RESEARCH ARTICLE

A prospective observational study of the effect of platelet transfusions on levels of platelet-derived cytokines, chemokines and interleukins in acute leukaemia patients with severe chemotherapy-induced cytopenia

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ABSTRACT. ***Background.*** **Platelet concentrates contain soluble mediators derived from both platelets and contaminating leukocytes. During platelet transfusion these mediators are transferred, and transfusion-induced modulation of the cytokine network may then occur, possibly contributing to transfusion reactions, immunomodulation, or affecting residual leukemic cells. In this prospective observational study, we investigate the effect of platelet transfusion on the systemic levels of platelet-derived cytokines, chemokines and interleukins in an unselected group of acute leukaemia patients with severe chemotherapy-induced cytopenia.** ***Study design and methods.*** **We investigated 31 platelet transfusions involving pre-storage, white blood cell-reduced, gamma-irradiated or pathogen-inactivated, photochemically-treated platelet concentrates received by 10 unselected patients. Peripheral blood plasma samples were collected before, immediately after, one hour, and 24 hours after the transfusions. Sampling from platelet concentrates was performed immediately before transfusion. A total of 31 soluble mediators were examined. Ten healthy controls matched for age and gender were included.** ***Results.*** **Despite heterogeneity in patients and platelet concentrates, significantly increased plasma concentrations were detected for the platelet-derived mediators, platelet-derived growth factor, β-thromboglobulin, transforming growth factor-β (TGF-β), CCL5, and CXCL4, 1 hour and/or immediately after platelet transfusions. The plasma levels of vascular endothelial growth factor and soluble CD40 ligand were not altered by platelet transfusion. Certain interleukins (IL-1β, IL-2, IL-4, IL-6, IL-9, and IL-12), as well as interferon-γ showed a minor, transient decrease in systemic plasma levels during the first hour following transfusion.** ***Conclusion.*** **Platelet transfusions modulate the systemic cytokine network in acute leukaemia patients with severe, chemotherapy-induced cytopenia.**

Key words: platelet transfusion, acute leukaemia, cytokines, chemokines, sCD40L

Acute leukaemia patients receiving intensive chemotherapy experience a period of severe, treatment-induced pancytopenia often lasting 1-3 weeks depending on the treatment regimen [1-4]. The therapeutic use of hematopoietic growth factors has only limited effects on the duration of severe neutropenia [5], and the treatment-related mortality observed in this patient group is mainly due to the neutropenia, with a high risk of complicating bacterial and fungal infections [3]. The patients also develop severe thrombocytopenia. It is now generally accepted that these patients should be treated with prophylactic platelet transfusions, thus reducing the incidence of fatal hemorrhagic complications [6-8].

Patients with acute leukaemia are treated with conventional, intensive chemotherapy, autologous stem cell transplantation or allogeneic stem cell transplantation if a suitable donor is available [3]. These therapeutic strategies are associated with the development of severe, treatment-induced pancytopenia before reconstitution or engraftment occurs. Clinical studies have, in addition, demonstrated that for all these therapeutic strategies, the risk of a subsequent leukaemia relapse is reduced for patients with early

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lymphoid reconstitution [9-11]. The same is true for several other malignancies [12-14]. For allotransplant patients, it has been shown that cytokine levels during cytopenia have an additional prognostic impact [15]. Thus, biological events during the early period of therapy-induced cytopenia are important for the later risk of relapse, and thereby the long-term survival of these patients.

Platelet concentrates contain soluble mediators derived from both platelets and contaminating leukocytes [16-18]. These mediators are transferred passively with donor plasma, or they are released from platelets or leukocytes during harvesting or *ex vivo* handling of the concentrates. Additional cytokine accumulation is observed during storage, and depends on the preparation techniques used, *i.e.* pre-storage white blood cell filtration, pathogen inactivation methods or differences in plasma/platelet additive solution ratios [17, 19-25]. Because variations in cytokine concentrations may be important for the development of transfusion reactions [26-28], or have immunomodulatory effects [22, 29, 30], or direct or indirect effects on remaining leukaemic cells in the patients [31], we investigate whether infusion of these mediators alters the cytokine network immediately after, one hour, and 24 hours after platelet transfusion in an unselected group of acute leukaemia patients with severe, chemotherapy-induced thrombocytopenia.

DONORS AND METHODS

Study design

This prospective observational study was approved by the local Research Ethics Committee (University of Bergen, Norway). Owing to the lack of previous publications on this subject, we were not able to perform sample size calculation before data collections. The study was therefore designed as a prospective observational study, and data collection was performed during a pre-defined timeframe (December 2006 to April 2007). The study unit was defined as the platelet transfusion, and several transfusions could be included for each patient. The study hypothesis was that platelet transfusions had a general effect on systemic levels of soluble mediators in acute leukaemia patients with chemotherapy-induced cytopenia. The study was not designed to include separate analysis of patient subsets, but to investigate a population of unselected patients. All patients were recruited from the Section for Hematology, Department of Medicine at Haukeland University Hospital. The healthy control group (n = 10) was recruited from blood donors and members of the hospital staff and were matched by age (±6 months) and gender. The intention of the investigation of healthy controls was to identify those cytokines that usually show strong variations, and using this to illustrate the extent of transfusion-induced alterations in plasma levels. The control samples were collected during the study period in order to avoid differences in storage time between patient and control samples. All patients and healthy controls were included following informed written consent.

