Non transferrin bound iron (NTBI) in acute leukemias throughout conventional intensive chemotherapy: Kinetics of its appearance and potential predictive role in infectious complications

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A B S T R A C T

We analyzed appearance of non transferrin bound iron (NTBI) in 30 transplant eligible patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) during conventional chemotherapy treatment program and evaluated possible relationship with transfusional body iron intake, iron parameters and clinical complications. For each course, serum samples for NTBI detection were taken prior to chemotherapy, during treatment and during subsequent bone marrow myelosuppression: NTBI was assessed by HPLC. Appearance of NTBI was observed from the start of induction treatment and was still detectable during bone marrow myelosuppression; the recovery of the bone marrow function coincided with the disappearance of NTBI. This kinetic was observed in all subsequent high doses chemotherapy courses, independently from confounding variables such as transfusional iron intake and transferrin saturation. NTBI seems to be a consequence of chemotherapy induced lysis of bone marrow cells and, partly, of hepatocytes after cytotoxic injury. The subsequent persistence of NTBI throughout bone marrow myelosuppression is related to the transient suspension of erythropoietic activity. Moreover, NTBI levels >2 μM at the beginning of iatrogenic myelosuppression were associated with higher risk of sepsis caused by Gram negative Bacilli (RR 2.571), also compared with other infectious complications (RR 1.954).

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1. Introduction

Transfusional iron overload (IO) pre-allogeneic hematopoietic stem cell transplantation (HSCT) has been associated with reduced overall survival (OS), increased non relapse mortality, increased incidence of graft-versus-host disease, infectious complications, and veno-occlusive disease [1]. Non transferrin bound iron (NTBI), a toxic low molecular weight fraction of iron, is usually detectable in iron loaded patients. Hydroxyl radical formation catalyzed by NTBI during conditioning regimen is one possible mechanism for tissue injury during cytotoxic therapy and might contribute to HSCT related complications [2]. Previous studies have shown that NTBI is present in patients undergoing cytotoxic chemotherapy and that this may exacerbate organ damage following chemotherapy [3–5], but few data regarding the kinetics of NTBI in acute leukemias during chemotherapy treatment are available in the literature, deriving from small heterogeneous cohorts and considering a single chemotherapy course. No data regarding direct correlations between NTBI and infections in this specific setting of patients are reported. We therefore analyzed serum NTBI in transplant eligible patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) throughout the whole conventional chemotherapy program, in order to investigate its appearance and to evaluate possible relationship with transfusional daily body iron intake, iron parameters and clinical complications.

2. Materials and methods

2.1. Patients

We prospectively analyzed 30 consecutive patients (mean age 48 years; 21–65) with transplant eligible acute leukemia (16 AML and 14 ALL, 14 females and 16...
males), referred to our Division between November 2012 and December 2013. Clinical follow-up was performed from diagnosis to the end of treatment program and written informed consent was provided by all patients, in accordance with the Declaration of Helsinki. Allogeneic transplantation was performed according to risk classes [6,7]: since transplant indication is not always known at diagnosis (e.g., holding on completion of molecular and cytogenetic risk assessment during induction chemotherapy, relapsed/refractory disease after conventional chemotherapy), we considered as transplant eligible all patients for whom age (i.e., under 65 years) and comorbidities do not represent contraindication to allogeneic transplantation, as such conditions may occur; on account of this, all patients included in our study are treated with curative intent with intensive chemotherapy regimens. AML patients were treated with conventional 3 + 7 regimen (idarubicin and cytarabine) followed by high doses (HD) cytarabine consolidation chemotherapy; 3 of them received high doses salvage treatment for relapsed/refractory disease with fludarabine, cytarabine and mitoxantrone. After a short pre-phase with cyclophosphamide and prednisolone, patients affected by ALL underwent induction therapy according to IVAP regimen (prednisone, cyclophosphamide, idarubicin, vincristine, l-asparaginase, dexamethasone), followed by sequential consolidation therapy: high dose MTX/cytarabine or MTX/l-asparaginase, alternated with low dose chemotherapy; 1 of them received a course of clofarabine and cyclophosphamide for relapsed disease. PH positive patients received imatinib in association with standard chemotherapy, omitting l-asparaginase. Allogeneic HSCT was performed in 12 patients (7 AML and 5 ALL patients). Transfusional daily body iron intake until HSCT or until the end of consolidation chemotherapy, expressed in mg of iron, was calculated as total amount of red blood cells (RBCs) transfused X 1.08 mg iron (mL RBCs) X the number of days of study period and adjusted for body weight. For each course, peripheral blood samples for NTBI detection were taken prior to chemotherapy, at the end of the course (an additional sample was taken on day 4 in induction treatment) and during subsequent bone marrow myelosuppression. Serum iron, transferrin, transferrin saturation (% TFSat) and ferritin were also monitored in the same timepoints of NTBI detection.

