Chapter 5

Cisplatin-based chemotherapy increased serum iron and non-transferrin bound iron in patients treated for testicular cancer

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ABSTRACT

Introduction: Iron overload can result in widespread organ dysfunction. Chemotherapy has been reported to induce a rise in serum iron. We aimed to assess the kinetics of serum iron and non-protein bound ("free") iron (NBPI) in patients with germ-cell tumours (GCT) during curative chemotherapy.

Methods: We studied 26 metastatic GCT patients (median age 39 years) undergoing cisplatin-based chemotherapy; 23 received BEP (cisplatin 20 mg/m2 day 1-5, etoposide 100 mg/m2 day 1-5, bleomycin 30 mg/m2 day 2, 8 and 15; q 21d), 2 also received Paclitaxel (175mg/m2 on day 3) and one patient received cisplatin and etoposide. Two baseline and 35 serial blood collections for iron parametre measurements were performed in 26 patients during the first and in 20 patients during the second chemotherapy cycle.

Results: Twenty-four hours after start of chemotherapy, serum iron (20±9 vs. 42±12; $p<0.001$) and NPBI (0.7±0.4 vs. 1.4±0.5; $p<0.001$) increased two-fold, whereas iron binding capacity (30±10 vs. 4±3; $p<0.001$) markedly decreased. Iron status remained unchanged between days 2-8. Ferritin increased two-fold after the first chemotherapy cycle, but serum transferrin remained unchanged.

Conclusions: These data indicate that chemotherapy treatment in metastatic GCT patients results in a steep rise in serum iron, full saturation of transferrin and appearance of highly toxic non-transferrin bound iron. Further studies are warranted to establish the role of chemotherapy-associated iron overload and short- and long-term chemotherapy-related toxicity in GCT patients.

INTRODUCTION

Iron is essential for many physiological processes and the amount in the human body is determined by the iron entering the circulation from the gut, whereas there is no physiological mechanism for excretion of excess iron. However, excess iron can lead to severe tissue damage by promoting the generation of reactive oxygen species by the Fenton reaction. The safe sequestration of such metals in non-redox-active forms, restricting the availability of catalytic iron ions in vivo, can be regarded as a component of the antioxidant defence network, this component being particularly important in the extracellular milieu. Iron homeostasis is tightly controlled and slight disturbances of iron metabolism will readily lead either to iron deficiency or to iron overload.

About two-thirds of body iron is found in hemoglobin, with smaller amounts in myoglobin, and various enzymes. Iron is also stored in ferritin, which can accommodate up to 4,500 atoms of iron.[145] Normally, only one third of transferrin is saturated with iron and the amount of "free" iron available in the serum is expected to be zero. Iron sequestration may be regarded as a contribution to antioxidant defences and by using transport proteins, e.g. transferring, and storage proteins, e.g. ferritin, the size of the intracellular iron pool is minimized to prevent organ damage.

When the iron-binding capacity of transferrin and other iron transport proteins in the serum of iron-overloaded patients is exceeded, redox reactive non-protein bound iron (NPBI), often referred to as non-transferrin bound iron (NTBI), or "free" iron, enters the circulation and becomes detectable [146-149] leading to a cascade of oxidative damage.[59] The consequences of inability to dispose of excess iron are seen in iron overload following multiple blood transfusions and in idiopathic hemochromatosis. Clinical sequelae of transfusional iron overload has been shown to result in iron accumulation in the liver and other organs, leading to cardiac, pancreas, and pituitary dysfunction [150], a pattern of organ dysfunction similar to idiopathic hemochromatosis.