Preparation of platelet concentrates

Platelet concentrates were selected for transfusion based on the blood bank's general guidelines and by personnel not involved in the study. The donors reported no intake of medication known to affect platelet function within the five days prior to platelet donation. All platelet concentrates fulfilled the European requirements, including white blood cell content below 1.0×10^6 per unit [32]. Platelet concentrates were prepared according to the manufacturer's instructions, either by single needle aphaeresis employing the elutriation principle to provide leukocyte-reduced platelets (Fenwal Amicus Cell Separator, Baxter Healthcare Corp., Deerfield, Illinois, USA), or by use of pre-storage, white blood cell-filtered buffy-coat concentrates produced by automated procedures (OrbiSac, CaridianBCT, Inc., Lakewood, Colorado, USA). Pathogen-inactivated platelet concentrates were photochemically treated using Amotosalen and UVA-light (Intercept Blood System for Platelets, Cerus Corporation, Concord, CA, USA). Pathogen-inactivated and conventional platelet concentrates were suspended in 35-37% plasma and 63-65% PASIII (Intersol, Fenwal Inc., Lake Zurich, Ill, USA), or 63-65% PAS II (T-sol, Fenwal Inc.) respectively. Conventional platelet concentrates were gamma-irradiated immediately before transfusion (25 Gy; Gammacell 3 000 Elan, Nordion International inc., Ottawa, Canada). Concentrates were stored for up to 168 hours at 22 ± 2^{o}C, under constant agitation, in a flatbed, platelet incubator (Helmer, Noblesville, IN, USA) before transfusion. Bacterial surveillance was performed for all conventional concentrates.

Collection and preparation of blood samples

All patients had central venous catheters that were used for intravenous injections/infusions, and for the collection of blood samples. For the samples collected immediately after the platelet transfusion, 3 ml of saline were injected through the catheter; 10 mL of blood were aspirated and discarded before samples were collected. For all other procedures (transfusions, infusions, blood sampling) the institutional guidelines were followed for collection of the 1 hour and 24 hour study samples: *(i)* all previous catheter procedures (infusions or blood sampling) were ended by injecting 10 mL of saline and thereafter 2 mL of heparin solution 100 IU/mL; *(ii)* later blood sampling was performed as described for the immediate samples with injection of 3 ml of saline, discarding the first 10 mL of aspirated blood, and finally blood sampling.

Citrated blood samples from patients were collected before (< 8 hours) transfusion, immediately after transfusion, 45-120 minutes after transfusion, and 18-24 hours after transfusions. An additional sample was drawn from each platelet concentrate immediately before transfusion using a sterile docking device. All samples were centrifuged within 30 minutes in a two-step procedure at 4°C; first 1,000 g for 15 minutes, and thereafter 10,000 g for 10 minutes. The supernatants were stored at -70°C until analyzed.

Analysis of soluble mediators

Commercially available ELISA kits were used for analysis of CXCL4 (platelet factor 4, PF4, Hyphen BioMed, Neuville-sur-Oise, France), β-thromboglobulin (β-TG, Asserachrom β-TG, Diagnostica Stago, Asnieres, France), sCD40L (human sCD40L high sensitivity ELISA, Bender

Table 1
Characteristics of acute leukaemia patients included in the study.

Patient number	10
Number of platelet transfusions	31
Males/females	4/6
Age (years; median and range)	53 (21-62)
Diagnosis	
AML (number of transfusions)	8 (27)
ALL (number of transfusions)	2 (4)
Number of chemotherapy cycles examined*	13
Induction treatment (number of transfusions)	5 (17)
Consolidation therapy (number of transfusions)	8 (14)
Body surface area (m^2; median and variation range)	1.81 (1.50-2.19)
Number of patients (transfusions) with HLA-antibodies	3 (9)
Number of patients (transfusions) with HPA-antibodies	1 (3)
Number of patients (transfusions) with premedication (antihistamines ± intravenous corticosteroids)	2 (7)

* Three patients were tested both after induction and consolidation treatment.

MedSystems GmbH, Vienna, Austria), and transforming growth factor-beta 1 (human TGF-β1 ELISA, Bender MedSystems GmbH) in plasma and platelet concentrate samples. Concentrations of interleukin (IL) 1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, CCL11(Eotaxin), FGF basic (bFGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (INF-γ), CXCL10 (IP-10), CCL2 (MCP-1MCAF), CCL3 (MIP-1α), CCL4 (MIP-1b), platelet-derived growth factor (PDGF)-BB, CCL5 (regulated on activation normal T-expressed and secreted, RANTES), tumour necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF) were analyzed using multiplex bead-based cytokine assays (Bio-Plex ™ Cytokine Assay, Bio-Rad Laboratories, Hercules, CA, USA). Samples from patients and healthy controls were analyzed using a premixed, 27-plex cytokine assay, whereas samples from platelet concentrates were analyzed by combining two singleplex assays (CCL5 and PDGF), and a premixed 11-plex cytokine assay (IL-1b, IL-1ra, IL-6, CXCL8, IL-10, G-CSF, IFN-γ, CXCL10, CCL2, CCL4, and VEGF). In accordance with the manufacturer's recommendations, the samples from platelet concentrates were diluted to achieve the same protein concentration as the standard before analysis. All samples were analyzed in duplicate.

Additional investigations of patients and transfusions

All patients were examined for human leukocyte antigen (HLA) antibodies (FlowPRA® Class 1 Screening Test, One Lambda Inc, Canoga Park, CA, USA) and human platelet antibodies (MAIPA) [33]; patients with HLA antibodies were transfused with HLA class I-matched platelet concentrates. Reticulated platelets were examined regularly up to three times weekly, and were used as a predictor of hematopoietic reconstitution in accordance with a previous report [34]. In addition, information on the following clinical parameters was obtained for each transfusion: fever (>38.0 °C), documented infection, peripheral blood leukocyte counts, serum C-reactive protein (CRP), medication, and occurrence of transfusion complications.

