Cardioprotective effect of metoprolol and enalapril in doxorubicin-treated lymphoma patients: A prospective, parallel-group, randomized, controlled study with 36-month follow-up

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Anthracyclines have contributed to a marked increase in survival in different types of cancer [1,2]. Unfortunately, they are associated with dose-dependent cardiotoxicity and heart failure (HF) [3–8]. Change to a weekly dosage schedule with slow infusions has been tested, a strategy that requires more frequent hospital visits and increased storage resources [7,9]. Liposomal anthracycline formulations with reduced drug exposure and lower plasma concentrations may still be cardiotoxic at higher cumulative doses [10]. Beta-blockers and angiotensin converting enzyme (ACE) inhibitors have been shown to reduce anthracycline-induced cardiotoxicity, but have not been tested in long-term prospective, randomized, controlled studies with well defined cardiotoxicity criteria and careful cardiac function monitoring [11–16]. We investigated doxorubicin-induced clinical or subclinical cardiotoxicity in lymphoma patients after concomitant prophylactic therapy with metoprolol or enalapril or no concomitant treatment. We examined whether cardiotoxicity was related to the treatment or any other variable. We found that HF was less frequent under concomitant treatment than no treatment, especially in the metorpolol group, but the differences were not significant. No association was found between the presence of cardiotoxicity and concomitant treatment or other variable apart of age that had a significant impact. The marginal benefit seen with metoprolol should be investigated further.

One-hundred forty-seven patients were randomized to the three study groups, of whom 125 were eligible for analysis. One-hundred nine patients completed 1 year of follow-up (primary end-point), 64 two years, and 45 three years (see Supporting Information Fig. 1). The mean age was 49 years and men and women were equally represented. 60 patients (48%) had diagnosis of Hodgkin lymphoma (HL) and 65 (52%) Non-Hodgkin lymphoma (NHL), and were equally distributed in the groups (Table I). Baseline characteristics were similar across groups (Table I). Mean follow-up was 31 months.

Early and Late Cardiotoxicity

HF developed in six (4.8%) patients. Three received no concomitant treatment, one received metoprolol, and two enalapril ($\chi^2 = 1.178$, $P = 0.555$). We observed 20 (16%) cases of early cardiotoxicity and 8 (7.3%) cases of late cardiotoxicity (Table II). No association was found between the presence of cardiotoxicity and concomitant treatment, type of lymphoma, gender, smoking status, or age and body mass index, nor was there any association with factors known to contribute to cardiotoxicity such as radiation and cyclophosphamide. Only age had a significant impact (age effect = 0.051, se = 0.0236, $P = 0.032$).

The mean changes for all echocardiographic variables between groups did not differ significantly at any measuring time up to the early cardiotoxicity endpoint 12 months after baseline (Table III). There were also no significant

TABLE I. Baseline Characteristics and Treatment Details

	Metoprolol group $n = 42$	Enalapril group $n = 43$	Control group $n = 40$	*P* Value
Age, mean, years (SD)	51.0 ± 18.0	47.4 ± 16.2	49.1 ± 19.4	0.61
Gender: Male, *n* (%)	22 (52)	22 (51)	21 (53)	0.72
Body Mass Index, Kg/m^2 (SD)	25.7 (4.7)	25.6 (5.1)	25.1 (4.2)	0.90
Baseline LVEF, % (SD)	67.7 (5.0)	65.2 (7.1)	67.6 (7.1)	0.40
Baseline FS, % (SD)	36.5 (4.8)	35.7 (5.8)	37.9 (5.7)	0.33
Diagnosis, *n* (%)				
HL	21 (50)	19 (44)	20 (50)	
NHL	21 (50)	24 (56)	20 (50)	
CT received, *n* (%)[a]				
-ABVD	21 (50)	19 (44)	20 (50)	
-R-CHOP	21 (50)	24 (56)	20 (50)	
Cumulative doxorubicin dose, mg/m^2 (SD)				
After 3rd cycle	157.1 (4.0)	152.3 (5.0)	160.1 (8.1)	0.21
After 6th cycle	295.0 (4.8)	285.7 (6.4)	286.9 (4.5)	0.15
After 8th cycle	387.5 (6.8)	373.1 (6.3)	386.4 (5.7)	0.33
Number of cycles				
6 cycles	6	10	8	
8 cycles	27	25	24	
Autologous stem cell transplantation, *n* (%)	1	4	2	
Cyclophosphamide dose, mg/m^2 (SD)	9006.8 (2285.4)	9073.2 (3171.6)	9074.7 (2356.8)	0.92
Hypertension, *n* (%)	10 (24)	14 (33)	6 (15)	0.17
Diabetes, *n* (%)	10 (24)	3 (7)	6 (15)	0.09
Hypercholesterolaemia, *n* (%)	14 (33)	11 (26)	10 (25)	0.77
Familial history of cardiac disease, *n* (%)	7 (16)	5 (12)	5 (13)	0.88
Smoking history, *n* (%)				
Past	8 (19)	12 (28)	9 (23)	0.72
Current	9 (21)	8 (19)	7 (18)	0.78
Radiotherapy, *n* (%)	8 (19)	9 (21)	9 (23)	0.90
Mediastinal radiation, Gy (SD)	37.3 (2.8)	38.0 (1.9)	39.5 (1.8)	0.85
Mean final enalapril dose, mg/d (SD)	–	11 (0.68)	–	
Mean final metoprolol dose	88.8 (3.1)	–	–	
Control of echocardiography time, months (SD)	29.1 (17.7)	32.5 (16.0)	30.9 (18.3)	0.57

[a]The CT regimen consisted of 6–8 cycles of the "ABVD schema" for HL: doxorubicin (25 mg/m^2), bleomycin (10 mg/m^2), vinblastin (6 mg/m^2), and decarbazine (375 mg/m^2) intravenously on day 1 and day 15 every 4 weeks. The NHL patients received the "R-CHOP schema": rituximab (375 mg/m^2), cyclophosphamide (750 mg/m^2), doxorubicin (50 mg/m^2), and vincristine (1.4 mg/m^2) intravenously on day 1 and prednisolone (100 mg/m^2) orally on days 1–5 every 3 weeks.

differences between the baseline and 12-month values in each group (data not shown). The findings for late cardiotoxicity were similar. Only the E/A ratio of diastolic function was statistically significantly increased in the metoprolol group 30 and 42 months after treatment initiation, but not after 40 months (data not shown) (Table III).

Adverse Events

No patients died or interrupted chemotherapy (CT) due to doxorubicin-induced cardiotoxicity. The 19 adverse events recorded were distributed evenly across study groups (see Supporting Information Table 1). Metoprolol and enalapril were tolerated well in most patients (84.8%, 106/125 patients).

This randomized, controlled study compared the protective role of no treatment, enalapril and metoprolol in the development of early and late doxorubicin-induced cardiotoxicity in patients with HL and NHL on CT. Our major finding was that HF was less frequent under metoprolol and enalapril than no treatment, although the differences were not significant and the number of patients affected was very small. However, echocardiographic parameters of LV function were not different between groups. Furthermore, the two cardioprotective agents enalapril and metoprolol did not affect the probability of developing cardiotoxicity.

Only few reports are available on the cardioprotective use of beta-blockers and ACE inhibitors in patients receiving anthracyclines. Jensen et al. [11] conducted an observational study in patients with advanced breast cancer with a cumulative dose of 1000 mg/m^2 epirubicin. Rapid relief of most symptoms of HF was achieved with furosemide and digoxin, but exertional dyspnea persisted. Nine patients with clinical deterioration were given ACE inhibitors which reversed HF and increased LVEF close to normal levels. Lipshultz et al. [12] conducted a retrospective study assessing the long-term effects of enalapril on LV function in 18 long-term survivors of childhood cancer who had been treated with doxorubicin at an average of 7 years before enalapril administration. Children were followed-up for a median of 10 years. Enalapril therapy resulted in early improvement in all cardiac parameters that was unfortunately transient as after 6 to 10 years LV dysfunction returned back as it was before enalapril administration. In addition enalapril did not prevent LV wall thinning that worsened during the studied period. Cardinale et al. [13] conducted a prospective, randomized, controlled study on the cardioprotective effect of enalapril after high doses of CT in 114 patients with plasma troponin I greater than 0.07 ng/mL, which is considered a predictor of LV dysfunction. Ten of the patients had HL and 39 NHL. After 12 months' follow-up, the incidence of an absolute decrease of >10% in LVEF associated with a decline below 50% was significantly lower in the enalapril group (long-rank $\chi^2 = 30.5$, $P < 0.001$).

Noori et al. [14] evaluated the effects of beta-blockers on anthracycline-induced cardiomyopathy (ACM) in a retrospective case-control study in 32 patients, in which the controls were patients with idiopathic dilated cardiomyopathy (IDM). LVEF was improved to a similar extent in both groups. The only randomized, controlled, study published was by Kalay et al. [15], who examined early cardiotoxicity in 50 patients randomized to receive either carvedilol or placebo. While LVEF, LVEDD, and LVESD did not change from baseline in the carvedilol group, in the control group LVEF was significantly lower ($P < 0.001$) and LVEDD and LVESD were significantly increased ($P = 0.008$ and 0.0001, respectively). Mitral E wave velocity and E/A ratio significantly decreased in the control group ($P = 0.02$), but remained similar in the carvedilol group.

Our study showed that metoprolol or enalapril did not reduce cardiotoxicity, as reported elsewhere. These differences may be due to different study designs, different types of malignancies studied, or the lower cumulative anthracycline dose in our study, as it is well documented that anthracycline-induced cardiotoxicity is dose-related. We observed a total rate of HF cases higher than that reported by Von Hoff et al. [3], who, however, felt they may have underestimated the incidence of HF incidence in their study. Swain et al. [4] reported a higher incidence than Von Hoff et al. in patients in prospective, randomized controlled trials, and also a rate of doxorubicin-related HF of 5.1% at a cumulative doxorubicin dose for the majority of events of 400 mg/m^2. Our findings were similar (4.8%; dose of about 380 mg/m^2). Swain et al. also reported that 6.5% of patients were at risk of developing a cardiac event at a cumulative dose of 150 mg/m^2. In support of this, we observed subclinical effects when the cumulative doxorubicin dose was nearly 160 mg/m^2.

We also recorded high levels of subclinical cardiotoxicity reflected by abnormalities in LVEF. A decrease in LVEF is not always predictive of HF [4,5], and this was confirmed in our study. Swain et al. [4] retrospectively analysed data of 630 patients from the placebo arms of three studies that evaluated cardiotoxicity after treatment with dexrazoxane and doxorubicin-containing CT for breast cancer or small cell lung cancer. They concluded that LVEF values were not good predictors of HF: not all patients who developed HF (21 of 32) had a reduction of <30% in LVEF, and LVEF changes occurred in many other patients who did not develop HF. Similarly Limat et al. [5] retrospectively analysed data from 135 patients with aggressive NHL treated with CHOP and found that half of the patients with subclinical cardiac events had clinical signs of HF. This was also seen in two population-based studies [17,18].