Also, experimental iron overload in animals was shown to result in oxidative cellular damage [56;57], manifested by vascular, renal and liver damage [58]. Animal data support a link between iron and atherosclerosis[151] whereas treatment of the animals with the iron chelator desferrioxamine has shown to delay the onset of both iron accumulation and atherosclerosis.[152]

Tissue injury also liberates catalytic metal ion and cells sense iron levels and subsequently respond by production of iron-sequestrating proteins, e.g. ferritin. Also during chemotherapy, release of iron as a result of cell injury has been observed

resulting in saturation of plasma transferrin and the appearance of non-protein bound iron in the circulation.[55] Release of iron may not have been recognized up till now as a side effect of standard chemotherapy regimens in cancer patients.

The prognosis of patients with germ-cell tumours (GCT) has become excellent since the introduction of cisplatin-based chemotherapy, with cure achieved in the vast majority of patients with metastatic disease.[21;153] Chemotherapy administered to these relatively young cancer patients is associated with acute as well as long-term toxicity, such as potentially life-threatening venous thrombo-embolic and arterial vascular complications during chemotherapy in about ten percent of patients, and an increased risk of cardiovascular disease and other late sequellae in long-term GCT survivors.[21;28;153;154] The aim of the current study was to investigate the kinetics of serum iron parameters in a homogenous group of male metastatic GCT patients receiving curative chemotherapy. These results may provide further insight in potential mechanism of treatment-related toxicity and tissue damage.

MATERIAL AND METHODS

Study Design

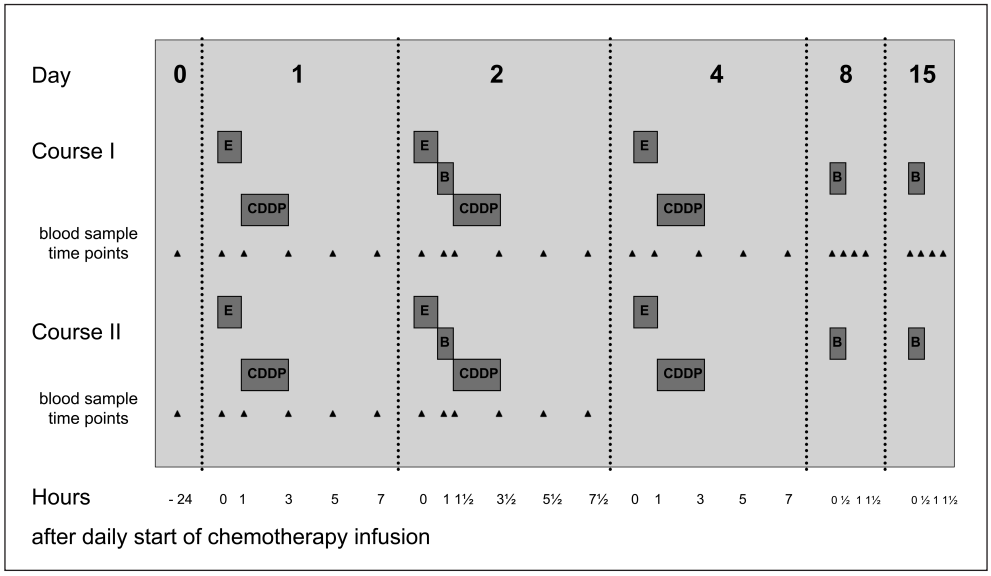
Between 2007 and 2009, we prospectively studied 26 metastatic GCT patients aged 18 to 58 years (median age 38 years), referred to the Department of Clinical Oncology of the Leiden University Medical Center for first-line cisplatin-based combination chemotherapy (Table 1). During the same period 14 patients could not participate for logistic reasons or non-eligibility. Twenty-three patients had a favorable prognosis, and 3 patients had an intermediate risk using the IGCCCG Classification [155]. Exclusion criteria were concomitant use of medication with known anti-oxidative activity, active cardiovascular diseases not related to the current malignancy, and elevated transaminases above three times the upper limit of normal. The majority of patients had an unremarkable medical history and no co-morbidity; one patient had a history of hypothyroidism and diabetes mellitus requiring oral medication and presented with venous thrombosis of pelvic veins as a result of local compression by lymph node metastases. Three patients used medication for mild hypertension. One patient received adjuvant single dose carboplatin (AUC7) for stage one seminoma 8 years before the start of BEP chemotherapy. Patients did not receive blood transfusions before chemotherapy or during chemotherapy.