Unsupervised clustering

To examine and visualize the global trends in the pre-transfusion cytokine levels, unsupervised clustering was performed in J-Express 2009 [35]. For TGF-β, the plasma levels were determined for only 13 transfusions, and this mediator was omitted from the analysis, together with CXCL4 and CCL3, as their levels were undetectable for all patients. As the values measured for the different molecules were quite varied, the data were preprocessed further, with a variance and mean normalization step for each molecule's measurement profile. The hierarchical clustering was performed using Pearson's correlation as distance measure and average (wpgma) linkage.

Statistics

Descriptive statistics, the exact Mann-Whitney test, and Pearson correlation analyses, not adjusted for individual patient effect, were performed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Changes in plasma concentrations of mediators before and after transfusion and comparison between healthy controls and patient pre-transfusion samples were analyzed after adjustment for individual patient effects, using the mixed effects model (nlme, R version 2.8.0, www.R-project.org, The R Foundation for Statistical Computing, Vienna, Austria) [36]. Differences were regarded as statistically significant when p-values were less than 0.05.

RESULTS

Patients and platelet concentrates

The patient characteristics are given in *table 1*. We studied a total of 31 routine platelet transfusions that were given to 10 unselected adult patients with acute leukaemia. During the study period, all acute leukaemia patients receiving intensive conventional chemotherapy were asked to participate, and all except one patient agreed to be included. All the included patients had a history of previous pregnancies and/or previous platelet

transfusions, none of them had disseminated intravascular coagulation. All except one patient were treated with conventional intravenous cytarabine-based chemotherapy. For most patients, cytarabine was used in conventional doses (100-200 mg/m^2), and combined with anthracycline, amsakrine, etoposide or mitoxantrone; but high-dose cytarabine alone (single doses of 3 g/m^2) was used for 10 consolidation cycles. All patients developed severe chemotherapy-induced pancytopenia, and they were all dependent on regular platelet transfusions to keep the peripheral blood platelet count above the individual transfusion trigger. All patients were treated according to the same institutional guidelines with regard to indications for platelet transfusions. Prophylactic platelet transfusions were given to non-febrile and clinically stable patients with peripheral blood platelet counts below 10×10^9/L, and to febrile patients when platelet counts were below 20×10^9/L. In patients with increased risk of bleeding (*e.g.* prior to invasive procedures or recent hemorrhage) or ongoing bleeding, platelet transfusions were administered when peripheral blood platelet counts were below 20-50 $\times 10^9$/L. All transfusions were ABO-compatible (recipient having no antibodies incompatible with the red cell ABO antigens of donor).

None of the patients showed signs of thrombopoietic reconstitution at the time of platelet transfusion and sampling. All patients depended on regular platelet transfusions to maintain platelet counts above the individual transfusion trigger. In 30 of 31 transfusions, the patients showed leukopenia, with neutrophil counts below 0.5×10^9/L. In one transfusion, the neutrophil count on the day of transfusion was 0.9×10^9/L. The median time from first day with neutropenia until platelet transfusion was nine days (range 1 to 26 days). The median pretransfusion percentage of circulating reticulated platelets was only 1.56 (range 0.31-6.73), and exceeded 4% only for one transfusion.

Both conventional (n = 22) and pathogen-inactivated (n = 9) platelet concentrates were included in the study. Fifteen of the transfused platelet concentrates were obtained by single needle aphaeresis, whereas sixteen were pre-storage, leukocyte-filtered buffy-coat platelet concentrates. The median age of the platelet concentrates at the time of transfusion was 70 (range 21-167) hours, and no significant difference was observed between storage time for conventional gamma-irradiated and pathogen-inactivated concentrates. The plasma concentrations of all mediators, apart from TGF-β, were investigated before, immediately after, and one hour following platelet transfusion for 31 platelet transfusions. The patient cytokine concentrations after 24 hours were measured for 11 unselected transfusions. For TGF-β, the plasma levels were investigated for only 13 transfusions apart from the 24-hour levels that were examined for five transfusions.

Systemic cytokine levels in acute leukaemia patients with severe chemotherapy-induced pancytopenia; examination of pretransfusion plasma levels

We investigated pretransfusion plasma cytokine levels for the 10 patients before 31 platelet transfusions. The pretransfusion cytokine concentrations for the 31 investigated cytokines and their classification are shown in *table 2*. Individual cytokines showed a wide variation, and from the overall results we observed:

– cytokines released by platelets showed relatively low plasma levels in cytopenic patients compared with healthy controls. Significantly lower plasma concentrations of β-thromboglobulin, PDGF-BB, and CCL5 were observed in patients. Statistical comparison could not be performed for CXCL4 owing to the high number of non-detectable levels;

– the levels of six additional CCL or CXCL chemokines were determined. In contrast to platelet-derived chemokines (CCL5, CXCL4), the patient pretransfusion levels were relatively high; the median plasma levels corresponding to the upper part of (CCL11) or exceeding the corresponding variation range for healthy controls (CCL2, CCL4, CXCL8, CXCL10). The only exception was CCL3 that showed undetectable levels. No significant difference was observed between pretransfusion patient samples and healthy controls;