A further major finding of our study was that older patients were more likely to develop cardiotoxicity, adding a new dimension to findings on the effect of age reported so far, since we included only patients with HL or NHL who are generally younger than other cancer patients and would therefore be expected to be less susceptible [3,7,19].

Major limitations of our study were the open-label design, although the echocardiographic evaluations were conducted by blinded examiners. Actual administration of a placebo in the control group within a double-blind design might have minimised any bias, especially in the questioning on adverse events. Our study's strengths were its prospective, randomized, controlled design, the clear endpoint definition and sample size calculation, the inclusion of only one type of cancer, and the repeated measurements of LV function in each CT cycle and periodically until the end of the study [20,21].

TABLE II. Number of Cases of Cardiotoxicity

Time points	Total, *n* (%) N = 125	Metoprolol group[a] N = 42	Enalapril group[a] N = 43	Control group[a] N = 40
Baseline	1 (0.8)	0 (2)	1 (0)	0 (1)
3CT cycles	4 (3.7)	2 (7)	2 (7)	0 (3)
6CT cycles	5 (6.9)	2 (20)	2 (17)	1 (16)
8CT cycles	5 (4.8)	1 (7)	3 (9)	1 (5)
12 months	5 (4.6)	2 (4)	2 (8)	1 (4)
18 months	3 (3.7)	1 (16)	2 (16)	0 (11)
24 months	3 (4.7)	1 (23)	2 (21)	0 (17)
30 months	2 (3.7)	0 (23)	2 (25)	0 (23)
Early cardiotoxicity cases[b]	20 (16.0)	7	10	3
Late cardiotoxicity cases[c]	8 (7.3)	2	6	0
Total cardiotoxicity cases[d]	28 (22.4)	9	16	3

[a]Figures represent number of cases and missing observation in parentheses.
[b]Cardiotoxicity cases recorded from baseline to 12th month of follow-up.
[c]Cardiotoxicity cases recorded after 12th month of follow-up and until the end of the entire follow-up period (approximately the 30th month).
[d]Cardiotoxicity cases recorded from baseline to the end of the study.

TABLE III. Mean Values for Subclinical Cardiotoxicity Variables at Baseline and After 12 Months

	Baseline				12 months			
	Metoprolol group	Enalapril group	Control group	*P* value	Metoprolol group	Enalapril group	Control group	*P* value
LVEDD, cm (SD)	4.7 (0.5)	4.9 (0.4)	4.8 (0.6)	0.19	4.9 (0.4)	5.0 (0.5)	4.8 (0.5)	0.34
LVESD, cm (SD)	2.9 (0.3)	3.1 (0.4)	3.0 (0.5)	0.16	3.2 (0.4)	3.2 (0.5)	3.0 (0.4)	0.13
LVEF, % (SD)	65.7 (5.0)	65.2 (7.1)	67.6 (7.1)	0.40	63.3 (7.4)	63.9 (7.5)	66.6 (6.7)	0.06
FS, % (SD)	36.5 (4.8)	35.7 (5.8)	37.9 (5.7)	0.33	34.6 (5.5)	35.4 (5.3)	37.0 (5.0)	0.17
E/A, ratio (SD)	1.1 (0.4)	1.1 (0.4)	1.0 (0.4)	0.62	1.1 (0.4)	1.0 (0.4)	1.0 (0.4)	0.34
$E/E_{a,}$ ratio (SD)	4.8 (1.9)	4.6 (1.3)	4.9 (1.4)	0.73	5.3 (2.7)	4.7 (1.3)	5.0 (1.5)	0.68

Our results demonstrate for the first time in a randomized, controlled trial that metoprol and enalapril do not reduce the risk of cardiotoxicity in patients treated with doxorubicin. The incidence of HF and subclinical cardiotoxicity—although not statistically significant different between-groups were lower in the treatment groups than in the control group, especially in the metoprolol group, and this should be further evaluated for its clinical importance.

Author Contributions

P. Georgakopoulos carried out the study and contributed to study design, analysed the data, and wrote the article. P. Roussou contributed to overall study management. E. Matsakas and A. Karavidas carried out and analysed the echocardiograms. N. Anagnostopoulos supervised the data management. A. Galanopoulos and T. Marinakis were involved in data validation and in selection of the cohort. F. Georgiakodis and S. Zimeras did statistical analysis. M. Kyriakidis was the principal investigator and was involved in the study design and in the writing of the article. A. Ahimastos judged the article.

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Additional Supporting Information may be found in the online version of this article.

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Predicting survival for diffuse large B-cell lymphoma patients using baseline neutrophil/lymphocyte ratio

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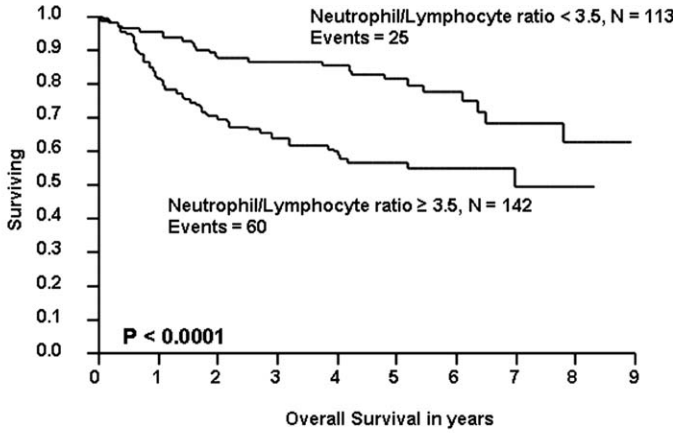
The neutrophil/lymphocyte (N/L) ratio at diagnosis has been shown to be a prognostic factor for survival in solid tumors. The N/L ratio at diagnosis as a prognostic factor for non-Hodgkin lymphoma (NHL) has not been studied. Thus, we studied N/L ratio at diagnosis as a prognostic factor for patients with diffuse large B-cell lymphoma (DLBCL) treated with R-CHOP. From 2000 until 2007, 255 consecutive DLBCL patients, originally diagnosed, treated with R-CHOP, and followed at Mayo Clinic, Rochester, were included in this study. With a median follow-up of 4.0 years (range: 0.3–9.0 years), patients with an N/L ratio $<$3.5 at diagnosis experienced a superior overall survival (OS) and progression-free survival (PFS) compared with those patient with an N/L ratio $\geq$3.5 at diagnosis. The median OS was not reached versus 6.8 years, $P < 0.0001$; and the median PFS was not reached versus 3.3 years, $P < 0.0001$, respectively. Multivariate analysis showed N/L ratio to be an independent prognostic factor for OS and PFS. This study suggests that baseline N/L ratio at diagnosis is a simple, inexpensive, standardized prognostic factor to assess clinical outcomes in DLBCL patients treated with R-CHOP.

Absolute lymphocyte count (ALC), as a surrogate marker of host immunity, has been reported to a prognostic factor for survival in patients with diffuse large B-cell lymphoma (DLBCL) at diagnosis [1–4], or after first relapse [5]. In solid tumor, baseline neutrophil count, as a surrogate marker of inflammation, has been associated with survival [6–8]. Furthermore, the neutrophil/lymphocyte (N/L) ratio at diagnosis in solid tumors has been reported to be a prognostic factor for clinical outcomes [9–13]. The rationale for the N/L ratio is to compare the inflammatory response (i.e., neutrophils) produced by cancer to the host immunity (i.e., lymphocytes). Thus we set out to investigate if N/L ratio at diagnosis is a predictor of survival in DLBCL patients treated with rituximab, cyclophosphamide, adriamycin, vincristine, and prednisone (R-CHOP).

The median age at the time of diagnosis for this cohort of 255 DLBCL patients was 64 years (range: 20–92). The distribution of additional baseline characteristics for the cohort are presented at on line Supporting Information Table I.

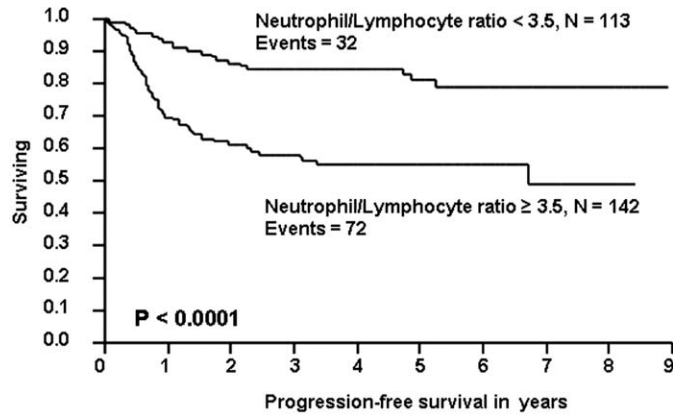
The median follow-up on living patients ($N = 170$) in this cohort was 59.1 months (range: 13.9–107.8). Fifty-eight patients died due to lymphoma recurrence and 27 patients due to non-lymphoma related causes.

As continuous variable N/L ratio at diagnosis was predictor for OS and PFS by univariate and multivariate analysis (on line Supporting Information Tables II and III). Using the cutoff for N/L ratio $\geq$3.5 at diagnosis, patients with an N/L ratio $<$3.5 at diagnosis experienced superior OS compared with patients with an N/L ratio $\geq$3.5 at diagnosis (see Fig. 1). The median OS was not reached



Numbers at risk										
Neutrophil/Lymphocyte ratio < 3.5	113	108	98	82	70	49	30	17	10	1
Neutrophil/Lymphocyte ratio ≥ 3.5	142	117	96	70	56	43	20	10	5	

Figure 1. Overall survival (OS) on N/L ratio. Patients with an N/L ratio <3.5 experienced superior OS compared with patients with an N/L ratio ≥3.5. The median OS was not reached versus 6.8 years and the OS 5-year rates were 87% versus 56%, respectively, $P < 0.0001$.



Numbers at risk										
Neutrophil/Lymphocyte ratio < 3.5	113	102	92	75	64	42	28	16	10	1
Neutrophil/Lymphocyte ratio ≥ 3.5	142	97	80	58	47	37	16	7	3	

Figure 2. Progression-free survival (PFS) based on N/L ratio. Patients with an N/L ratio <3.5 experienced superior PFS compared with patients with an N/L ratio ≥3.5. The median PFS was not reached versus 3.3 years and the PFS 5-year rates were 72% versus 45%, respectively, $P < 0.0001$.

versus 6.8 years and the OS 5-year rates were 87% versus 56%, respectively, $P < 0.0001$. In similar fashion, superior PFS was observed in patients with an N/L ratio <3.5 at diagnosis compared with patients with an N/L ratio ≥3.5 at diagnosis (see Fig. 2). The median PFS was not reached versus 3.3 years and the PFS 5-year rates were 72% versus 45%, respectively, $P < 0.0001$.