Table 1. Demographics and tumour characteristics.

Demographics (median, min - max)	
Age (years)	38.4 (18.4 -57.7)
Δ Time after orchidectomy (years)	0.1 (0.01-8.1)
Characteristics (mean ± SD)	
Height (m)	1.83 ± 0.08
Weight (kg)	85.7 ± 12.2
BMI (kg/m^2)	25.4 ± 3.2
Systolic blood pressure (mmHg)	137 ± 20
Renal function (Cockroft clearance ml/min)	138 ± 34
Life style; n (%)	
Smoking (packyears)	9 (34.6)
Alcohol use (>20 U/week)	5 (19.2)
Histology; n (%)	
Seminoma	12 (46.1)
Non-seminoma	8 (30.8)
Combined tumour	6 (23.1)
TNM Tumour Staging; n (%)	
Stage II (para-aortic lymph node metastasis)	16 (61.5)
Stage III (distant metastasis)	10 (38.5)

Dependent on the prognostic risk group, 23 patients were scheduled to receive 3 or 4 courses of 3-weekly BEP chemotherapy consisting of intravenously administered etoposide (100 mg/m2 over 1 hr, days 1-5), cisplatin (20mg/m2 over 2 hr, days 1-5), and bleomycin (30 IUSP over 30 min), days 2, 8, and 15 (Figure 1). Two patients in addition received paclitaxel, 175 mg/m2 on day 1). One patient, a sport diver, did not receive bleomycin because of the potential pulmonary toxicity and was treated with etoposide and cisplatin (EP). The Medical Ethical Committee of Leiden University Medical Center (LUMC) approved the study protocol, and all subjects gave written informed consent before participation.

Figure 1. Time table of chemotherapy courses and blood sampling (triangles) on days 0, 1, 2, 4, 8 and 15 of the 1st chemotherapy course (course I) and days 0, 1 and 2 of the 2nd chemotherapy course (course II) in 26 patients with a testicular germ-cell tumour (TGCT).



E: etoposide, CDDP: cisplatin and B: bleomycin. The dotted line is used to indicate the separation between the sampling days.

Blood Sampling

To investigate the kinetics of NPBI and related iron parameters blood was drawn at various time points during the first cycle (n=26) and the second cycle (n=20) of chemotherapy. Blood was put on ice and centrifuged immediately at 2000g at 4°C for 10 min. Samples were kept below -70°C until analysis. Samples obtained from individual patients were analysed in the same run to minimize assay variability. All assays were performed at the Central Laboratories of Leiden University Medical Center.

During the first course, blood was drawn at 25 different time points: before pre-hydration (Day 0) and 24 blood samples drawn at fixed time points on days 1, 2 and 4, prior to each individual infusion of etoposide, bleomycin and cisplatin (Figure 1); on days 1, 2 and 4 at 2, 4 and 6 hours after the start of the cisplatin infusion (Figure 1) and on days 8 and 15; before and 30, 60 and 90 minutes after the start of the bleomycin infusion. During the second course blood was drawn on days 0, 1 and 2 at similar time points as during course 1 (Figure 1).

