are used in high doses to rapidly eradicate tumor cells (Babiak et al., 1998; Vagace and Gervasini, 2011). Corticosteroids play a crucial role in potentially promoting tumor cell apoptotic gene expression (Tissing et al., 2007). ALL response to prednisone only (exclusively used from D0 to D8) is an important prognostic factor and is defined by a positive blast count on D8 (Meissner et al., 2004).
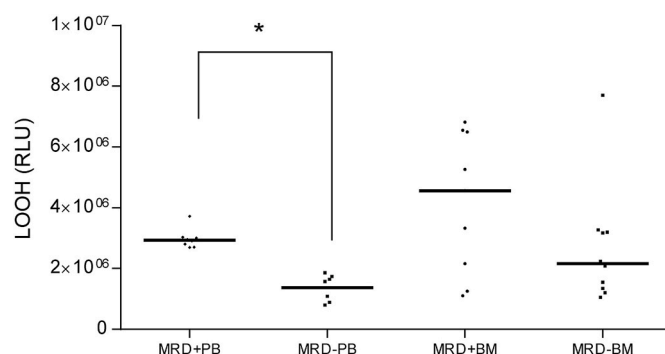
Fig. 3. Comparative analysis of oxidative stress markers in peripheral blood (PB) and bone marrow (BM) plasma concerning the minimal residual disease status (MRD) at D28. MRD = minimal residual disease, PB = peripheral blood, BM = bone marrow, RLU = relative light unities. + = positive, - = negative, LOOH (hydroperoxides); R.L.U = relative light unities; * indicates p < 0.05. Dots represents LOOH level for each individual.

Table 2
Oxidative status of peripheral blood (PB) and bone marrow (BM) plasma in patients with B-LLA at D0 and D28 during the induction phase of treatment.

PERIPHERAL BLOOD	NOx (μM)	SH (μM)	TRAP (μM)	LOOH (R.L.U)
D0	6.54	354.95	907.98	966 × 103
	(2.50–16.16)	(148.81–436.29)	(690.24–1390.30)	(690 × 103–398 × 104)
D28	8.54	308.56	691.35	1740 × 103
	(5.19–24.73)	(205.32–403.68)	(574.56–1234.0)	(799 × 103–467 × 104)
BONE MARROW				
D0	5.13	330.75	857.17	243 × 104
	(3.17–17.41)	(72.70–418.33)	(619.64–1291.9)	(948 × 103–531 × 104)
D28	8.03	294,06	775,16	2200 × 104*
	(4.38–27.47)	(238.96–324.80)	(496.80–1114.90)	(105 × 103–771 × 105)

Legend: The results are presented as median (min-max). Statistics – Wilcoxon completed with Spearman test. PB = peripheral blood plasma; BM = bone marrow plasma; B-LLA = Acute lymphocytic leukemia B; D = day of treatment; NOx (nitric oxide products); SH (sulfhydryl groups); TRAP (total radical-trapping antioxidant parameter); LOOH (hydroperoxides); R.L.U = relative light unities. Symbols indicates p < 0.05: ● D0 vs D28.

LOOH was augmented in PB on D28 concerning the diagnosis. Peripheral changes may reflect systemic catabolism and/or stress. LOOH are intermediate metabolites generated by lipid peroxidation in redox reactions, persisting from oxidative cellular damage to apoptosis induction. In leukemia, systemic LOOH has been reported as either normal (Devi et al., 2000) or augmented (Battisti et al., 2008) during treatment. The present data indicate that LOOH increased in PB during treatment, probably reflecting the cumulative free radicals generated by ALL-related chemotherapy, since most of the drugs used here are known pro-oxidants (Sentürker et al., 1997; Sarmento-Ribeiro et al., 2012). This observation is following a previous finding (Naz et al., 2013), wherein oxidative stress was higher at the end of the induction phase of treatment in acute leukemia patients regarding their diagnostic status.