– G-CSF was the only hematopoietic growth factor that showed relatively high plasma levels in patients with cytopenia. There were significantly higher levels in pretransfusion patient samples compared to healthy controls. G-CSF therapy was used in 11 platelet transfusions: three of these transfusions were given to ALL patients receiving routine G-CSF therapy and eight transfusions were given to AML patients treated with G-CSF as a consequence of severe bacterial infections. The G-CSF concentrations were significantly higher in patients receiving G-CSF therapy (median level 3,526 pg/mL, range 489-13,870), compared to pretransfusion plasma samples collected from patients not receiving G-CSF (median level 486 pg/mL, range 51-4,762; $p < 0.001$, exact Mann-Whitney test, SPSS 15.0). G-CSF levels before transfusion showed a significant correlation with serum CRP levels ($p = 0.004$) in patients not receiving G-CSF therapy, suggesting that these high levels are, at least partly, caused by the acute phase reaction. GM-CSF and bFGF showed low or undetectable levels;

– the majority of monocyte- and T cell-secreted cytokines showed relatively low levels, both in patients and healthy controls; this was also true for IL-7 and IL-15. The only exceptions were IL-1RA and IL-6 that are released by several cell types including monocytes and T cells. Plasma IL-1RA and IL-6 levels correlated significantly with the CRP levels ($p < 0.001$ and $p = 0.007$ respectively) in patients not receiving G-CSF therapy, and significant correlations were also detected when all transfusions were included in the analyses. These observations suggest that high IL-1RA and IL-6 levels are part of the acute phase reaction similar to the high G-CSF levels.

Unsupervised clustering analysis was used to further analyze pretransfusion cytokine levels. The results are summarized in *figure 1A*. Pretransfusion levels for the same patient generally clustered together; an observation strongly suggesting that the pretransfusion levels are mainly determined by patient-dependent characteristics. The main trends that can be observed are the clear distinctions between to major cytokine clusters. One cluster included several chemokines and another cluster included several interleukins *(figure 1B, C)*. Thus, acute leukaemia patients with chemotherapy-induced cytopenia show individual differences in their plasma cytokine

Table 2
Plasma levels of soluble mediators in healthy controls compared to pretransfusion levels in acute leukaemia patients.

Soluble mediator	Healthy control samples n = 10	Pretransfusion patient samples n = 31	P-value (*)
Platelet-derived mediators			
β-thromboglobulin (IU/mL)	1825 (540-3393)	154 (ND-632)	< 0.001
TGF-β (ng/mL) (†)	0.34 (0.14-0.42)	0.19 (0.02-2.7)	Ns
PDGF-BB	24 (ND-151)	ND (ND-35)	0.007
VEGF	1 (ND-22)	4 (ND-55)	Ns
CCL5	624 (148-1742)	8 (ND-112)	0.001
CXCL4 (ng/mL)	28 (ND-106.8)	ND	NP
sCD40L (ng/mL)	0.60 (0.10-2.54)	0.91 (ND-1.76)	Ns
Chemokines			
CCL2	48 (23-70)	367 (138-11975)	Ns
CCL3	ND (ND-7)	ND	Ns
CCL4	37 (26-70)	79 (22-749)	Ns
CCL11	87 (51-130)	67 (23-551)	Ns
CXCL8	ND (ND-5)	152 (ND-8240)	Ns
CXCL10	400 (220-752)	880 (117-6465)	Ns
Regulators of hematopoiesis and angiogenesis			
G-CSF	0.3 (ND-1028)	1266 (51-13870)	0.0450
GM-CSF	ND (ND-17)	ND (ND-146)	Ns
bFGF	ND	ND (ND-185)	NP
Monocyte-secreted cytokines			
IL-1β	ND (ND-6.5)	0.6 (ND-16.1)	Ns
IL-1RA	ND (ND-109)	100 (ND-11058)	Ns
IL-12	0.5 (ND-390)	ND (ND-56)	Ns
TNF-α	ND (ND-8)	ND (ND-223)	Ns
T cell-derived cytokines			
IL-2	ND (ND-3)	ND (ND-44)	NP
IL-4	1 (ND-9)	2 (ND-34)	Ns
IL-5	ND (ND-1)	4 (ND-27)	0.0225
IL-6	ND (ND-9)	96 (ND-5195)	Ns
IL-7	ND	ND (ND-22)	NP
IL-9	ND	ND (ND-223)	NP
IL-10	ND (ND-3)	8 (ND-80)	Ns
IL-13	0.1 (ND-3)	1 (ND-169)	Ns
IFN-γ	12 (ND-91)	25 (ND-518)	Ns
Other cytokines			
IL-15	ND	10 (ND-42)	NP
IL-17	ND	ND (ND-182)	NP

Results presented as median (range). If the median level was not detectable (ND), this is indicated, if the median value reached a detectable level then the variation range is also presented. The levels are presented as pg/mL if not otherwise stated.
* Normal levels compared with patient levels before transfusion (mixed effects model, nlme, R). For seven mediators, the analysis could not be performed (NP) due to the high number of "not detectable" (ND) values.
† For TGF-β pretranfusion patient samples: n = 13.
Ns : non-significant.

profile, but these differences are observed mainly for a subset of chemokines and interleukins. The analysis illustrates the heterogeneity of systemic cytokine levels in acute leukaemia patients and illustrates that cytokine concentrations vary both during cytopenia for the individual patient and between patients.

Platelet transfusions increase plasma levels of several platelet-derived cytokines

The patient plasma concentrations of platelet-derived cytokines and sCD40L before and after platelet transfusion are given in *table 3*. sCD40L and VEGF were not significantly altered following the transfusions, and PDGF-BB levels were only increased immediately after the transfusion. In contrast, the levels of the other four platelet-derived mediators (β-thromboglobulin, TGF-β, CCL5, and CXCL4), showed significantly increased plasma concentrations immediately after and one hour following transfusion *(table 3)*. However, the plasma levels 24 hours after the transfusion did not differ from the pretransfusion levels.