To evaluate the relevance of N/L ratio ≥3.5 at diagnosis in DLBCL patients, patients were divided into patients with an N/L ratio ≥3.5 versus <3.5 at diagnosis. Patients with an N/L ratio <3.5 at diagnosis presented with higher levels of LDH, neutrophilia, lymphopenia, poor performance status, and high International Prognostic Index (IPI) score. Stage was borderline statistically significant. Extranodal disease, number of cycles of R-CHOP, post-chemotherapy radiation therapy, and the reasons for post-chemotherapy radiation therapy was similar in both groups (on line Supporting Information Table IV).

Patients with an N/L ratio <3.5 at diagnosis were associated with prognostic factors related to inflammation (i.e., neutrophilia and B-symptoms) and tumor burden (i.e., LDH). Thus, in an attempt to understand how N/L ratio affects survival in DLBCL, we studied the relationships between N/L ratio and prognostic factors associated with inflammation/tumor burden. We identified a higher N/L ratio with higher IPI scores: IPI score of 0 (median N/L ratio = 2.5; IPI score of 1 (median N/L ratio = 2.9); IPI score of 2 (median N/L ratio = 4.4); IPI score of 3 (median N/L ratio = 4.5); IPI score of 4 (median N/L ratio = 4.6); and IPI score of 6 (median N/L ratio = 6.1) ($P < 0.002$). Higher N/L ratio was also associated with B-symptoms ($P < 0.003$), Stage III/IV ($P < 0.002$), and with higher LDH ($r_s = 0.4$, $P < 0.0001$). LDH released into the circulation is due to tissue breakdown. In lymphomas, LDH production has been mainly attributed to tumor burden. However, the association between N/L ratio and LDH in DLBCL argues that another source of LDH production in DLBCL could be due to another tissue breakdown rather than lymphoma produced by the inflammatory reaction produced by the tumor. To assess this hypothesis, we studied factors associated with LDH in DLBCL. Univariate logistic regression model identified the following factors associated with LDH: age ($P < 0.04$); B-symptoms ($P < 0.0003$); extranodal disease ($P < 0.001$); stage ($P < 0.0001$); and N/L ratio ($P < 0.0001$). In the multivariate logistic regression model, only N/L ratio ($P < 0.03$) and stage ($P < 0.0001$) remained significantly associated with LDH.

The current risk factors used to assess prognosis in DLBCL patients treated with standard therapy are identified prior to treatment, such as gene expression profiling [14,15] and the IPI [16]. A limitation of the DLBCL cell of origin gene expression profiling or the IPI is that neither of these prognostic models take into consideration the role of the host immunity (i.e., ALC) and the inflammatory environment produced by the tumor (i.e., neutrophil count). Therefore we set up to investigate if the N/L ratio at diagnosis, a biomarker comparing tumor inflammation and host immunity, affects survival in DLBCL patients.

Our study shows that DLBCL patients with an N/L ratio $\geq$3.5 at diagnosis were associated with worst clinical outcomes compared with patients with a low N/L ratio $<$3.5. N/L ratio at diagnosis was identified to be an independent prognostic indicator for survival. N/L ratio at diagnosis was associated with prognostic factors of tumor burden such stage and LDH, as well as biomarkers of inflammation such as B-symptoms (fever, and night sweats). LDH production in lymphoma has been attributed to the tumor burden. However, we identified a positive correlation between N/L ratio at diagnosis and LDH. In addition, N/L ratio at diagnosis was an independent predictive factor besides stage for LDH production. Blatt et al. [17] reported no correlation between the intracellular lymphoma LDH isoenzymes and the serum LDH isoenzymes. From the clinical standpoint, the sensitivity of LDH post treatment to detect lymphoma relapse has been reported to be only 45% [18]. These findings suggests that other sources of tissue breakdown (i.e., the reason for release of LDH into the circulation) such as the inflammatory environment produced by the tumor besides tumor burden could be an important factor for the LDH production in lymphomas.

The association of poor clinical outcomes with a high N/L ratio could be the result of tumor-associated events that in turn produce inflammatory mediators of immune suppression manifesting as a decrease in host immunity (i.e., ALC). Key inflammatory transcription factors NF-kB, HIF-1α and STAT-3 have been associated with cancer development [19]. Besides antibody-dependent cell cytotoxicity and direct apoptosis against lymphoma cells, another possible mechanism of action of rituximab is to target the inflammatory component of the tumor by causing neutropenia [20] and direct inhibition of NF-kB [21] and STAT-3 [22]. Infiltrating tumor-associated macrophages (TAM) has been associated with poor prognosis in lymphomas as well as solid tumors. M2 macrophages has been associated with polarizing the host immunity from a Th1 to a Th2, leading to host immunity suppression [19]. However, despite these associations, a mechanistic explanation for the association between N/L ratio at diagnosis and clinical outcomes in DLBCL can only be addressed in an appropriately designed prospective clinical trial where relevant analyses of both systemic immunity and tumor phenotype can be studied. Nevertheless, based on the presented data, the association between N/L ratio at diagnosis and DLBCL survival seems clinically useful in judging survival risk for DLBCL patients treated with R-CHOP.

This study identifies a worldwide, standardized; low cost risk factor to assess clinical outcomes in DLBCL patients treated with R-CHOP. To our knowledge, this study is the first to identify N/L ratio as a prognostic factor for survival in DLBCL patients treated with R-CHOP. Thus, our study suggests that the N/L ratio can be used as a simple, inexpensive tool to assess survival outcomes in DLBCL treated with immunochemotherapy.

Methods

Patient population. To participate in this study, patients were required to have the diagnosis of de novo DLBCL, be treated with R-CHOP with or without consolidation radiation therapy at the discretion of the attending physician, and be followed at Mayo Clinic Rochester. Patients with primary DLBCL central nervous system (CNS) lymphoma, transformed NHL, post-solid organ transplant lymphoproliferative disorder, or positive human immunodeficiency virus were excluded from the study. From December 20, 2000 until December 27, 2007, 255 consecutive DLBCL patients were qualified for the study. Data from DLBCL patients were collected prospectively and entered into a computerized database. No patients were lost to follow-up. All patients gave written, informed consent allowing the use of their medical records for medical research. Approval for the retrospective review of these records was obtained from the Mayo Clinic Institutional Review Board and was in accordance with US federal regulations and the Declaration of Helsinki.

Endpoint. The primary endpoint of the study was to assess if baseline N/L ratio at diagnosis predicts survival in DLBCL patients treated with R-CHOP. The ALC and the neutrophil count were obtained from the complete blood cell count [23] at diagnosis.

Risk factors for relapse. Risk factors tested in the study included B symptoms (Fevers $>$38°C; drenching sweats; and weight loss $>$10% of normal body weight), baseline N/L ratio, IPI index [16]; [Age $\geq$ 60, extranodal sites $\geq$2, LDH (abnormal versus normal levels), performance status $\geq$2, and stage (I/II versus III/IV)], and postchemotherapy consolidation radiation.

Chemotherapy. All patients received rituximab 375 mg m^{-2}; cyclophosphamide 750 mg m^{-2}; doxorubicin 50 mg m^{-2}; vincristine 1.4 mg m^{-2}; and prednisone 100 mg m^{-2} $\times$ 5 days, every 21 days.

Response. Response criteria were based on criteria from the Lymphoma International Workshop [24]. Overall survival (OS) was defined as the time from diagnosis to death as a result of any causes or last follow-up. Progression-free survival (PFS) was defined as the time from diagnosis to disease progression, death as a result of any causes, or last follow-up.

Statistical analysis. OS and PFS times were analyzed using the method described by Kaplan and Meier [25]. Differences between survival curves were tested for statistical significance using the two-tailed log-rank test. The Cox proportional hazards model [26] was used to assess N/L ratio at diagnosis as a prognostic factor for OS and PFS times as well as to adjust for other known prognostic factors. The cutoff of N/L ratio $\geq$3.5 at diagnosis was supported by the data because it yielded the greatest differential in survival at N/L ratio $\geq$3.5 at diagnosis based on χ^2 values analyzed at different cut-points between the 25th and 75th quartiles (2.4–6.3) from log-rank tests (χ^2 = 15.6 for OS and χ^2 = 18.7 for PFS).

χ^2-tests were used to determine relationships between categorical variables. The Wilcoxon/Kruskal-Wallis rank tests were used to determine associations between continuous variables and categories, and Spearman correlation coefficients were used to evaluate associations for continuous variables. Logistic regression models were also used to assess associations between prognostic variables. All *P* values represented were two-sided, and statistical significance was declared at $P < 0.05$.

Author Contributions

LFP had the original idea for the study, designed the study, analyzed and interpreted data, did statistical analysis, and wrote the manuscript. KR collected the data and wrote the manuscript. TH, DJI, INM, wrote the manuscript. SNM designed the study, analyzed and interpreted data, and wrote manuscript.

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Comparison of real-time microvascular abnormalities in pediatric and adult sickle cell anemia patients

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The conjunctival microcirculation in 14 pediatric and eight adult sickle cell anemia (SCA) patients was studied using computer-assisted intravital microscopy. The bulbar conjunctiva in SCA patients in both age groups exhibited a blanched/avascular appearance characterized by decreased vascularity. SCA patients from both age groups had many of the same abnormal morphometric [vessel diameter, vessel distribution, morphometry (shape), tortuosity, arteriole:venule (A:V) ratio, and hemosiderin deposits] and dynamic [vessel sludging/sludged flow, boxcar blood (trickled) flow, and abnormal flow velocity] abnormalities. A severity index (SI) was computed to quantify the degree of vasculopathy for comparison between groups. The severity of vasculopathy differed significantly between the pediatric and adult patients (SI: 4.2 ± 1.8 vs. 6.6 ± 2.4; $P = 0.028$), indicative of a lesser degree of overall severity in the pediatric patients. Specific abnormalities that were less prominent in the pediatric patients included abnormal vessel morphometry and tortuosity. Sludged flow, abnormal vessel distribution, abnormal A:V ratio, and boxcar flow appeared in high prevalence in both age groups. The results indicate that SCA microvascular abnormalities develop in childhood and the severity of vasculopathy likely progresses with age. Intervention and effective treatment/management modalities should target pediatric patients to ameliorate, slow down, or prevent progressive microvascular deterioration.