Analytical methods of iron status

All iron parameters were measured by routine procedures according to the manufacturer's instructions. For total iron and latent iron-binding capacity (LIBC), measurements were made with a Cobas Integra 800 instrument (Roche Diagnostics, Basel, Switzerland) with lower limits of detection (total assay variability between brackets) were 0.24 µmol/L (<2.8%) for total iron and 4.2 µmol/L (<4.3%) for LIBC. Transferrin and ferritin were measured with a Elecsys E170 analyser (Roche Diagnostics, Basel, Switzerland) with lower limits of detection (total assay variability) for ferritin and transferrin of 13 mg/L (<1.2%) and 0.5 g/L (<5.4%) respectively. NPBI concentrations were measured using a colourimetric method as described previously by Kolb et al.[156] Briefly, the serum samples were mixed 9:1 with a 40 mM NTA containing buffer of 5 mM Tris-HCl pH 6.5. After filtration and centrifugation thioglycolic acid sodium salt (3 mM) was added. Measurements were done using a Reader Spectra Max 250 plate reader at 537 nm. The (pooled) sera used for repeated experiments and patient sera were stored at -80 ºC until measurements. No influence of storage on the NPBI results was found. The lower limit of detection (total assay variability between brackets) was 0.01 µmol/L (<9.2%).

Routine hematology and chemistry

Blood samples were collected between 8.00 and 10.00 a.m. after an overnight fast for routine hematology (hemoglobin and white blood cell count) and chemistry [LDH, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphate (AP) and gamma glutamyl transferase (y-GT), creatinine, and albumin] assays before each course and when clinically indicated. Normal laboratory reference ranges are shown in Table 2.

Statistical analysis

To assess the changes within the first course (course 1) measurements of total iron, LIBC, NPBI at baseline, after pre-hydration, at the end of the first day, before and after the fourth day, before the fifteenth day, and before the second course were analysed using a mixed model analysis of variance (SAS proc. mixed) with visit as repeated factor within subject and time as fixed effects, and subject as random effect. To assess long-term treatment effects the course baseline and follow up measurements of all variables were analysed the same way. The time course of ferritin (after log-transformation because of non-normal distribution of the data)

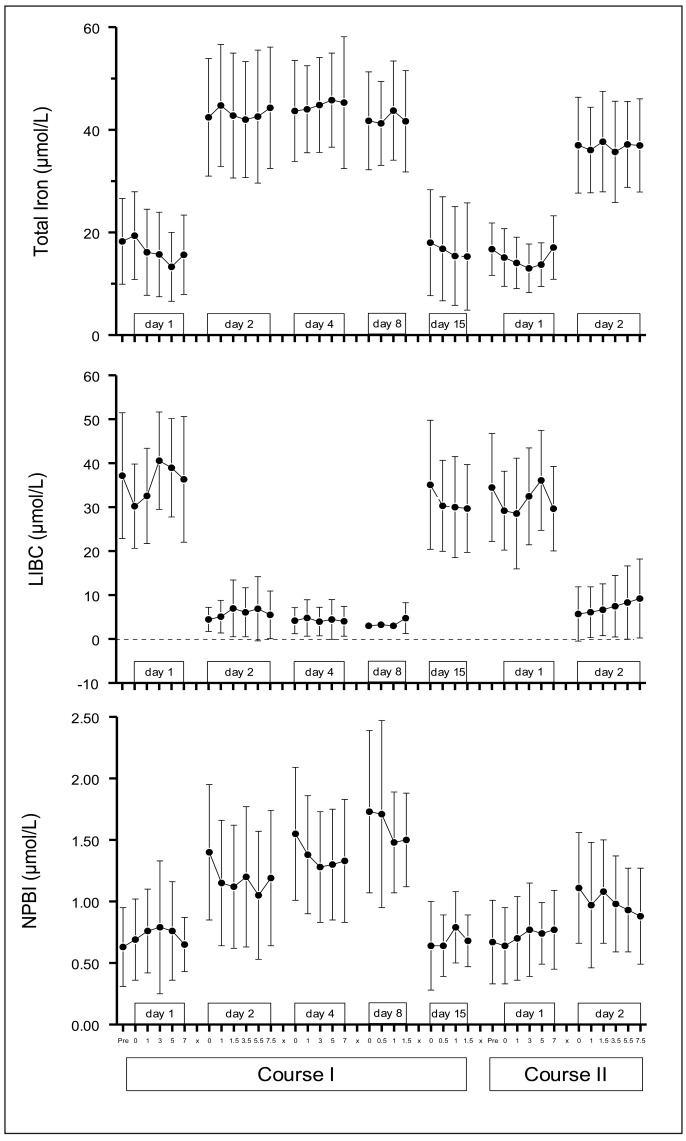
and transferrin concentrations were analysed with repeated measures analysis of variance and after overall statistical significance was found, individual time points were compared within the model. The routine laboratory parameters obtained before the first and second course were compared using paired t-tests with Bonferroni-Holm adjustment for multiple testing. All statistical analyses were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA). All p-values are adjusted for multiple comparison.