When investigating the correlations between pretransfusion cytokine levels and pos-transfusion levels determined

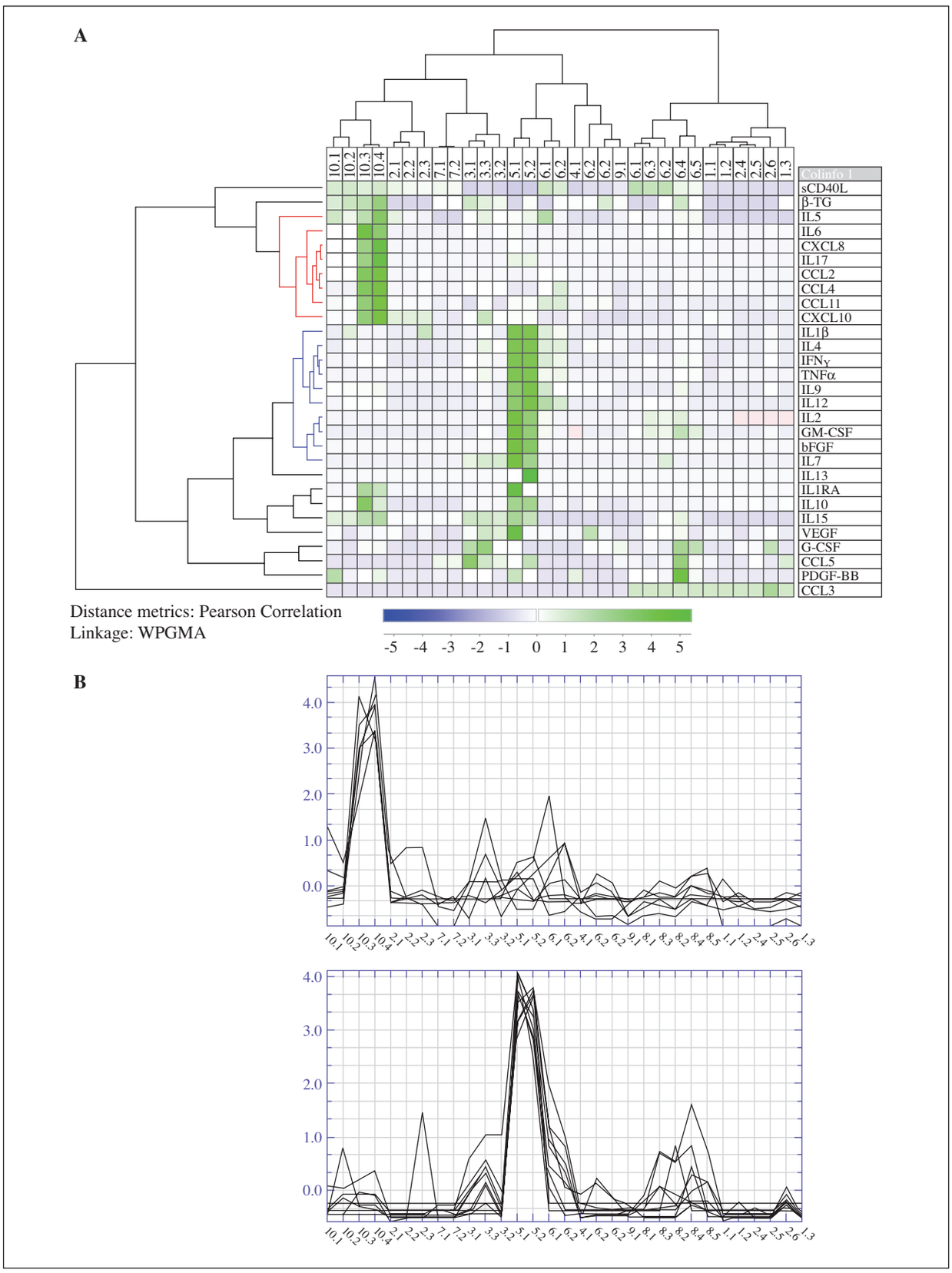

Figure 1

Clustering analysis of pretransfusion cytokine levels in acute leukaemia patients with severe, treatment-induced pancytopenia. The analysis included cytokines with detectable plasma levels: TGF-β was excluded because of missing data.

A) The unsupervised clustering is presented as the clustering of platelet transfusions (patient number. transfusion number) at the top of the figure: cytokine clustering is shown at the left margin of the figure. Pre-transfusion levels determined in the same patient generally clustered together. The major differences between patients were determined by two cytokine clusters, one including several chemokines and the other including certain interleukins (**B, C**). The detailed analysis of the two cytokine clusters demonstrates that the high levels of these cytokines were only detected for a limited number of transfusions, in certain patients.

Table 3
Plasma levels of soluble mediators in acute leukaemia patients with chemotherapy-induced thrombocytopenia before and after platelet transfusion.