2.2. NTBI quantitation

Serum NTBI content was assayed by high performance liquid chromatography [10]. Briefly, 450 μl of serum was added to 50 μl of nitritotriacetic acid (NTA) 800 mol/l (pH 7.0) to scavenge NTBI which was quantitatively converted to Fe–NTA complex. After ultrafiltration, the ultrafiltrate (20 μl), which contained the Fe–NTA complex, was injected directly into the HPLC system (Perkin-Elmer series 200 IC cation pump module; Perkin-Elmer Life Science, Boston, MA, USA). Standards were run routinely from 0 to 10 μmol/l (in 1 μmol/l steps). Under these conditions, the 0.01 μmol/l standard corresponded to 80 μmol/l of NTBI. Normal individuals always have negative NTBI values because samples are measured in parallel with a corresponding blank formed by water and NTA. Water per se contains small amounts of iron that are not bound by transferrin, whereas in samples, transferrin that is not completely saturated captures some iron from the Fe–NTA complex. Therefore, the blank subtraction makes the NTBI value in some samples negative.

2.3. Statistical analysis

Descriptive statistics are expressed as means ± standard deviation (SD), unless otherwise stated, or percentages where appropriate. Student’s t-test was performed for continuous variables and Fisher’s exact test for categorical variables. All P-values are two sided with the level of significance set at <0.05. Relations between NTBI and other variables were evaluated with linear regression. Statistical analysis was also performed after adjusting NTBI levels for confounding variables such as transfusion iron intake and iron parameter in order to better evaluate the influence of chemotherapy on NTBI appearance.

3. Results

3.1. Appearance of NTBI and iron parameters

Median follow up was 4.7 months (0.5–10.6). Fig. 1 shows the variation in mean NTBI levels throughout induction treatment and subsequent high doses chemotherapy courses. Appearance and increase of NTBI levels respect to pre-chemotherapy values was statistically significant for each timepoint, except for the 4th high doses chemotherapy course, where NTBI was detectable pre-chemotherapy. Persistence of NTBI was observed during bone marrow myelosuppression; NTBI disappearance coincided with the recovery of bone marrow function. Of note, NTBI was already detectable at diagnosis in 9 (30%) patients.