Table 2. Serum concentrations of routine laboratory parameters.

Parameter (mean±SD)	Before first course	Before second course	p-value*	Normal Range
Hematologic parameters				
Hemoglobin (mmol/l)	9.1 ± 0.8	8.1 ± 1.0	<0.001	8.5 – 11.0
Hematocrit (l/l)	0.432 ± 0.037	0.381 ± 0.043	<0.001	0.400 – 0.540
WBC (x10^9/l)	6.4 ± 1.9	3.3 ± 1.6	<0.001	4.5 – 10.0
Iron parameters				
Iron (µmol/l)	18 ± 8	17 ± 5	NS	11 – 28
LIBC (µmol/l)	35 ± 11	35 ±12	NS	27 – 54
NPBI (µmol/l)	0.63 ± 0.33	0.66 ± 0.34	NS	< 2.0
Transferrin (g/l)	2.44 ± 0.47	2.28 ± 0.39	NS	2.04 – 3.60
Ferritin (µg/l)	224.2 ± 170.6	460.3 ± 261.2	<0.001	35 – 260
Biochemical parameters				
Creatinine (µmol/l)	79.7 ± 12.3	72.9 ± 10.3	0.03	62 – 106
Albumin (g/l)	45.8 ± 3.6	43.5 ± 3.9	NS	34 – 48
AP (U/l)	87 ± 25	79 ± 20	NS	40 – 120
γ-GT (U/l)	36 ± 32	41 ± 28	NS	5 – 55
ASAT (U/l)	33 ± 10	29 ± 8	NS	5 – 35
ALAT (U/l)	33 ± 19	37 ± 18	NS	5 – 45

WBC: White blood cell count, NPBI: Non-protein bound iron, LIBC: Latent iron binding capacity, AP: Alkaline phosphatase, γ-GT: gamma glutamyl transferase, ASAT: Aspartate aminotransferase. ALAT: Alanine aminotransferase.

Figure 2. Time course of total serum iron (upper panel), latent iron-binding capacity (LIBC; middle panel) and non-protein bound iron (NPBI; lower panel) before the start of treatment ("pre-chemotherapy"), during the 1st BEP chemotherapy course, before the start of the 2nd chemotherapy course and during the first two days of the 2nd BEP chemotherapy course.



The data on the X-axis indicate the time (in hrs) at each observation day and the 'X' is used to indicate the separation between the sampling days. Sample times are noted according to the number of hours after start of daily chemotherapy infusion e.g. day one pre-infusion sample is noted as "Day 1, 0 hr sample" and day four last sample is noted as "day 4, 7 hr sample".

RESULTS

Baseline characteristics

The demographics of the 26 patients and their tumour characteristics are shown in Table 1. Twenty-four patients obtained a complete remission after planned first-line chemotherapy, whereas one patient required additional surgical resection ofresidual masses confirming a complete remission. Two patients developed major vascular complication during the 3rd chemotherapy cycle; one patient, aged 41 years, developed hemiplegia in the 2nd week of his 3rd BEP cycle as a result of a thrombotic mass in the artery cerebri media leading to a large area of infarction (CT-scan confirmed), and died of massive brain edema. Another patient, aged 34 years, was diagnosed with acute deep venous thrombosis of the popliteal vein and multiple pulmonary emboli four weeks after the start of his third and last BEP chemotherapy cycle. These events occurred in the absence of any known risk factor for vascular complications, hypomagnesia, and without any warning signs during the previous two chemotherapy courses.