Soluble mediator	Before transfusion (n = 31)	Immediate after transfusion (n = 31)	p-value (*)	1 hour after transfusion (n = 31)	p-value (**)	24 hours after transfusion (n = 31)	p-value (***)
Platelet-derived mediators							
β-thromboglobulin (IU/mL)	154 (ND-632)	3,629 (1,867-4,069)	< 0.001	1 694 (466-2,944)	< 0.001	170 (0-632)	Ns
TGF-β (ng/mL) (†)	0.19 (0.02-2.7)	0.60 (0.12-2.6)	< 0.001	0.45 (0.18-2.58)	0.024	0.03 (0-2.16)	Ns
PDGF-BB	ND (ND-35)	61 (4-700)	< 0.001	16 (0-308)	Ns	1 (0-11)	Ns
VEGF	4 (ND-55)	7 (ND-30)	Ns	8 (ND-32)	Ns	4 (0-16)	Ns
CCL5	8 (ND-112)	522 (82-1451)	< 0.001	83 (1-469)	0.015	12 (0-46)	Ns
CXCL4 (ng/mL)	ND	33.5 (10.9-118)	< 0.001	5.7 (ND-37.8)	0.030	ND	Ns
sCD40L (ng/mL)	0.91 (ND-1.76)	0.70 (ND-1.71)	Ns	0.70 (ND-1.71)	Ns	0.96 (0.11-1.70)	Ns
Chemokines							
CCL2	367 (138-11,975)	417 (125-12,204)	Ns	424 (127-13,244)	Ns	456 (133-11 975)	Ns
CCL3	ND	ND	Ns	ND (ND-6)	Ns	ND (ND-4)	Ns
CCL4	79 (22-749)	84 (20-1,163)	Ns	88 (23-2,481)	Ns	78 (43-749)	Ns
CCL11	67 (23-551)	72 (22-533)	Ns	77 (27-448)	Ns	57 (28-551)	Ns
CXCL8	152 (ND-8,240)	166 (ND-6,830)	Ns	166 (ND-6,044)	Ns	161 (24-8 240)	Ns
CXCL10	880 (117-6,465)	827 (109-6,059)	Ns	891 (109-5,859)	Ns	765 (398-6 465)	Ns
Regulators of hematopoiesis and angiogenesis							
G-CSF	1266 (51-13,870)	1271 (46-11,307)	Ns	1128 (58-11,927)	Ns	950 (63-9 457)	Ns
GM-CSF	ND (ND-146)	ND (ND-101)	Ns	ND (ND-101)	Ns	ND (ND-43)	Ns
bFGF	ND (ND-185)	ND (ND-154)	Ns	ND (ND-161)	Ns	ND	Ns
Monocyte-secreted cytokines							
IL-1β	0.6 (ND-16.1)	0.4 (ND-13.8)	Ns	0.1 (ND-12.9)	0.009	0.1 (ND-5.0)	Ns
IL-1RA	100 (ND-11,058)	91 (ND-6910)	Ns	84 (ND-6,523)	Ns	84 (ND-3 953)	Ns
IL-12	ND (ND-56)	ND (ND-41)	Ns	0.8 (ND-41)	0.018	1.3 (ND-7.0)	Ns
TNF-α	ND (ND-223)	ND (ND-165)	Ns	ND (ND-182)	Ns	ND (ND-27)	Ns
T cell-derived cytokines							
IL-2	ND (ND-44)	ND (ND-29)	0.010	ND (ND-30)	0.039	ND (ND-12)	Ns
IL-4	2 (ND-34)	1 (ND-25)	Ns	1 (ND-24)	0.039	2 (ND-4)	Ns
IL-5	4 (ND-27)	3 (ND-27)	Ns	4 (ND-41)	Ns	4 (ND-27)	Ns
IL-6	96 (ND-5195)	77 (ND-3436)	Ns	81 (ND-1986)	0.031	260 (ND-4 107)	Ns
IL-7	ND (ND-22)	ND (ND-16)	Ns	ND (ND-16)	Ns	ND (ND-7)	Ns
IL-9	ND (ND-223)	ND (ND-184)	0.025	ND (ND-162)	Ns	ND (ND-45)	Ns
IL-10	8 (ND-80)	8 (1-107)	Ns	7 (1-121)	Ns	6 (ND-30)	Ns
IL-13	1 (ND-169)	1 (ND-15)	Ns	1 (ND-16)	Ns	0.1 (ND-4)	Ns
IFN-γ	25 (ND-518)	26 (ND-455)	Ns	21 (ND-430)	0.007	15 (6-91)	Ns
Other cytokines							
IL-15	10 (ND-42)	9 (ND-33)	Ns	11 (ND-39)	Ns	13 (ND-38)	Ns
IL-17	ND (ND-182)	ND (ND-161)	Ns	ND (ND-154)	Ns	ND (ND-182)	Ns

Results presented as median (range). If the median level was not detectable (ND), this is indicated, if the range value reached a detectable level then the variation range is also presented. The levels are presented as pg/ml if not otherwise stated.

* Comparison between plasma levels before and immediate after transfusion (mixed effects model).

** Comparison between plasma levels before and 1 hour after transfusion (mixed effects model).

*** Comparison between plasma levels before and 24 hours after transfusion (mixed effects model).

† For TGF-β: N=13 (5 after 24 hours).

Ns: non-significant.

Table 4
Concentration of platelet- and leukocyte-derived cytokines in platelet concentrate supernatants at the time of transfusion*.

Soluble mediator	Number of transfusions	Concentration (pg per unit)
Platelet-derived cytokines		
β-thromboglobulin (IU per unit)	30	709×10^5 (190×10^5-$1{,}741 \times 10^5$)
TGF-β (ng per unit)	13	26,492 (9,413-36,729)
PDGF-BB	27	10.3×10^5 (4.2×10^5-18.9×10^5)
VEGF	27	9,805 (1,696-35,269)
CCL5	27	88.7×10^5(28.0×10^5-310.0×10^5)
CXCL4 (ng per unit)	30	15.7×10^5 (3.0×10^5-32.5×10^5)
sCD40L (ng per unit)	30	1,439 (447-2,835)
Chemokines		
CCL2	27	8,236 (1,675-12,003)
CCL4	27	12,494 (8,439-28,605)
CXCL8	27	3,049 (821-6,606)
CXCL10	27	31,371 (3,749-64,719)
Leukocyte-derived cytokines		
G-CSF	27	37,259 (23,263-68,977)
IL-1β	27	565 (28-1,793)
IL-1RA	27	2.3×10^5 (1.1×10^5-4.5×10^5)
IL-6	27	7,111 (3,217-14,091)
IL-10	27	3,435 (1,473-7,189)
IFN-γ	27	36,939 (17,181-75,558)