Mean ferritin level at diagnosis was 1199 μg/L (95% CI: 725–1674), with no differences with ferritin levels at the end of the study (1605 μg/L; 95% CI: 936–2112). Serum ferritin concentration varied widely between patients and during the observation period, with no correlation with NTBI levels at any stage. Linear relation was found between NTBI and serum iron (p < 0.0001, r² 0.4782), although NTBI was detectable in many cases with serum iron within the normal range. Baseline transferrin saturation was 52 ± 22.1%: there was a sudden rise in transferrin saturation after chemotherapy start. Increase in transferrin saturation was statistically significant with respect to pre-chemotherapy values for each cycle, except for the 4th high doses chemotherapy course. Of note, mean maximum peak in transferrin saturation was 80.6 ± 8.9% after the third HD chemotherapy course. Linear relation was found between transferrin saturation and NTBI levels (p < 0.0001, r² 0.6153): appearance of NTBI was observed in 90.4% of the samples with transferrin saturation >80%, but NTBI was also detectable in 28.5% of the samples with transferrin saturation <80%. Only in 2 samples throughout the whole observation study transferrin was fully saturated. Absolute transferrin levels fell from pre-chemotherapy levels during each course and the subsequent bone marrow transient myelosuppression, with recovery of serum levels at the time of the next chemotherapy course. Of note, mean serum transferrin levels were always under the lower normal range of 200 mg/dl of our laboratory. No correlation between NTBI and serum transferrin were found, whereas linear relation between NTBI and absolute mg/dl of saturated transferrin was observed (p < 0.0001, r² 0.4853). Mean daily body iron intake at the end of the study was 0.70 ± 0.41 mg/kg per day. No correlation between transfusion burden and NTBI appearance was found. In particular, we analyzed variation in NTBI levels between consecutive timepoints of serum collection and calculated the respective daily body iron intake of each interval: after adjusting each NTBI variation for the respective iron intake no relationship between NTBI formation and transfusion burden was observed; moreover, appearance of NTBI between two consecutive timepoints was observed in many cases in the absence of active transfusion during the interval. In order to evaluate the influence of absolute saturated transferrin as a confounding variable in the appearance of NTBI, adjustment of NTBI levels with respective absolute saturated transferrin values was performed: significant influence of chemotherapy and bone marrow myelosuppression as independent variables on the appearance and disappearance of NTBI was maintained. Comparing AML and ALL patients, no significant differences regarding NTBI behavior throughout chemotherapy treatment were found, although the sample size is probably too small for a comparison of different treatment regimens. Considering patients who underwent allogeneic HSCT, occurrence of NTBI was observed at the end of conditioning regimen, comparing with pre-treatment samples (p = 0.0008); of note, in 2 of the 12 patients NTBI was detectable in the pre-conditioning regimen serum sample.

3.2. NTBI levels and infectious complications

Clinical follow-up among the whole observation period revealed 64 infective events, including 11 clinically documented infection (CDI) in the absence of microbial isolation, and 6 invasive fungal infection (IFI), according to diagnostic EORTC/MSG 2008 criteria [11]. Of them, 32 events were sepsis caused by Gram negative Bacilli, while the remaining 15 events were caused by other microorganisms, as reported in Table 1. Serum samples collected at the beginning of bone marrow suppression of each course with absolute neutrophil count (ANC) <500 mm⁻³ revealed that mean NTBI levels were significantly higher in patients who underwent sepsis caused by Gram negative bacilli respect to all others: 2.115 μM (95% CI: 1844–2387) vs 0.8424 (95% CI: 0.4496–1.235), p = 0.0004. Statistical significance was maintained considering both mean NTBI levels of patients with no infective complications (0.9334 μM; 95% CI: 0.5594–1.308, p < 0.0001) and
mean NTBI levels of patients who underwent other kinds of infections (0.7313 μM; 95% CI: 0.2642–1.198, p < 0.0001), as shown in Fig. 2. We arbitrarily considered a cut off value of 2 μM and we found that NTBI levels >2 μM measured at the beginning of bone marrow suppression were associated with an increased risk of sepsis caused by Gram Negative Bacilli (RR 2.571), also compared with other infectious complications (RR 1.954).

Appearance of NTBI was observed from the start of induction treatment and was still detectable during bone marrow myelosuppression; the recovery of the bone marrow function coincided with the disappearance of NTBI. This kinetic was observed in all subsequent high doses chemotherapy courses, independently from confounding variables such as transfusion iron intake and transferrin saturation. No relations with ferritin levels were found. NTBI levels >2 μM at the beginning of iatrogenic myelosuppression were associated with higher risk of sepsis caused by Gram negative Bacilli (RR 2.571), also compared with other infectious complications (RR 1.954).