Figure 3. Time course of serum transferrin (left axis) and serum ferritin (right axis) concentrations during the 1st chemotherapy course.

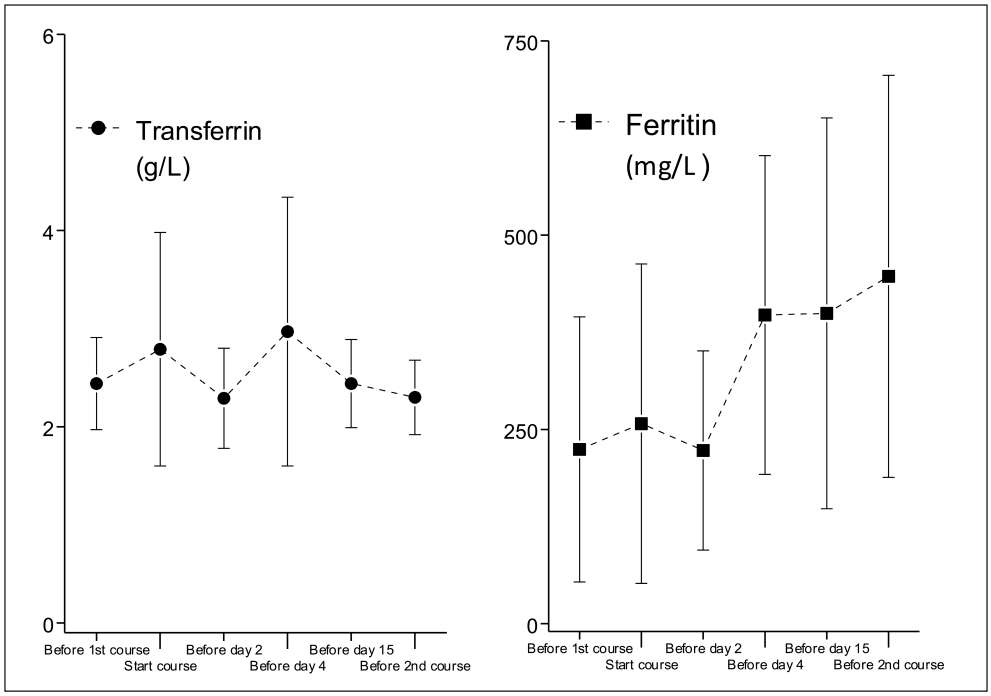


Table 3. Time course of estimated differences, 95% confidence intervals (95% CI) and corresponding p-values for total iron, latent iron-binding capacity (LIBC) and non-protein bound iron (NPBI) following the first course of chemotherapy in GCT patients.

	Total Iron (mmol/l)		**LIBC (µmol/l)**		**NPBI (mmol/l)**	
Contrast	Difference (95% CI)	p-value	Difference (95% CI)	p-value	Difference (95% CI)	p-value
Day 1, 0 hr sample vs. Day 0, -24 hr sample	1.97 (-2.08 / +6.01)	NS	-6.94 (-11.15 / -2.74)	0.001	0.06 (-0.16 / +0.28)	NS
Day 1, 7 hr sample vs. Day 1, 0 hr sample	-4.03 (-8.04 / -0.03)	0.05	6.16 (+2.00 / +10.32)	0.004	-0.04 (-0.27 / + 0.18)	NS
Day 2, 0 hr sample vs. Day 1, 0 hr sample	24.70 (20.64 / 28.75)	<0.0001	-24.99 (-29.20 / -20.78)	<0.0001	0.44 (+0.22 / +0.67)	0.0001
Day 4, 0 hr sample vs. Day 1, 0 hr sample	23.96 (19.80 / 28.11)	<0.0001	-26.70 (-30.96 / -22.44)	<0.0001	0.86 (+0.63 / +1.09)	<0.0001
Day 4, 7 hr sample vs. Day 1, 0 hr sample	26.18 (22.17 / 30.19)	<0.0001	-25.99 (-30.15 / -21.83)	<0.0001	0.61 (+0.39 / +0.83)	<0.0001
Day 15, 0 hr sample vs. Day 1, 0 hr sample	-1.65 (-6.85 / 3.55)	NS	5.04 (-0.36 / +10.44)	NS	-0.02 (-0.31 / + 0.28)	NS
Day 21, -24 hr sample vs. Day 1, 0 hr sample	-1.67 (-5.82 / 2.48)	NS	4.54 (+0.28 / +8.80)	0.04	-0.01 (-0.23 / + 0.21)	NS