Sickle cell anemia (SCA) is a genetic disorder that affects millions of people worldwide, for which there is no cure despite substantial understanding of its underlying pathogenesis [1,2]. Anemia caused by ineffective erythropoiesis and hemolysis is a contributing factor, but vascular complications and abnormal blood flow dynamics account for much of SCA morbidity and mortality. However, there are few real-time in vivo studies on the microcirculation in SCA patients, except for the work by Lipowsky et al. [3] on intravital microscopy of nailfold capillary hemodynamics in SCA.

We have previously reported three real-time in vivo studies on the microcirculation of the bulbar conjunctiva in SCA patients using computer-assisted intravital microscopy (CAIM) [4–6]. The microvascular bed of the bulbar conjunctiva offers a readily accessible site for noninvasive measurements from which it is possible to extrapolate the in vivo condition of the microvasculature within soft tissues, and to quantify changes in microvascular condition of critical end organs over time. Using our imaging studies of the bulbar conjunctiva in SCA patients, we have characterized and quantified the morphometric and dynamic microvascular abnormalities (vasculopathy) of the disease [4], demonstrated that abnormal microvascular blood flow dynamics correlate with intracranial blood flow velocity in the Circle of Willis measured by transcranial Doppler ultrasonography [5], and evaluated the efficacy of the drug Poloxamer 188 (RheothRx® and Flocor™) on vasoocclusion [6]. Thus, microvascular characteristics from image analysis of the bulbar conjunctiva can serve as a reliable surrogate biomarker of the severity of microvascular pathology and the efficacy of interventions designed to treat and ameliorate complications resulting from SCA-associated vasculopathy.

These real-time in vivo studies using CAIM have included both adult [4,6] and pediatric SCA patients [5,6]. However, in pediatric patients, these studies have focused primarily on the measurements of vessel diameter and blood flow velocity, and assessments of vasculopathy have not been reported. Moreover, there have been no direct comparisons of microvascular abnormalities and severity of vasculopathy between pediatric and adult SCA patients. Accordingly, the goal of this study was to characterize and compare real-time measurements on the degree of in vivo vasculopathy in pediatric and adult SCA patients, and to test the hypothesis that the severity of vasculopathy increases with age as a natural course of the disease.

Fourteen pediatric and eight adult SCA patients participated in the study. Mean ages of the two groups were significantly different (13.6 ± 4.4 years vs. 36.8 ± 11.9 years, $P < 0.001$). Conjunctival microvasculature was compared between the pediatric and adults patients, and contrasted with that of healthy, non-SCA control subjects analyzed in previous studies [4,7,8]. Figure 1A shows a typical image of the conjunctival microvasculature in a non-SCA subject frame captured from a videotape sequence from an unrelated study [7,9]. There is an orderly presence of anastomosing networks of capillaries, arterioles, and venules without the presence of ischemic (avascular) zones (Fig. 1A). The normal A:V ratio is typically ~1:2, and the arterioles and venules exhibit an even distribution without the presence of dilations, narrowing, distension, microaneurysm, sacculated (beaded) vessels, broken/damaged vessels, or hemosiderin deposits. Normal conjunctival blood flow, though variable in red cell velocity, is smooth and nonintermittent. Blood sludging, tortuous vessels, and boxcar blood flow (trickled flow) patterns are typically not observed.

The conjunctival microcirculation in the pediatric and adult SCA patients uniquely differs from those found in non-SCA control subjects [See Supporting Information]. There is a lower amount of vascularity (diminished presence of conjunctival vessels) and abnormal vascular distribution in most patients in both age groups, giving the bulbar conjunctiva a "blanched" avas-

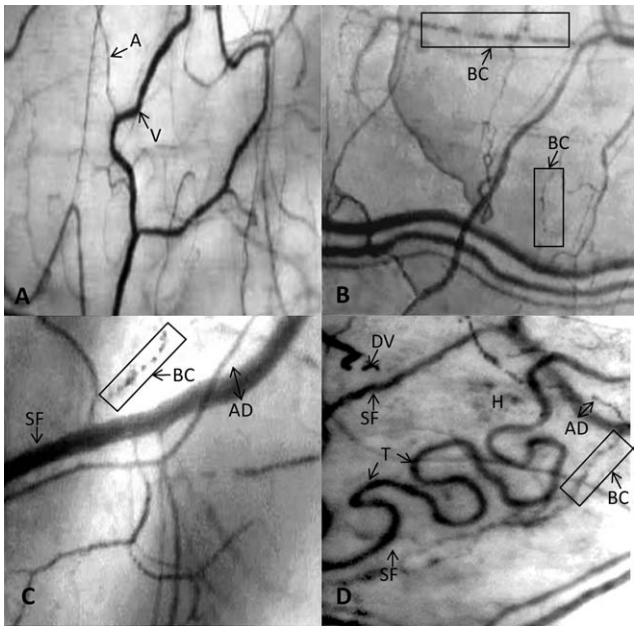

Figure 1. A: A frame-captured image of the conjunctival microcirculation in a healthy non-SCA control subject [7,9]. Optical magnification 4.5; onscreen magnification ×125. This image illustrates a typical view of the conjunctival microcirculation in a healthy (non-SCA) control subject who has no history of any vascular disease. Note the even and orderly distribution of normal-sized arterioles, venules, and capillaries in a richly vascularized network. B: A frame-captured image of the conjunctival microcirculation in a pediatric SCA patient (Patient #P-3; age 8 years). Optical magnification ×4.5; onscreen magnification ×125. The SI of this patient is 3 and the microvascular abnormalities include only sludged blood flow (vessel sludging), boxcar (trickled) blood flow, and abnormal A:V ratio. Overall, the vasculopathy observed is mild. C: A frame-captured image of the conjunctival microcirculation in another pediatric SCA patient (Patient #P-8; age 15 years). Optical magnification ×4.5; onscreen magnification ×125. Patient P-8 is 7 years older than the patient described in Figure 1B. The microcirculation shows a greater level of vasculopathy, which includes abnormal vessel diameter, sludged blood flow, boxcar (trickled) blood flow, abnormal vessel distribution, hemosiderin deposits, and abnormal A:V ratio in this captured frame. The overall vasculopathy in this pediatric patient is severe, with an SI of 7 (compared with the SI of 3 in the pediatric patient described in Figure 1B). D: A frame-captured image of the conjunctival microcirculation in an adult SCA patient (Patient #A-7; age 58 years). Optical magnification ×4.5; onscreen magnification ×125. The microvascular abnormalities in this adult patient include abnormal vessel diameter, pronounced vessel tortuosity, abnormal vessel distribution, abnormal A:V ratio, sludged (trickled) blood flow, boxcar flow pattern, damaged vessel, and hemosiderin deposits. A, arteriole; V, venule; BC, boxcar (trickled) blood flow; SF, sludged blood flow (stop-and-go pattern of blood flow as evidenced by area(s) of darker or uneven coloration within the vessel); AD, abnormal diameter (wide); DV, damaged vessel; H, hemosiderin deposits; T, tortuosity.

cular appearance. The prevalence of specific microvascular abnormalities in both patient groups is summarized in Tables I and II [See Supporting Information] and some of the abnormalities are shown in Figure 1B–D. SCA patients from both age groups exhibit, to varying degrees, the same morphometric and dynamic abnormalities, including abnormal vessel diameter, abnormal vessel distribution, abnormal vessel morphometry (shape), sludged flow, vessel tortuosity, abnormal A:V ratio, boxcar blood flow, hemosiderin deposits, and abnormal flow (red cell) velocity. These microvascular abnormalities are rarely found in the bulbar conjunctiva of healthy non-SCA subjects [4,7,8]. The severity of vasculopathy, as indicated by the severity index (SI), was significantly lower in the pediatric patients than in the adult patients (4.2 ± 1.8 vs. 6.6 ± 2.4, $P = 0.028$). For comparison, the mean SI values for both the pediatric and adult SCA patients were significantly higher than the mean SI value determined for a previous cohort of healthy non-SCA subjects ($n = 10$; SI = 0.31 ± 0.72; $P < 0.05$) [8]. In comparing the prevalence of microvascular abnormalities between pediatric and adult SCA groups, the following significant differences were observed:

- Abnormal vessel morphometry was observed in three out of eight adult patients (38%) but was not observed in any of the pediatric patients. The odds ratio (OR) [95% confidence interval (CI)] for the difference in prevalence was ∞ (1.2, ∞) ($P = 0.036$).
- Vessel tortuosity was observed in seven out of eight adult patients (88%) compared with only three out of 14 pediatric patients (21%). The OR (95% CI) for the difference in prevalence was 25.7 (1.7, 1258) ($P = 0.006$).

In addition, several microvascular abnormalities were highly prevalent in both the pediatric and adult patients. Ten out of 14 pediatric patients (71%) and seven out of eight adult patients (88%) had vessel sludging. Ten out of 14 pediatric patients (71%) and eight out of eight adult patients (100%) had an abnormal A:V ratio. Eight out of 14 pediatric patients (57%) and seven out of eight adult patients (88%) had an abnormal vessel distribution. Eleven out of 14 pediatric patients (79%) and six out of eight adult patients (75%) exhibited boxcar flow patterns.

CAIM is a real-time technology that can be used to noninvasively videotape, analyze and quantify real-time microvascular abnormalities in vascular diseases. The technique has been used successfully in our laboratory to assess microvascular abnormalities in type-1 and type-2 diabetes, Alzheimer's disease, and SCA [4–12]. The in vivo microvascular bed of the bulbar conjunctiva (conjunctival microcirculation) is particularly amenable to the use of CAIM because it is noninvasively and easily accessible, and yields images of excellent quality and clarity. Results from some of the studies on the identification and quantification of microvascular abnormalities in the conjunctival microcirculation [4,11] have been used as a basis for subsequent translational research and interventional efficacy studies [6,10].

This study was designed to extend our knowledge base on real-time vasculopathy in pediatric and adult SCA patients. Our overall goal is to understand the ontogeny of vasculopathy based on the hypothesis that, as a genetic disorder, SCA microvascular complications and vasculopathy begin to develop after birth and continue to progress into adulthood as part of the natural course of the disease. Results from this study support this hypothesis: the severity of microvascular abnormalities in the pediatric patients was significantly lower than that observed in the adult patients. Secondary analyses of specific microvascular abnormalities revealed that the observed difference in severity was primarily due to a lower prevalence of abnormal vessel morphometry and vessel tortuosity in the pediatric patients compared with the adults. These findings suggest that these two specific abnormalities develop at a slower rate than other microvascular abnormalities.