A comparative analysis of PB and BM revealed that LOOH status is completely different at diagnosis, suggesting an oxidative lipid-rich environment in BM. The intensity of the induction phase of treatment is crucial for reducing the number of tumor cells; however, it also affects normal blood precursors present in the BM niche (El-Sabagh et al., 2011). On further analysis of total lipid content from all BM samples, no difference was detected among groups during treatment (data not shown), despite the potential replacement of cellular lipids in BM cells during treatment. These data suggest that lipid peroxidation in B-ALL is possibly dependent on both lipoproteins in PB and cell membrane in BM.

Regarding the hematopoietic cytokine environment observed in B-ALL biology, it is known that some of them are significantly modified by induction chemotherapy, especially IFN-γ and TNF-α (Broto et al., 2020). We found increased levels of IFN-γ in the BM and augmented TNF-α in the PB at the end of induction treatment (D28 vs D0). This finding indicates that the cytokine dynamics between both compartments change distinctly in response to induction chemotherapy. At D0, the predominant cell phenotype is malign, which is progressively replaced by healthy cells as treatment advances. It seems that corticotherapy is responsible for TNF-α reduction and INF-γ increase in ALL, and these changes are reported as accompanied by neutrophil count increase and augmented G-CSF and GM-CSF (Tuncer et al., 1996). It may be associated with the recovery of the normal bone marrow observed at the end of the induction phase. Further, TNF- is a pro-inflammatory cytokine that regulates oxidative stress production, as observed by the high LOOH in PB detected in our patients.

Concerning the clinical impact of our findings, it was observed that MRD + patients at the end of the treatment presented significant changes in LOOH levels. On D28, MDR + patients presented higher LOOH levels in PB and no significant fluctuations in BM. These findings suggest that positive MRD status, i.e., the presence of residual treatment-resistant tumor cells at the end of the treatment may be required for the production of lipoperoxides observed in this study. MRD occurs significantly in some cases of ALL (Ozcicek et al., 2016) and its assessment is primordial to classify the risk of patient relapse at this stage (Li et al., 2009). An in vitro study (Reddy et al., 2011) identified that oxidative stress generation in mitochondria is linearly correlated with chemotherapeutic resistance in acute lymphoblastic leukemia, which allows for redox adaptation and an increase in MRD in patients. This information reinforces our finding regarding LOOH in MRD + patients since lipid peroxidation metabolites can contribute substantially to promote and sustain oxidative stress. As far as we know, this is the first time that oxidative stress products are pointed as putative markers associated with MRD in the B-ALL context.

Our study has limitations. First, the sample size was limited, since the study was initiated at the diagnosis stage and the same patients were followed up through the induction phase of treatment. Second, there was a lack of healthy controls to evaluate changes in both PB and BM parameters. Further studies with larger sample sizes and appropriately matched healthy controls are demanded.

5. Conclusions

Our data indicate that distinct inflammatory mediators are produced in response to induction chemotherapy from diagnosis to the end of treatment and showed LOOH as a potential marker with clinical meaning in children with MRD. Such findings could have clinical implications in the future, suggesting that the measurement of lipid peroxides at diagnosis and the end of the induction phase could help clinicians predict patients who will not respond to ALL-B treatment and present MRD.

Availability of data and material

Datasets of this study are available from the corresponding author upon request.

Ethics approval

CAAE number 24498213.0.0000.5231 – Plataforma Brasil.

Consent to participate

All participants have consent forms signed by their parents or guardians.

Consent for publication

All data presented in the manuscript was obtained after authorization for publication by parents or guardians of patients.

CRediT authorship contribution statement

G.E. Broto: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **P.R.B. Silva:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **F.C. Trigo:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **V.J. Victorino:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **K.L. Bonifácio:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **W.R. Pavanelli:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **F. Tomiotto-Pelissier:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **M.R. Garbim:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **S.T. Oliveira:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **J.J. Jumes:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **C. Panis:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review &

editing. **D.S. Barbosa:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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