* The cytokine concentration levels determined in platelet concentrate supernatants immediately before transfusion. The levels are presented as pg per unit if not otherwise stated.

immediately after, one hour and 24 hours after the transfusion for the seven main, platelet-secreted mediators, the mediators could be classified into different groups:
– pretransfusion levels showed significant correlations with all three post-transfusion time points for TGF-β and sCD40L;
– β-thromboglobulin showed a significant correlation only between pretransfusion and 24 hours post-transfusion levels;
– PDGF-BB, and VEGF showed significant correlations with pretransfusion levels both immediately and one hour post-transfusion, whereas CCL5 showed significant correlation only after 1 hour.
CXCL4 showed non-detectable levels prior to transfusions, and for this reason correlation analyses could not be performed.

Platelet concentrates show high levels of platelet-derived cytokines

The amount of 17 platelet- and leukocyte-derived cytokines in the supernatants of unselected platelet concentrates are presented in *table 4*. High levels of platelet-derived mediators were detected in the platelet concentrate supernatant. For cytokines showing significantly increased levels after transfusion, correlations between the levels of transferred cytokine in the platelet concentrates and the corresponding increase in patient plasma cytokine concentrations was investigated. Significant correlations were then observed for β-thromboglobulin immediately after (correlation coefficient 0.563, p-value = 0.001), and one hour after transfusion (correlation coefficient 0.787, p-value $\leq$ 0.001). For the other mediators (*i.e.* TGF-β, CCL5, PDGF-BB and CXCL4), no significant correlation was observed between increases in patient plasma levels and the amount of cytokine transfused.

We also investigated the levels of various leukocyte-derived cytokines in the platelet concentrate supernatants. The leukocyte-released cytokines generally showed lower levels than the platelet-derived cytokines *(table 4)*. Even though relatively high levels of several mediators were detected in the concentrates, none of these cytokines showed significantly altered levels in patients early after the transfusions *(table 3)*. To conclude, the plasma levels of leukocyte-derived cytokines showed a wide variation between patients before transfusion, and this variation persisted after the platelet transfusions, in spite of transference of relatively high amounts of mediators.

Plasma levels of several leukocyte-derived cytokines are decreased one hour after platelet transfusion

When comparing the pretransfusion and post-transfusion concentrations of the various leukocyte-derived cytokines we found that several interleukins, as well as IFN-γ, showed significantly decreased levels one hour after transfusion *(table 3)*. In addition significantly decreased levels of IL-2 and IL-9 were observed immediately after the transfusion. IL-2 and IL-9 showed detectable cytokine levels in plasma for only a small number of transfusions; 3 and 10 respectively. These differences were relatively small, and all levels were normalized 24 hours post-transfusion
When investigating the correlations between pretransfusion plasma cytokine concentrations and post-transfusion levels determined for the 24 leukocyte-related cytokines (see *table 3*), we observed generally strong and statistically significant correlations between pretransfusion levels and all post-transfusion levels. The exceptions were:
– immediately after platelet transfusion; the only cytokine not showing a significant correlation was CCL3;

– one hour after transfusion; the only exception was CCL4;
– 24 hours after transfusion; no significant correlations were observed for bFGF, IL-1β, IL-2, IL-9 and IL-7.

Occurrence of transfusion complications

Only one minor transfusion complication (flushing) was reported. No premedication was administered before this transfusion. No significant differences in plasma cytokine levels were observed immediate or one hour after the actual transfusion compared to other, non-symptomatic study transfusions given to the same patient (n = 3). This patient had however, experienced flushing on several previous platelet transfusions, and reported that the symptoms were related to the rate of infusion.

DISCUSSION

Platelet concentrates contain both platelets, a small number of contaminating, normal blood cells and a supernatant phase. All these components are transfused into the patients. Regular platelet transfusions are common in cancer patients with severe chemotherapy-induced thrombocytopenia, and our present study demonstrates that in unselected, acute leukaemia patients, the transfusions influence systemic cytokine levels, both for platelet-derived cytokines (including angioregulatory β-thromboglobulin, PDGF-BB, CXCL4), and the non-platelet cytokines IL-1β, IL-2, IL-4, IL-6, I-L9, IL-12, and IFN-γ.

Our present study included consecutive and thereby unselected patients. By using this methodological approach, our results should be regarded as reflecting routine clinical handling of such patients. Our acute leukaemia patients have in common pre-treatment bone marrow infiltration by immature leukemic cells: they all received intensive conventional chemotherapy and developed severe, treatment-induced cytopenia as described in previous studies [1, 31, 37]. Additionally, all transfusions were given during the period of pancytopenia when the patients show similarities in treatment-induced effects as regards systemic levels of both cytokines and soluble adhesion molecules [1, 37]. It should however be emphasized that due to the study design our patients are heterogeneous with regard to diagnosis (AML and ALL), G-CSF therapy, chemotherapy, and preparation technique of the transfused platelet concentrate. The observed transfusion-induced variations in plasma cytokine levels were, however, sufficiently strong to reach statistical significance, even in this context of patient heterogeneity. Owing to the study design, we cannot exclude the possibilities that *(i)* the magnitude of the described differences vary between patient subsets, *(ii)* certain cytokines may show transfusion-dependent variations only in certain patient subsets, or *(iii)* additional cytokines may show smaller variations that do not reach statistical significance in our heterogeneous patient group.