The primary limitation of this study is that it is cross-sectional. The observed difference in the severity of vasculopathy between the pediatric and adult patients could be attributable to advances in management of the disease that were not available to the adult patients during their childhood. A longitudinal study in which the microvasculature of SCA patients is evaluated at regular intervals from childhood to adulthood would be required to definitively test the hypothesis that the severity of vasculopathy progresses with age. If confirmed, the results of this study suggest that the pediatric years represent a window of opportunity during which effective treatment and management modalities may slow or ameliorate complications of SCA caused by vasculopathy that arises as a natural progression of the disease from childhood to adulthood. Specific abnormalities, e.g., abnormal vessel morphometry and vessel tortuosity, may serve as landmark biomarkers to evaluate the efficacy of treatment and disease management modalities over time. Moreover, the high prevalence of other abnormalities in both pediatric and adult patients, including vessel sludging, abnormal A:V ratio, abnormal vessel distribution, and boxcar flow patterns—indicative of rapid development of vasculopathy in childhood—suggests an urgency to identify better interventions and treatments that ameliorate or slow the progression of microvascular abnormalities and can be used to treat pediatric SCA patients more aggressively.

Conjunctival vessels have unique shapes and forms (Fig. 1A–D) and can be easily reidentified for follow-up studies using CAIM—each individual vessel can serve as its own baseline (reference) control and then relocalized and reassessed in longitudinal studies [6,10]. This makes the conjunctival microcirculation an ideal arena and CAIM an excellent noninvasive real-time technology for longitudinal studies of SCA disease progression and evaluations of the efficacy of medications and other treatment or management modalities. At this time, CAIM is not yet widely used as a research tool. However, two identical CAIM systems have been built recently and are functional in other laboratories. A blinded interventional collaborative study to compare independently obtained real-time in vivo vasculopathy data is in progress. These studies will eventually allow for independent confirmation of our results at other institutions and will validate the utility of CAIM as a clinical tool to objectively and noninvasively study vasculopathy in SCA and other vascular diseases.

Methods

Patient groups studied. The University of California Davis Institutional Review Board approved the study, and written informed consent was obtained from all patients or from their parents or guardians. Pediatric SCA patients (HbSS; ages 6–18 years) were recruited from the Pediatric Sickle Cell Clinic at the University of California Davis Medical Center (UCDMC). Adult SCA patients (HbSS; ages 27–58 years) were recruited from the Adult Sickle Cell Clinic at UCDMC. Before initiation of the study, all patient records were evaluated to ensure that each patient was not having any sickling complications (i.e., in steady-state condition) and had not suffered a vasoocclusive (painful) crisis for at least a month before the study. SCA patients on chronic transfusion were allowed to participate in this study.

Computer-assisted intravital microscopy. A CAIM system substantially modified and adapted from the earlier prototype originally designed to study the conjunctival microcirculation in adult subjects [4,10] has been utilized successfully thereafter to study pediatric patients [5,6,11]. The CAIM system uses macro-optics in which image acquisition is based on real-time video documentation of selected regions in the in vivo conjunctival microcirculation. The procedural details of this technique have been described in detail in previous publications [7–9].

Quantification of severity of vasculopathy and prevalence of microvascular abnormalities. Videotape sequences made of the conjunctival microcirculation in each patient were coded for subsequent viewing and analysis to ensure objectivity, with the medical history and identity of each pediatric and adult patient blinded to the investigators prior to and during data analysis. Data analysis, which was described in detail in previous reports [4,7–9], was conducted in two phases:

1. Visualization phase—Identification of morphometric characteristics. Videotape sequences of each patient were viewed in their entirety. Key landmark features (characteristics), including comma signs, vessel sludging (sludged flow), boxcar (trickled) blood flow pattern, microaneurysms (micropools), ischemia, vessel morphometry (pattern or shape), vessel distribution, distended vessels, tortuous vessels, sacculated (beaded) vessels, damaged vessels, and hemosiderin deposits were identified and tabulated for their presence in each experimental subject [See Tables I and II in Supporting Information]. The same coded videotape sequences were analyzed by at least two observers. Differences in the identification of the morphometric features, though infrequent, were discussed and reconciled through a third adjudicator.
2. Quantification phase—computer-assisted image analysis. Four to five short coded videotape sequences of ~30 sec each from each experimental subject were selected and frame captured for data quantification, including vessel diameter, total lengths of arterioles and venules per area for arteriole-venule (A:V) ratio computation, and measurement of red cell flow velocity [4,7–9].

Based on previous studies on microvascular abnormalities in various vascular diseases, 15 possible aberrations can be found in the conjunctival microvasculature [7–11]. A SI is computed to quantify the degree (severity) of vasculopathy in each patient, based on the arithmetic summation of the presence of any of the 15 microvascular abnormalities listed above on a binary (yes = 1; no = 0) basis. The SI ranges from a score of 0 (no abnormalities present) to 15 (all 15 abnormalities present). This SI computation methodology has been validated in previous studies [7–11] and has an inter-investigator variation coefficient of $<5\%$.

Statistical analysis. Results were reported as means ± standard deviation and medians with ranges. The two-sided Wilcoxon rank-sum test was used to compare SI, which is a numerical variable, between the two groups. The two-sided Fisher's exact test was used to compare the prevalence of each of the 15 microvascular abnormalities between the two groups, which can be constructed as a 2×2 contingency table. An OR for each abnormality in the adult patients with 95% CI was reported with the pediatric patients serving as the reference group. This OR is the ratio of the odds of an abnormality appearing in the adult patient group to the odds of it appearing in the pediatric patient group. An OR with 95% CI represents a statistically significant difference in the appearance of a specific microvascular abnormality between the two patient groups. All statistical analyses in this study were performed using the SAS v9.2 software (SAS Institute, Cary, NC). A *P*-value ≤ 0.05 was considered statistically significant.

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Author Contributions

ATWC developed the intravital microscope and CAIM methodology, designed and conducted the study, analyzed and critically interpreted the data, and wrote the manuscript. JWM contributed to study design and interpretation of the data and critically reviewed and edited the manuscript. SMC, PLT, and XL performed and interpreted the CAIM analysis and reviewed the manuscript. SLS coordinated the study, recruited patients, and reviewed the manuscript. PCYC co-designed the intravital microscope and CAIM methodology with ATWC and independently verified the CAIM results off-site. TZ and TW provided access to and assisted in patient recruitment, contributed to study design and interpretation of data, and critically reviewed the manuscript. CSL served as biostatistician for the study and critically reviewed the manuscript. RG, who is principal investigator of NIH grant R01 HL83276 which funded this project, contributed to study design and interpretation of data and critically reviewed and edited the manuscript.

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Urinary markers of bone resorption, pyridinoline and deoxypyridinoline, are increased in sickle cell patients with further increments during painful crisis

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The painful crisis is the hallmark of sickle-cell disease (SCD). Bone resorption, as part of physiological bone turnover, results in release into the circulation with subsequent urinary excretion of the collagen cross-links pyridinoline (PYD) and deoxypyridinoline (DPD). Urinary PYD and DPD concentrations could reflect the extent of bone infarction during painful sickle-cell crisis. Urinary concentrations of PYD and DPD, adjusted for urine creatinine, were measured in sickle-cell patients (38 clinically asymptomatics and 27 during painful crisis) and healthy controls ($n = 25$) using high-performance liquid chromatography (HPLC). PYD and DPD concentrations were higher in asymptomatic HbSS/HbSβ^0-thalassemia patients compared to controls ($P < 0.05$) with further increments during painful crisis in both HbSS/HbSβ^0-thalassemia and HbSC/HbSβ^+-thalassemia patients ($P < 0.05$). In the asymptomatic HbSS/HbSβ^0-thalassemia patients, there was a statistically significant positive correlation between DPD and hemolytic rate. Based on urinary PYD and DPD concentrations, bone degradation is increased in asymptomatic sickle-cell patients, with further increments during painful crisis. Urinary PYD and DPD concentrations are potentially diagnostic and prognostic tools in SCD.

Sickle-cell disease (SCD) affects millions worldwide. One of the most frequently occurring complications is the painful crisis [1]. The painful crisis manifests as acute musculo-skeletal (usually juxta-articular) and/or visceral pain mostly associated with mild pyrexia, which often necessitates treatment with parenteral opiates and thus hospital-based medical care [2]. The pathophysiology involves multiple mechanisms ultimately leading to the obstruction of microvasculature with subsequent tissue ischemia and infarction as result [3–5]. Even though mostly self-limiting, painful crises are associated with severe complications such as the acute chest syndrome, stroke, multiorgan failure, and sudden death [6–8]. Furthermore, patients experiencing three or more painful crises per year requiring medical attention carry a higher risk of early death [1,9,10].

One of the most challenging aspects in the management of patients with acute painful crises is the lack of objective laboratory tools to confirm the diagnosis and to estimate its severity. Next to parameters such as reticulocyte and leukocyte counts and lactate dehydrogenase (LDH) levels, studies have focused on laboratory markers involved in the pathophysiological processes of SCD such as markers of endothelial activation, cytokine profiles, and coagulation proteins [11–18], none of which have made the transition to the clinic. The lack of diagnostic tools to diagnose vaso-occlusion can contribute to misinterpretation of symptoms, unjust withholding of adequate analgesia, distorted communication, and doctor–patient relationships with unjust stigmatization of patients as drug addicts. Therefore, objective laboratory tools accurately reflecting the vaso-occlusive process would be of great value to those caring for patients with SCD.

As marrow ischemia and infarction potentially leads to bone degradation, laboratory markers of bone resorption may be of value in monitoring vaso-occlusion in SCD. Pyridinoline (PYD) and deoxypyridinoline (DPD) are collagen cross-links, and their urinary concentrations have been proven accurate markers of bone resorption [19–22]. After bone degradation, PYD and DPD are released in the circulation and excreted directly into urine without further systemic metabolism. Pyridinium-based cross-links are an important part of the extracellular collagen fibrils in most connective tissue types. However, unlike other connective tissue types, bone is continuously remodeled and therefore forms the main source of urinary cross-links. Furthermore, the ratio of PYD to DPD in urine is approximately the same as in adult human bone (3.5:1), further supporting bones as the predominant source of urinary PYD and DPD [23]. Given the above, we set out to determine profiles of urinary PYD and DPD in SCD in both the clinically asymptomatic state as well as during painful crisis.

Twenty-seven HbSS/HbSβ^0-thal [median age (interquartile range [IQR]) 26 (18–52) years; 23 HbSS and 4 HbSβ^0-thalassemia] and 11 HbSC/HbSβ^+-thal [age 29 (21–42); all HbSC] sickle-cell patients during clinically asymptomatic state and 21 HbSS/HbSβ^0-thal [age 25 (19–48); all HbSS] and 6 HbSC/HbSβ^+-thal [age 25 (21–33); 1 HbSβ^+-thalassemia and 5 HbSC] during painful crisis were included. Twenty-five race- and age-matched healthy HbAA [age 28 (18–41)] volunteers were included as controls. See Supporting Information 1 for detailed baseline characteristics.