Table 1

<table>
<thead>
<tr>
<th>Etiology of infective complications observed during the study period. IFI: invasive fungal infection; CDI: clinically documented infection.</th>
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<tbody>
<tr>
<td><strong>Microorganism/infective events</strong></td>
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<tr>
<td>Escherichia coli</td>
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<td>Pseudomonas aeruginosa</td>
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<td>Klebsiella pneumoniae</td>
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<td>Enterobacter cloacae</td>
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<td>Serratia marcescens</td>
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<td>Stenotrophomonas maltophilia</td>
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<td>Acinetobacter baumannii</td>
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<td>Proteus penneri</td>
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<td>Ochrobactrum anthropi</td>
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<td>Enterococcus faecium</td>
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<td>Enterococcus faecalis</td>
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<td>Bacillus cereus</td>
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<td>Clostridium septicum</td>
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<tr>
<td>Clostridium difficile</td>
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<tr>
<td>IFI (EORTC/MSG 2008 criteria [11])</td>
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<td>CDI</td>
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</tbody>
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Fig. 1. NTBI levels (mean with 95% CI) throughout chemotherapy program. Timepoints for each course are detailed on the graph. P-values are referred to comparisons with respective pre-chemotherapy NTBI levels.

Fig. 2. NTBI levels (mean with 95% CI) at the beginning of bone marrow suppression according to infective complications.
4. Discussion

Our study analyzed the kinetics of the appearance and disappearance of NTBI in a prospective cohort of AML and ALL patients treated with conventional chemotherapy. Our observations regarding chemotherapy-related changes in serum parameters are partially consistent with previous study performed on heterogeneous cohorts of patients with different hematologic diseases and treated with a single course of different chemotherapy regimens [3–5]. Chemotherapy repeatedly induces a transient decrease in serum transferrin and its rapid saturation; those alterations persisted during bone marrow suppression and disappeared after recovery of bone marrow function, again occurring at the subsequent chemotherapeutical course. In contrast with previous reports, occurrence of NTBI was observed in 28.5% of the samples with transferrin saturation <80%. Conventional intensive chemotherapy and subsequent bone marrow suppression causes the transient appearance of NTBI, independently from confounding variables such as transfusion daily body iron intake and low transferrin levels throughout the study. This observation suggests that formation of NTBI is, firstly, a consequence of chemotherapy induced lysis of bone marrow cells and, partly, of hepatocytes after cytotoxic injury. The subsequent persistence of detectable NTBI throughout bone marrow myelosuppression is related to the transient suspension of erythropoietic activity. To the best of our knowledge, no other study has investigated NTBI biology and clinical implications throughout the whole first line chemotherapy treatment, preceding HSCT: our data suggest that iron toxicity in these patients is not primarily related to IO per se but to serum free iron, which may increase oxidative tissue damage. Moreover, according to our data serum NTBI levels >2 μM at the beginning of iatrogenic myelosuppression seems to be associated with increased risk of sepsis caused by Gram negative Bacilli. It is known from different experimental models of infections that bacterial virulence is greatly enhanced by iron [12]: the mechanisms whereby potential pathogens acquire the iron necessary for growth in vivo, and the expression of virulence, are extremely complex and diverse; particularly, Gram negative Bacilli such as the genera Escherichia can uptake freely available iron by the production of iron chelators enterobactin and aerobactin, while Pseudomonas species possesses effective siderophores in pyochelin and pyoverdin. If confirmed, our observation suggests that monitoring of NTBI at the end of chemotherapy may predict patients at risk of sepsis caused by Gram negative Bacilli. Moreover, our data reinforce the rationale for early iron chelation strategies in order to prevent such complications; of note, a preliminary attempt regarding feasibility of early chelation therapy in acute leukemias during induction and consolidation chemotherapies was unsuccessful in a clinical trial [13]: warnings regarding safety and interactions with chemotherapy require careful investigations.

Conflict of interest statement

The authors have no competing interests.

Acknowledgments

Contributions: A.B. designed parts of the study, analyzed data and wrote the paper; L.D. performed the HPLC assay; L.B. designed parts of the study and discussed the data; S.R. collected parts of the settings; R.K. collected parts of the data; M.P. collected parts of the serum samples; P.P. revised statistical analysis and discussed the results; E.P. designed parts of the study; M.D.C. provided the HPLC, designed parts of the study and discussed the data.

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