Samples taken on days 1, 2, 4 and 15 of chemotherapy course I and day 1 and 2 of chemotherapy course II are also depicted in Figure 1 and 2. Sample 1.0 refers to day 1 and 0 hr, i.e. before the start of the first cytotoxic agent infusion (etoposide) whereas sample 1.7 refers to the blood sample collected on day 1, 7 hr after the start of the chemotherapy infusion (see also Figure 1).

Routine laboratory measurements

The first course of chemotherapy led to a significant decrease in serum hemoglobin, hematocrit, white blood cell count and creatinine and a significant increase in serum ferritin (Table 2).

Baseline variables and serial iron measurements

At baseline, all iron parameters, including total serum iron, latent iron binding capacity, transferrin and ferritin were within the normal range of the laboratory measurements (Table 2, Figure 2 and 3). In all patients detectable serum concentrations of NPBI were present before dosing ranging from 0.10 to 1.58 µmol/l with a mean value of 0.63 µmol/l (Table 2, Figure 2).

The pre-hydration preceding the administration of the cytostatics did not significantly alter total serum iron (p=0.34) and NPBI concentrations (p=0.59) (Table 3), but a 6.94 µmol/l decrease (19%) in iron binding capacity was noted (Table 3, p=0.001) which was restored to baseline levels after the completion of the first day of chemotherapy. Transferrin (2.44±0.47 vs. 2.83±1.24; p=0.16) and ferritin (224±171 vs. 259±216; p=0.55) concentrations did not change significantly after pre-hydration (Figure 3).

Serum iron concentrations increased within 24 hr following the start of the first infusions of etoposide and cisplatin and remained elevated during days 2-8 (Figure 2, Table 3). The distinct rise in total iron was accompanied by saturation of the iron-binding proteins, as evidenced by a decrease in LIBC and rise of NPBI. NPBI remained elevated at days 2-4. Iron parameters returned to baseline levels on day 15, and remained unchanged up till 1.5 hrs post outpatient bleomycin infusion. Transferrin concentrations varied slightly with a mean of 2.44±0.47 g/l during the first course of chemotherapy and were slightly lower (2.28±0.39 g/l; p=0.22) at day 21 at the beginning of second chemotherapy course (Table 2, Figure 3). Serum ferritin concentrations slightly increased from day 4 onwards and serum ferritin concentrations at the start of the 2nd chemotherapy course was significantly higher than at baseline (460±261 vs. 224±171 µg/l; p<0.001; Table 2, Figure 3).

Similar observations were made in the first days of the second course of chemotherapy, i.e. a rapid increase in total serum iron, depletion of the latent iron-binding capacity and increase in NPBI.

DISCUSSION

We identify a striking increase in total serum iron, saturation of iron binding proteins and rise of NPBI within 24 hours after a single administration of etoposide and cisplatin in all GCT patients studied. These changes persisted during the following days of cytostatic infusions up till day 8 and iron parameters returned to baseline values on day 15 of the first cycle. Similar changes in iron parameters were observed during the second chemotherapy course at the time points measured. The changes in iron parameters were identical in the two patients who received paclitaxel on day 3, and in the patient who did not receive bleomycin. Two patients developed severe vascular toxicity during the 3rd respectively 4th cycle, with fatal outcome in one patient.