Although urinary PYD and DPD to creatinine ratios were comparable between healthy controls and HbSC/HbSβ^+-thal patients in steady state, they were significantly higher in asymptomatic state HbSS/HbSβ^0-thal patients (Fig. 1A,B). Further increments were observed during painful crisis, although the differences did not reach statistical significance for PYD in both groups and for DPD in HbSC/HbSβ^+-thal patients. In a paired analysis of 19 patients who were included both during asymptomatic state and painful crisis, the urinary cross-links increased during painful crisis, although the difference was only statistically significant for DPD (see Fig. 2).

Urinary concentrations of PYD and DPD were significantly related to hemolytic rate in asymptomatic state HbSS/HbSβ^0-thal patients but not HbSC/HbSβ^+-thal patients (Supporting Information 2). No correlations between the cross-links and the hemolytic rate were observed during painful crisis. Gender was not related to the degree of urinary excretion of PYD and DPD (data not shown). PYD and DPD after the first night of hospital admission were not related to the duration of hospital stay (days) for the treatment of painful crisis (data not shown).

The findings in this study indicate a higher degree of chronic bone degradation in SCD with exacerbations during painful crisis. Increased urinary excretion of PYD and DPD during painful crisis primarily suggests that bone ischemia and subsequent necrosis due to microvascular occlusion induces bone degradation. However, increased metabolic bone turnover during painful crisis could also contribute. The degree of increments in PYD and DPD concentrations during painful crisis in most patients of the paired analysis was comparable. Because of interpatient variation in baseline concentrations during asymptomatic state, it was not possible to determine a normal cut-off value.

Baseline PYD and DPD values were elevated in HbSS/HbSβ^0-thal patients when compared with healthy controls. Given the fact that sickle-cell patients are characterized by an increased renal creatinine excretion [24], the expressed cross-links to creatinine ratios are likely to be underestimated in these patients. PYD and DPD concentrations were highest in patients with the greatest hemolytic rate, and it may well be possible that hemolytic anemia-induced bone marrow expansion contributes to bone degradation. SCD is characterized by a continuous state of inflammation, which could also be a significant contributory factor to increased bone resorption [25]. Ongoing clinically silent vaso-occlusion is another potential explanation of increased bone ischemic damage and degradation [26]. Hemolysis-induced vasculopathy in SCD, mediated by oxidative stress, reduced nitric oxide bioavailability, hemostatic activation, adhesion of activated leukocytes, and platelets to endothelial cells might result in ischemia-reperfusion injury and thus bone degradation, especially in genotypes characterized by severe hemolysis (HbSS/HbSβ^0-thalassemia) [27,28]. It is very likely that a combination of these factors in addition to bone volume and bone metabolism related factors such as calcium, phosphate, vitamin D, parathyroid hormone, and age ultimately determines (the variation in) base-line PYD and DPD values in the clinically asymptomatic state. Associations with biomarkers of inflammation and bone metabolism were out of the scope of this study. Currently, it is unknown whether each individual patient has a stable base-line value over time.

In interpreting these data, some pitfalls should be considered. As there is no gold standard for diagnosing a sickle-cell crisis (let alone its severity), one should be cautious to relate the urinary concentrations of PYD and

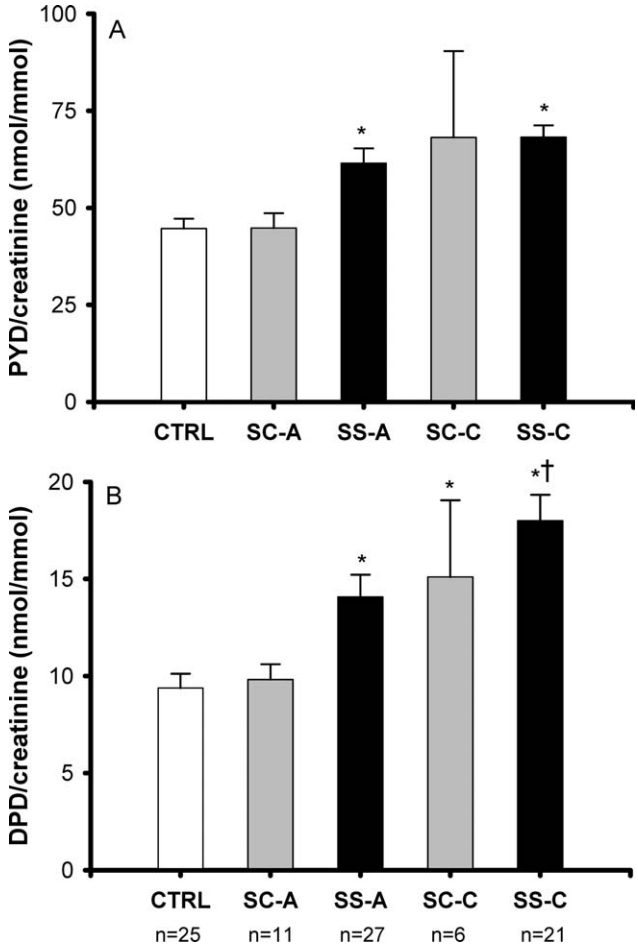

Figure 1. Urinary concentrations of pyridinoline (PYD) and deoxypyridinoline (DPD), adjusted for urine creatinine, in 25 healthy controls with normal HbAA hemoglobin (CTRL), 27 HbSS/HbSβ^0-thalassemia (SS), and 11 HbSC/HbSβ$^+$-thalassemia (SC) sickle-cell patients during asymptomatic state (A) and 21 HbSS/HbSβ^0-thal and 6 HbSC/HbSβ$^+$-thal patients during painful crisis (C). A: PYD concentrations. B: DPD concentrations. *Significantly different when compared with healthy controls ($P < 0.05$). †Significantly different when compared with asymptomatic state ($P < 0.05$). Bars indicate means ± SEM.

DPD to the severity of a vaso-occlusive crisis. We could not find a correlation between the urinary PYD and DPD concentrations and crisis duration. The number of patients included was low, and these preliminary findings need confirmation in a large, prospective study where serial measurements of PYD and DPD during painful crisis are studied in relation to clinical outcomes such as pain score. In the paired analysis, DPD, but not PYD, increased significantly during painful crisis. This could be explained by the greater bone specificity of DPD, as PYD is also a major component of collagen fibrils in other tissue types [23].

In conclusion, the findings of increased PYD and DPD in asymptomatic state sickle-cell patients with further increments during painful crisis seem not only of potential diagnostic importance but of pathophysiological importance as well, since sickle-cell patients may have an increased risk of osteoporosis due to continuously increased bone degradation, with further increments during each painful crisis [3,29]. Urinary PYD and DPD concentrations can be determined rapidly with a widely available technique, and their value as potential diagnostic tools of the painful sickle cell crisis is now subject of further study.

Methods

Study population. Consecutive clinically asymptomatic adult (≥18 years old) patients with SCD [HbSS, HbSβ^0-thalassemia, HbSβ$^+$-thalassemia, and HbSC confirmed by high-performance liquid chromatography (HPLC)], visiting the outpatient clinic and patients admitted with a painful crisis at the Academic Medical Centre and Slotervaart Hospital, Amsterdam, the Netherlands, were eligible for the study. A painful crisis was

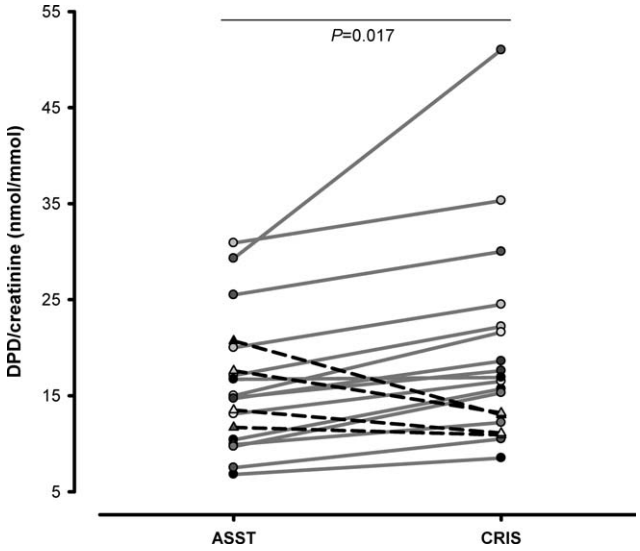

Figure 2. Deoxypyridinoline (DPD) in 19 patients included both during asymptomatic state (ASST) and painful crisis (CRIS). Urinary concentrations of DPD are higher during painful crisis as compared to the asymptomatic state.

defined as musculo-skeletal pain not otherwise explained and recognized as such by the patient. The clinically asymptomatic state was defined as being free of SCD-related acute events, such as painful crises, priapism, acute chest syndromes, and strokes during at least 4 months before study participation. Exclusion criteria were autoimmune inflammatory diseases, active infection, pregnancy, and women in (post-) menopausal stage. Healthy race and age-matched HbAA volunteers served as controls. All participants gave written informed consent. The protocol was reviewed and approved by the local medical ethical committee and conducted in agreement with the Helsinki declaration of 2000.

Blood and urine samples. Blood samples were drawn via venipuncture. Standard blood counts were performed in EDTA-anticoagulated blood (Cell-Dyn 4000, Abbott, IL). LDH and total bilirubin levels were measured in heparinized plasma with spectrophotometry (P800 Modular, Roche, Basel, Switzerland). Urine samples were collected in the morning between 8 and 10 a.m. after overnight fasting of at least 8 hr and stored at −80°C until measurements.

Laboratory analysis. Cross-links are determined by HPLC using commercial reagents (ChromSystems, München, Germany). To adjust for the degree of urine concentration, urinary creatinine is measured, and the cross-links are expressed as PYD to creatinine and DPD to creatinine ratios.

Statistical analysis. For data analysis, patients were divided in two groups with patients with the relatively severe genotypes HbSS and HbSß^0-thalassemia grouped in one group (HbSS/HbSβ^0-thal) and patients with the relatively milder HbSC and HbSß$^+$-thalassemia genotypes collected in the other group (HbSC/HbSβ$^+$-thal) [30]. As data were not normally distributed, statistical tests for nonparametric data were used. For multiple-group comparisons of continuous variables, the Kruskal–Wallis test was used. The Mann–Whitney *U*-test was used for comparison between two groups. The Wilcoxon Signed Rank Test was used for a paired analysis of patients included both during asymptomatic state and painful crisis. For correlation studies, the Spearman Rank correlation coefficient (r_S) was determined. Continuous data are presented as medians with corresponding IQR, unless stated otherwise. $P < 0.05$ was considered statistically significant (SPSS 16.0, SPSS, Chicago, IL).