The pretransfusion plasma cytokine concentrations varied between the mediators investigated. The levels of platelet-derived cytokines were low, and this is explained by the severe thrombocytopenia that was observed for all these patients. Most chemokines showed relatively high plasma levels in patients, but this overlapped significantly with the controls; this was observed both for CCL and CXCL chemokines and is thus independent of this classification. The detection of high G-CSF levels in patients not receiving growth hormone therapy is not surprising because these levels are generally high in neutropenic patients and can be further increased by acute phase reactions [38]. Finally, the relatively high levels of IL-1RA and IL-6 are possibly caused by contributions from different cells, including monocytes. In our study, we chose to investigate one control per patient. The intention of the investigation of healthy controls was to identify those cytokines that usually show strong interindividual variations and by this, to illustrate the extent of transfusion-induced alterations in plasma levels.

Platelet-derived as well as leukocyte-associated mediators (CCL5, CXCL8, TNF-α, IL-6, IL-1β, and sCD40L) have previously been reported to be associated with non-hemolytic transfusion reactions.[26, 27, 29, 39, 40] When comparing pre- and post-transfusion plasma cytokine levels, increased post-transfusions levels were detected for most of the platelet-derived cytokines, the only exceptions being VEGF and sCD40. In our study, no serious transfusion reactions were observed and only one acute transfusion reaction (flushing) was reported in a patient not receiving premedication. The observation of cytokine modulation in patients not receiving premedication and experiencing no transfusion reactions suggests that the transfusion-induced modulation of the cytokine network is not sufficient for development of acute transfusion reactions, and indicates that additional mechanisms may be important.

We examined cytokine levels in the platelet concentrate supernatants. In these studies, we included platelet-derived cytokines and selected leukocyte-derived cytokines known to be released by various activated leukocytes (*e.g.* T cells, monocytes, macrophages, natural killer cells). According to previous publications [17, 22], the platelet-derived cytokines were generally present at high levels. Despite prestorage leukocyte depletion and prolonged storage time, low but detectable levels of several leukocyte-derived cytokines were still observed. Thus, platelet transfusions include the infusion of significant amounts of platelet-derived and to a lesser extent leukocyte-derived soluble mediators. A statistically significant correlation between the amount of infused platelet-derived cytokines and the increase in plasma cytokine levels was, however, observed only for β-thromboglobulin and not for the other platelet-derived cytokines. Thus, the mechanisms causing increased plasma levels of β-thromboglobulin are probably different from those driving the other mediators. The results from the correlation analyses between amount of cytokine transfused and post-transfusion plasma concentration show that the systemic cytokine levels are not only determined by the amount of infused mediators, but also by other factors, *e.g.* differences in binding/degradation caused by infections and/or acute phase reactions and differences in distribution volume between the patients.

We investigated the levels of 17 selected cytokines in the platelet concentrates, including:

– cytokines released during platelet activation;

– cytokines that seem to be a part of the acute phase reaction in acute leukaemia patients with chemotherapy-induced cytopenia (G-CSF, IL-1β/IL-1RA, IL-6);

– cytokines that can be released during activation of various immunocompetent cells, including monocytes/macrophages (CCL2, CCL4, CXCL8, CXCL10, IL-1β, IL-10), T cells (CCL4, IL-6, IL-10, IFN-γ), B cells CCL4, IL-1β) and dendritic cells (IL-1β) [1, 31, 39].

The selected cytokines also represent those cytokines with the largest variation ranges in pretransfusion plasma levels. Our results demonstrated that a wide range of leukocyte-released cytokines could be detected in the supernatants of platelet concentrates. However, in contrast to most of the platelet-derived cytokines, infusion of leukocyte-derived cytokines did not increase the *in vivo* plasma levels, *i.e*. the wide variation ranges persisted after the transfusions. This difference may be due to the generally lower amounts of leukocyte-derived cytokines, and possibly also to differences in post-transfusion binding or degradation.

By examination of non-platelet cytokine levels, we detected a relatively small, but statistically significant, decrease in the plasma levels for several of the proinflammatory leukocyte-derived cytokines. This decrease was observed one hour after the platelet transfusion, and this delay is consistent with the hypothesis that the transfusions induce increased *in vivo* expression of certain cytokine receptors thereby increasing the binding/degradation and decreasing the plasma levels of these cytokines. Another possibility would be decreased endogenous release of these cytokines in response to the transfusions: a dilution effect due to the transfusion-induced increase in plasma volume is unlikely because one would then expect similar effects for all leukocyte-derived cytokines.

The significant correlation between pre- and post-transfusion cytokine levels suggests that patient characteristics have a major influence on the plasma levels even after the transfusions. Our observations indicate that for leukocyte-released cytokines, patient characteristics have a major influence on the post-transfusion plasma concentration of the different mediators, whereas transfusion-associated mechanisms are less important and have only a minor influence.

For most platelet-derived mediators (sCD40L, TGF-β, PDGF-BB, and VEGF), significant correlations between pre- and post-transfusion concentrations indicate that patient-dependent factors are the most important ones for the systemic levels. For some platelet-derived cytokines such as β-thromboglobulin and CCL5 however, we observed no significant correlations between pre-transfusion plasma samples and samples investigated immediately after and/or one hour following transfusion. This last observation supports our hypothesis that a transient effect on plasma cytokine levels may be seen in patients receiving platelet transfusions, and that this effect might be caused by the platelet transfusion itself

To conclude, platelet transfusions seem to modulate the systemic cytokine network in acute leukaemia patients with severe chemotherapy-induced thrombocytopenia.

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