The observed rise in serum iron and NPBI at 24 hr post infusion can be attributed to the infusion of etoposide, cisplatin or both. We have not collected blood samples at 24 hr post bleomycin infusion on days 8 and 15, and are thus not able to assess whether a similar two-fold increase in serum iron occurred post single agent bleomycin administration. The observed closely linked changes in serum iron parameters indicate that measurement of only serum iron and NPBI suffices to assess whether iron overload occurs.

Few reports exist concerning iron overload and release of catalytic non-protein-bound iron following chemotherapy.[56;157-161] Most studies concern hematological malignancy patients who received multiple blood transfusions and underwent chemotherapy with a variety of cytostatic agents often followed by stem cell transplantation, and these studies have not measured NPBI at multiple time points after the start of chemotherapy. Gordon *et al.*[162] also reported a rise in total serum iron in patients with hematological and solid tumours treated with chemotherapy or radiation, whereas Gordeuk *et al.*[163] described a rise of bleomycin-reactive iron in six out of nine patients treated for acute non-lymphocytic leukemia. Although hematological malignancy patients may have abnormal serum iron parameters related to the inherent bone marrow abnormalities and frequent red blood cell transfusions, the variety of cytostatic agents used point to a common effect of different chemotherapeutic agents to induce release of highly toxic excess iron.

A previous study reported about vascular events during cisplatin-based chemotherapy [21], we also encountered serious vascular toxicity during first-line chemotherapy in two of the current 26 patients studied. Acute and long-term vascular toxicity may be related to the chemotherapy-associated increase in iron; excess iron has been

associated with an increased risk of development of thrombo-embolic events and vascular dysfunction in animal experiments [160;164] and in humans. Excess iron increases the levels of adhesion molecules [161], and catalyses the oxidation of LDL-cholesterol [159] thus providing a plausible explanation by which excess iron may be involved in the development and progression of atherosclerotic lesions.

The marked changes in iron status during cisplatin-based chemotherapy could be caused by various mechanisms. Cytostatic drugs, in particular cisplatin, are known to inhibit erythropoiesis by blocking the biosynthesis of porphyrins and haem and iron incorporation into erythroid progenitor cells [157] resulting in subsequent release of free iron into the circulation.[158;165] Binding of cisplatin to hemoglobin, leading to dissociation of haem from the hemoglobin molecule is another mechanism suggested by *in vitro* experiments.[166] Preclinical studies and a few case reports suggest that lysis of iron-rich liver tissue or erythrocytes occurs during chemotherapy, but several studies have failed to detect significant liver damage or hemolysis [167;168], and this concurs with our own experience. However, chemotherapy-induced tumour lysis, specifically in chemosensitive tumours, and cellular damage of other tissues are a possible source of excess iron.

The current first-line cisplatin-based chemotherapy in patients with intermediate and good prognosis metastatic GCT leads to a cure rate exceeding respectively 80 and 90%. Thus, chemotherapy-associated iron overload and release of potentially harmful catalytic non-protein bound iron in these healthy young men may hold significant clinical implications in view of the reported acute and long-term morbidity and mortality in cured long-term survivors. Moreover, particularly cisplatin-based chemotherapy is known to be accompanied by anemia requiring blood transfusion, supplementation with erythropoietin and even oral or intravenous iron, which may further aggravate the cyclical iron overload. Before our findings can be translated to other groups of cancer patients receiving chemotherapy, these disturbances of iron metabolism should be monitored in other centers. A prospective trial investigating the prophylactic use of iron chelating agents aiming at prevention of chemotherapy-associated side effects is warranted.