The CURAMA study group is a collaborative effort studying sickle cell disease in the Netherlands Antilles and the Netherlands. Participating centers: The Red Cross Blood Bank Foundation, Curacao, Netherlands Antilles; The Antillean Institute for Health Research, Curacao, Netherlands Antilles, The Department of Internal Medicine, Slotervaart Hospital, Amsterdam, the Netherlands, The Departments of Hematology and Vascular Medicine, Academic Medical Center, Amsterdam, the Netherlands; the Department of Hematology, Erasmus Medical Center, Rotterdam, the Netherlands; Pathology and Laboratory Medicine,

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Additional Supporting Information may be found in the online version of this article.

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Allogeneic hematopoietic cell transplantation for myelofibrosis: A 10-year experience at single institution

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Allogeneic hematopoietic cell transplantation (HCT) remains the only curative approach for patients with myelofibrosis [1,2]; however, it can result in substantial transplant related mortality. We retrospectively examined the outcomes of 18 myelofibrosis patients who underwent allogeneic HCT between 2000 and 2008. Eleven (61%) had high-risk prognostic scores. Fourteen (78%) received a reduced-toxicity regimen of pharmacokinetically-targeted (a median AUC of 5,300 $\mu m\ min^{-1}$; range, 3,500–6,000) intravenous busulfan plus fludarabine (t-IV Bu/Flu). Source of hematopoietic cells were from HLA matched-related (50%), matched-unrelated (44%), or mismatched-unrelated (6%) donors. Fourteen (78%) received G-CSF-mobilized peripheral blood stem cells. Median time to neutrophil and platelet engraftment were 17 (range, 15–28) and 18 (range, 8–213) days, respectively. There were two cases of secondary graft failure after myeloablative conditioning and one case following t-IV Bu/Flu. Overall survival was 50.2% at 54 months (95% confidence interval [C: 24.1–71.6%) with a median follow-up of 15 (range, 3–63) months. Nonrelapsed mortality (NRM) at 100 days and at 1-year were 0 and 22.6% (95% CI: 9.1–49.7%), respectively. The causes of NRM comprised of infections (56%) and graft-versus-host-disease (44%). t-IV Bu/Flu is a feasible conditioning regimen for allogeneic HCT in myelofibrosis showing encouraging engraftment with 7% graft failure rate.

Myelofibrosis is a clonal hematopoietic disorder characterized by bone marrow fibrosis, leukoerythroblastic features, splenomegaly, constitutional symptoms, and extramedullary hematopoiesis [3–5]. The Lille scoring system is a prognostic model that helps stratify patients with myelofibrosis [6]. Intermediate-risk group (1 factor) and high-risk group (2 factors) have a median survival of 26 months and 13 months, respectively. Allogeneic HCT is a potentially curative treatment option for patients with primary myelofibrosis or myelofibrosis secondary to polycythemia vera (PV) or essential thrombocythemia (ET) [1,2]. Wider applicability of allogeneic HCT had been limited by the significant risks of transplant-related mortality resulting from myeloablative conditioning regimens, especially in the older patient population [7–10]. Reduced toxicity regimens have been used to facilitate allogeneic

TABLE I. Patient and Transplant Characteristics

Characteristics	Value
Patient median age, year (range)	55.5 (23–65)
Donor → patient gender combination	
Male → male	5
Male → female	3
Female → male	6
Female → female	4
Myelofibrosis	
Primary	7
Secondary[a]	11
Risk profile according to Lille score	
Intermediate	7
High	11
Risk profile according to IPSS-MF by IWG-MRT	
Low	1
Intermediate-1	5
Intermediate-2	10
High	2
Spleen size at transplant	
Enlarged	13
Not enlarged	3
Unknown	1
Splenectomy	1
Cytogenetics	
Abnormal[b]	7
Normal	9
Unknown	2
Blast percentages, % (range)	
Peripheral blood	2.5% (0–13.9%)
Bone marrow	2.5% (1–9%)
HLA-matching	
HLA-identical sibling	9
Matched unrelated	8
Mismatched unrelated (9/10)	1
Stem cell source	
Peripheral blood stem cells	14
Bone marrow	4
Median number of $CD34^+$ cells/kg BW (range)[c]	9.07×10^6 ($2.58–10.52 \times 10^6$)
Conditioning regimens	
t-IV Bu/Flu	14
Others[d]	4
GVHD prophylaxis	
TAC/MTX	11
TAC/MMF	4
CSA-based regimen	3

$n = 18$ patients. IPSS-MF indicates International Prognostic Scoring System for myelofibrosis; IWG-MRT, the International Working Group for Myelofibrosis Research and Treatment; HLA, human leukocyte antigen; t-IV Bu/Flu, targeted intravenous busulfan and fludarabine; GVHD, graft-versus-host-disease; TAC, tacrolimus; MMF, mycophenolate mofetil; MTX, methotrexate; CSA, cyclosporine; BW, body weight.

[a]Secondary myelofibrosis includes post-essential thrombocythemia, post-polycythemia vera, unclassifiable myeloproliferative neoplasms, and unclassifiable myelodysplastic/myeloproliferative neoplasms.

[b]Cytogenetic changes include deletion 13 ($n = 3$), trisomy 9 ($n = 2$), chromosome 12 abnormalities ($n = 2$), and chromosome 18 abnormalities ($n = 2$).

[c]Available for 16 patients.

[d]Other conditioning regimens include cyclophosphamide + total body irradiation, busulfan + cyclophosphamide, and fludarabine + melphalan.

TABLE II. Prior Therapies

Regimens	Number
Hydroxyurea	8
Prednisone	6
Thalidomide	5
Phlebotomy	5
Splenic irradiation	3
Lenalidomide	2
G-CSF	2
Anagrelide	2
Danazol	2
High dose busulfan (1 mg kg^{-1} orally every 6 hr for 16 doses) followed by autologous stem cell transplantation	1
IVIG	1
Cyclosporine	1
Rituximab	1
Decitibine	1
Interferon	1
Imatinib mesylate	1

$n = 18$ patients. G-CSF: granulocyte-colony stimulating factor; IVIG: intravenous immunoglobulin.

HCT in older myelofibrosis patients with encouraging results [11–16]. A combination of intravenous busulfan and fludarabine (IV Bu/Flu) has been demonstrated safe and effective as conditioning regimen in myeloid malignancies [17–19]. Pharmacokinetic analysis has been performed with IV busulfan administration, however, no large-scale trials have been undertaken to examine the safety and efficacy of t-IV Bu/Flu, specifically in myelofibrosis.

Eighteen patients received allogeneic HCT for myelofibrosis between 2000 and 2008 at Moffitt Cancer Center. The baseline characteristics of these patients are listed in Table I. There were 10 (56%) men and 8 (44%) women. The Lille and International Prognostic Scoring System for myelofibrosis (IPSS-MF) scores were calculated at the time of transplant [6,20]. Three (17%) patients had constitutional symptoms. Therapy received prior to allogeneic HCT is shown in Table II. Seventeen patients had bone marrow fibrosis information available pre-transplant and all had increased reticulin fibers. Five patients had osteosclerosis. Median time to neutrophil and platelet engraftment was 17 (range, 15–28) days and 18 (range, 8–213) days, respectively. All patients engrafted neutrophils initially. All but one patient conditioned with t-IV Bu/Flu achieved platelet engraftment within 30 days. One patient had pre-transplant platelet count of over 1,000,000 μL and platelet count nadir was at 40,000 μL. There were three cases of delayed platelet engraftment (> 30 days). One patient who was conditioned with busulfan and cyclophosphamide (Bu/Cy) experienced both delayed WBC (day +28) and platelet (day +213) engraftment. Three patients experienced secondary graft failure: one patient with fludarabine and melphalan (Flu/Mel) conditioning received second infusion of cells on day +89 due to secondary graft failure, another patient conditioned with cyclophosphamide with total body irradiation (Cy/TBI) developed secondary graft failure at 3 months, and a third patient who received t-IV Bu/Flu developed secondary graft failure at 11 months.

Median donor bone marrow (BM) chimerism (unsorted) was 97.5% (range, 91–100: $n = 14$) on day +30, 99.5% (range, 92–100: $n = 12$) on day +90, and 100% (range, 67–100: $n = 10$) on day +180. Median $CD3^+$ peripheral blood (PB) chimerisms were 58.5% (range, 44–100) on day +30, 64.5% (range, 45–100) on day +90, and 83.5% (range, 37–100) on day +180. Median $CD33^+$ PB chimerisms were 100% (range, 100–100) on day +30, 100% (range, 100–100) on day +90, and 100% (range, 45–100) on day +180.

Fourteen (74%) patients developed Grade II–IV acute graft-versus-host disease (GVHD). Maximum acute GVHD overall grades were: Grade I = 3 (17%), Grade II = 8 (44%), and Grade III = 6 (39%). There was no Grade IV acute GVHD. Fourteen (78%) patients developed chronic GVHD (limited = 8, extensive = 6). Overall, marrow fibrosis improved or resolved in 13 (76%) of 17 cases. Three patients had stable marrow fibrosis and there were no relapses of marrow fibrosis. One patient did not have assessment on the degree of marrow fibrosis post-allograft. Splenomegaly improved in 7 (54%) of 13 patients. There were three patients with $JAK2^{V617F}$ mutation and all became negative post-transplantation.

Overall survival (OS) by Kaplan–Meier method is 50.2% at 54 months (95% CI: 24.1–71.6%) with a median follow-up of 15 (range, 3–63) months, as shown in the Figure 1A. There was a trend toward improved probability of survival in patients with intermediate Lille risk group compared to high Lille risk group ($P = 0.124$ by Log Rank test; Fig. 1B). There was no association with IPSS-MF (not shown). NRM at 100 days and at 12 months were 0 and 22.6% (95% CI: 9.1–49.7%), respectively (Fig. 1C). The causes of NRM comprised of infections ($n = 5$, 56%) and GVHD ($n = 4$, 44%). OS curve of 14 patients who received t-IV Bu/Flu conditioning regimen is shown in Fig. 1D. One patient progressed to acute myeloid leukemia 1-year post-transplant and later received second matched unrelated donor hematopoietic cell transplant. However, the patient developed steroid-refractory GVHD with multi-organ failure and expired on day +51 after second transplant.

One patient conditioned with Cy/TBI developed idiopathic pneumonia syndrome (IPS) on day +23 post transplant requiring high-dose methylprednisolone. The patient required supplemental oxygen but no ventilator support and ultimately recovered from IPS. Another patient who was conditioned with Bu/Cy developed diffuse alveolar hemorrhage (DAH) on day +30 requiring high-dose steroid treatment with subsequent improvement. None of the patients treated with t-IV Bu/Flu experienced significant toxicities including seizure, hepatic sinusoidal obstruction syndrome/veno-occlusive disease (SOS/VOD) or IPS related to busulfan. Overall toxicities observed in

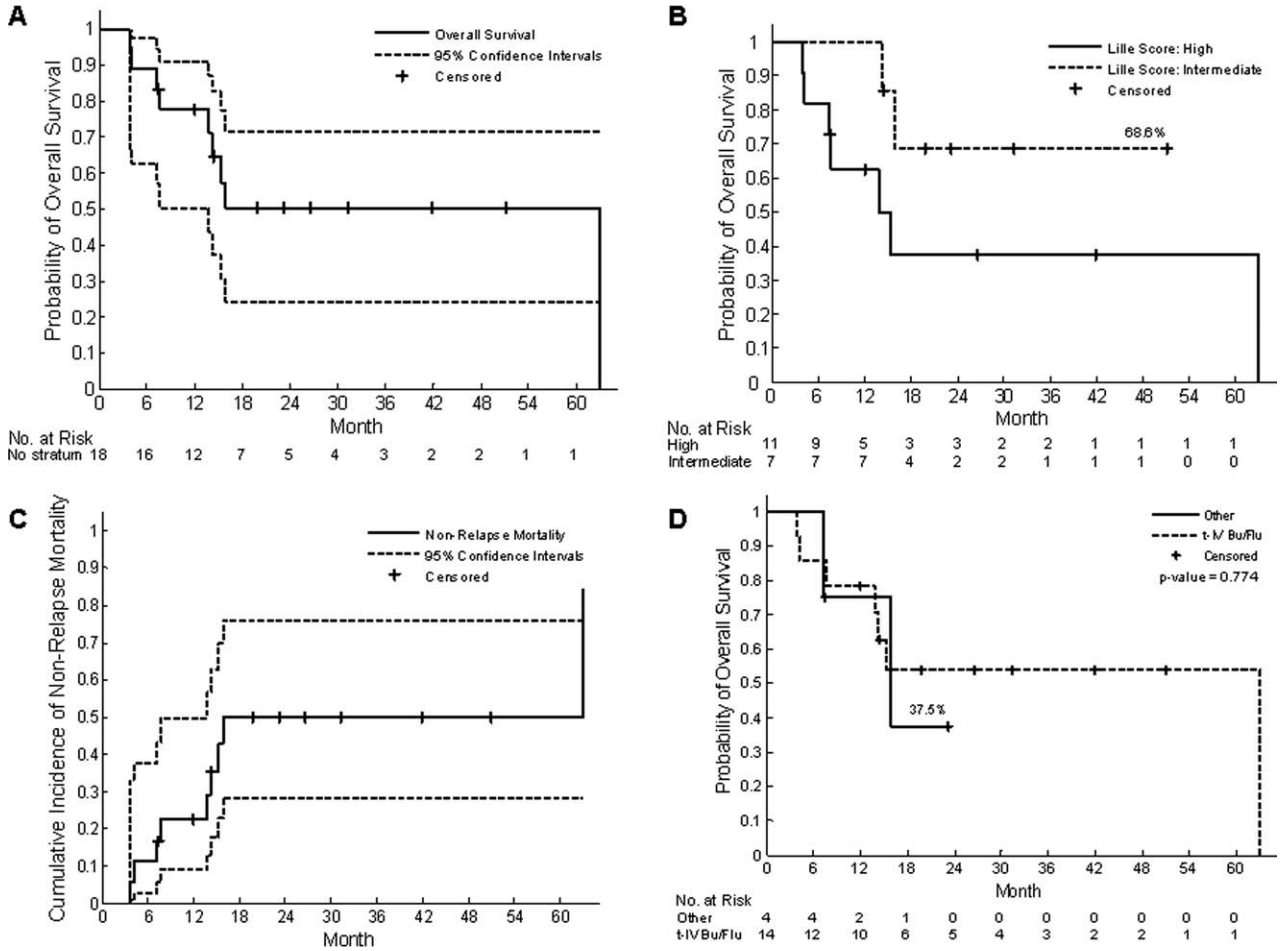

Figure 1. Overall survival after allogeneic stem cell transplantation (Panel A). Impact of Lille risk category on survival (Panel B). Cumulative incidence of nonrelapse mortality (Panel C). Overall survival curves based on conditioning regimens (Panel D).

all patients included Grade 2 and 3 mucositis in 7 (39%) and 11 patients (61%), respectively. Two patients (11%) developed Grade 2 renal failure which was reversible. There was one case of Grade 3 cyclophosphamide-induced hemorrhagic cystitis.

In summary, we describe a single center experience of patients with myelofibrosis who underwent allogeneic HCT with mostly t-IV Bu/Flu regimen. Although limited by the heterogeneity and size of the dataset, this series demonstrates a relatively low graft failure rate in patients with myelofibrosis. This study shows a trend toward increased probability of survival after allogeneic HCT in patients with intermediate Lille risk group compared to those with high risk. The International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) developed a new prognostic scoring system for myelofibrosis, IPSS-MF, which would facilitate therapeutic decision making for patients with myelofibrosis [20]. It was proposed that it would be reasonable to consider allogeneic HCT in patients with intermediate-2 risk category or higher, however, this remains to be validated in a prospective setting and our dataset did not show significant survival associations with IPSS-MF risk categories likely due to small sample size (data not shown).

Reduced toxicity regimens were introduced for patients with myelofibrosis due to low regimen-related morbidity, mortality, applicability to older patients and to those with significant comorbidities. The successful application of such regimens in patients with myelofibrosis were first reported by Hessling et al. [21] and Devine et al. [13]. These preliminary reports suggested that the conditioning regimen was well tolerated and offers effective therapy. The first retrospective analysis of 21 patients with a median age of 54 years, who received various reduced toxicity regimens with matched related donors showed the NRM at 1 year of 10% and the OS at 2.5 years of 85% [11]. Similarly, a prospective study of 21 patients, with a median age of 53 years, who received a conditioning regimen of Bu/Flu and anti-thymocyte globulin (ATG) demonstrated the NRM at 1 year of 16% and OS of 84% at 3 years [12]. The experience was later extended to unrelated donors [12,16,22]. Overall, the most commonly used regimens were Bu/Flu or Flu/Mel based. The median age of patients was, in general, a decade older than those with myeloablative conditioning. The NRM was less than 20%, and the OS was 55–85% range [11–13,21,23]. Our result is comparable in terms of OS; however, it showed considerably higher rates of GVHD and NRM. This may, in part, be explained due to use of unrelated donor sources in half of the cases, high Lille risk scores in the majority and extensive prior treatment history, among others.

IV Bu/Flu regimen has been previously demonstrated safe and effective in myeloid malignancies [17–19], although, pharmacokinetic targeted IV Bu/Flu has not been investigated in large scale randomized studies. Other reported reduced toxicity regimens for patients with myelofibrosis include a combination of low dose busulfan, fludarabine, and ATG [12,14,21–23], as well as Bu/Flu [24]. Recently, the European Group for Blood and Marrow Transplantation (EBMT) reported a prospective multicenter Phase II trial on a busulfan (10 mg kg^{-1})/fludarabine (180 mg m^{-2})-based reduced toxicity regimen in 103 patients with myelofibrosis [15]. The estimated 5-year event-free survival and OS was 51 and 67%, respectively. None of the studies have incorporated consistent pharmacokinetic targeting of busulfan dosing. Our result underscores the feasibility of t-IV Bu/Flu in older patients with myelofibrosis with low morbidity. Pharmacokinetically targeted IV Bu/Flu is a viable option for both related and unrelated donor allografting. Engraftment kinetics is encouraging despite the presence of splenomegaly and myelofibrosis. The regimen has a potential for application to a broader patient population.

Methods

Patients. Eighteen patients were treated with allogeneic HCT for myelofibrosis between February 25, 2000 and December 19, 2008 at Moffitt Cancer Center. The analysis was performed in March 2010. Restaging for mye-

lofibrosis occurred at days +30, +90, +180, +360, and yearly thereafter posttransplant. All patients signed inform consents for a long-term follow-up study of their transplant outcomes, approved by the University of South Florida (USF) Institutional Review Board (IRB). The present retrospective review of patient outcomes was also approved by the USF IRB.

Preparative regimens and GVHD prophylaxis. Fourteen patients received with t-IV Bu/Flu consisting of fludarabine 40 mg m^{-2} IV infused over 30 min on days -6 to -3, followed by IV busulfan 130–170 mg m^{-2} over 3 hr daily on the same days. Busulfan pharmacokinetic samples were obtained on day -6 and analyzed by gas chromatography/mass spectrometry by the University of Pennsylvania clinical toxicology laboratory; the busulfan dose was adjusted for the remaining two doses (on days -4 and -3) to target a median AUC of 5300 (±10%) $\mu m\ min^{-1}$ (range, 3,500–6,000 $\mu m\ min^{-1}$) for each of the 4 days. Antithymocyte globulin (ATG) was administered to one patient with HLA-B mismatched allograft with t-IV Bu/Flu. One other patient received fludarabine 40 mg m^{-2} per day × 4 days and melphalan 70 mg m^{-2} per day × 2 days. Other regimens included busulfan 0.8–1.0 mg kg^{-1} orally every 6 hr for 16 doses and cyclophosphamide 60 mg kg^{-1} day^{-1} IV × 2 days (two patients), and cyclophosphamide 60 mg kg^{-1} day^{-1} IV × 2 days and total body irradiation (1,320 cGy in 11 fractions) (one patient). GVHD prophylaxis consisted of tacrolimus and methotrexate ($n = 11$), tacrolimus plus mycophenolate mofetil ($n = 4$), and a cyclosporine-based regimen ($n = 3$).

Analysis of donor chimerism. All patients except one were evaluated for chimerism at days +30, +90, +180, and +360 after transplantation by polymerase chain reaction (PCR). The sources of material included unsorted BM and PB. In 14 patients (78%), STR-PCR analyses were also performed on cell subsets with CD3 and CD33 positivity. One patient who received Cy/TBI was evaluated for chimerism by fluorescence in situ hybridization (FISH) only on BM.

Endpoint definitions and statistical evaluation. Neutrophil engraftment is defined by the first of three successive days with an absolute neutrophil count (ANC) > 500 μL. Platelet engraftment is defined by the first of three successive days with a nontransfused platelet count >20,000 μL. Primary graft failure is defined as failure to achieve an ANC of 500 μL within 28 days following transplantation for patients who have not undergone a second transplant procedure. Secondary graft failure is defined as decline of an ANC <500 μL after having engrafted that is unresponsive to growth factors and unrelated to effects of medications or infection. Acute GHVD (aGVHD) was scored per modified Glucksberg criteria within 100 days from transplant [25]. Chronic GVHD (cGVHD) was staged per previous standard criteria [26,27]. OS was estimated from date of transplantation using the Kaplan–Meier method [28]. Survival curves stratified by Lille score and donor relation were compared using the log-rank test. Since there was no relapsed patient, nonrelapse mortality (NRM) is equal to 1 – OS.

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