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Neutrophil Elastase Activity as a Surrogate Marker for Neutrophil Extracellular Trap Formation following Hematopoietic Stem Cell Transplantation

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Impaired neutrophil extracellular trap (NET) formation compromises the host defense after engraftment following hematopoietic stem cell transplantation (HSCT) despite adequate neutrophil counts. The aims of the present study were to determine reference ranges for the activity of key enzymes of NET formation—neutrophil elastase (NE) and myeloperoxidase (MPO)—in a healthy population and to unravel the recovery dynamics of NET formation over time following HSCT, along with NE and MPO enzymatic activities. Reference ranges of NE and MPO activity were derived from 50 healthy volunteers. During 2017 to 2018, 11 consecutive pediatric patients undergoing allogeneic or autologous HSCT were recruited at a single referral center for pediatric hemato-oncology. Patients were followed for up to 1 year following engraftment. The mean reference value was 7.5 ± .4 mU for NE activity and 2.17 ± .4 U for MPO activity in the healthy population, and enzymatic activity of MPO was significantly higher in males. At 3 weeks following neutrophil engraftment, all study participants demonstrated extremely low enzymatic NE activity, whereas MPO activity was above the lower normal reference range at all time points. Reduced NE activity corresponded to the inability to form NETs. Neutrophil function improved over time, but partial impairment persisted for 7 months following transplantation. The ability of neutrophils to form NETs was significantly impaired for 3 weeks after engraftment in the setting of HSCT, exposing patients to bacterial infections. NE activity might serve as a surrogate marker for the capacity of neutrophils to form NETs.

INTRODUCTION

Infections constitute a major source of morbidity and mortality following hematopoietic stem cell transplantation (HSCT) [1]. The reduced protection against infections is due mainly to neutropenia and impaired neutrophil function. Studies investigating neutrophil function following HSCT have reported different patterns between autologous and allogeneic transplantations. In autologous setting, respiratory burst and phagocytosis were generally decreased within the first 1 to 3 months; however, phagocytosis was not impaired. In allogeneic setting, respiratory burst and chemotaxis were generally decreased for 4 to 6 months post-transplantation, whereas chemotaxis was severely decreased depending on the occurrence of graft-versus-host disease (GVHD) and use of antithymocyte globulin treatment [2–4]. However, the fate of neutrophil extracellular trap (NET) formation, an additional strategy for neutrophils to eliminate pathogens, has not been fully characterized following HSCT [5–7].

In the process of NET formation, neutrophils release extracellular fibers composed of chromatin and granule-derived proteins that bind and trap gram-positive and gram-negative bacteria as well as fungi and provide a high local concentration of antimicrobial molecules that effectively kill microbes [8]. NET formation might be specifically important in cases of fungi that are too large to be phagocytized [9] or in cases of bacteria, such as *Pseudomonas aeruginosa*, that can circumvent phagocytosis via formation of biofilms [10]. Both are known life-threatening pathogens following transplantation.

The aim of this study was to determine the ability of engrafted neutrophils to form NETs along a 12-month time course following HSCT, as well as the activities of the 2 key enzymes in the NETosis mechanism, neutrophil elastase (NE) and myeloperoxidase (MPO). To compare those activities with normal ones, we studied reference values of NE and MPO activity in 50 healthy individuals for what we believe to be the first time.

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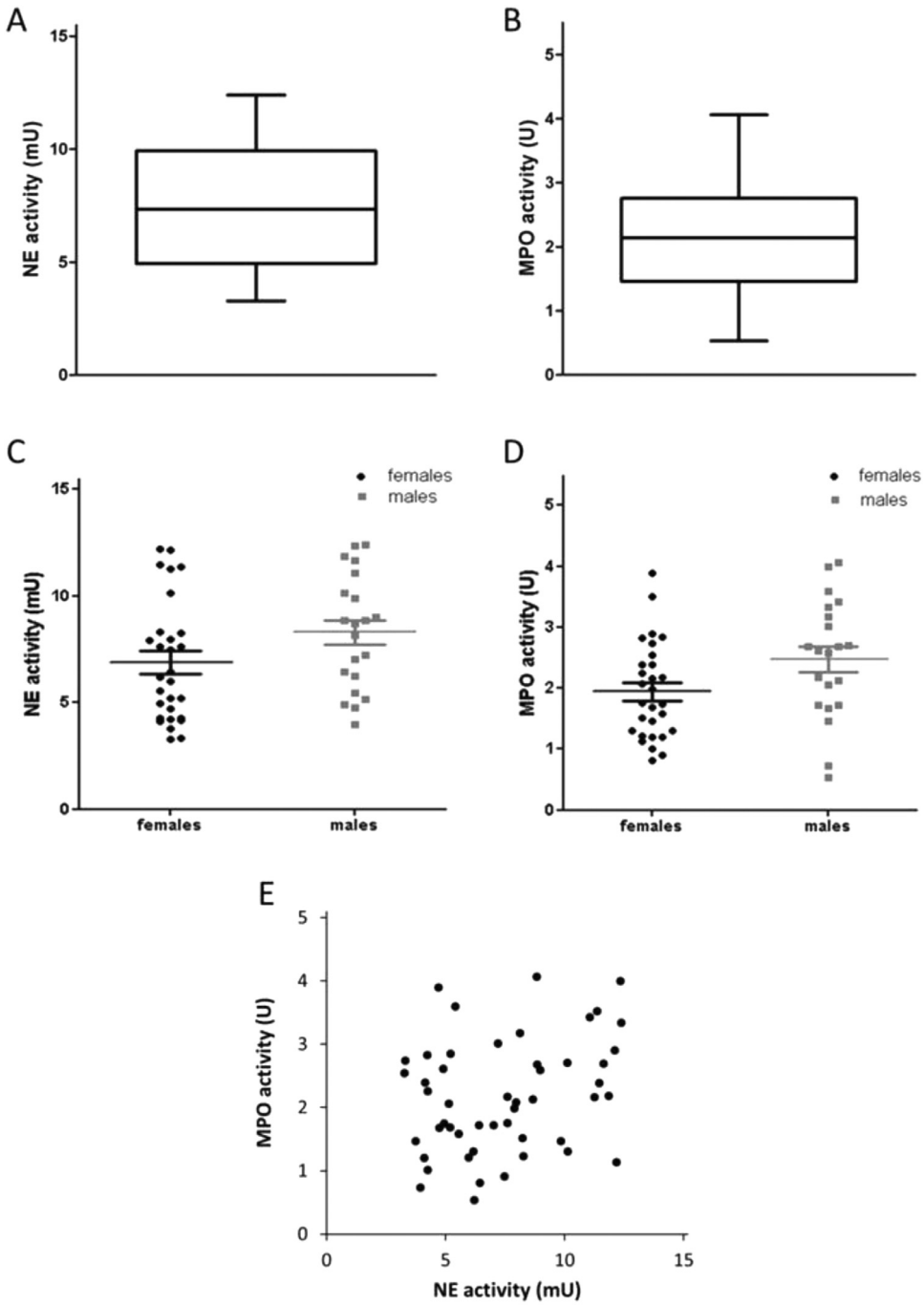

Figure 1. (A and B) Reference ranges for NE activity (A) and MPO activity (B) in healthy volunteers. (C and D) Enzymatic activity of NE (C) and MPO (D) in females and males. (E) No correlation between NE and MPO activities in 50 healthy controls (R^2 = .0799).

METHODS

Study Population

Eleven patients undergoing allogeneic or autologous HSCT were investigated. Blood samples were obtained at several time points following HSCT: at engraftment (neutrophil count $>.5 \times 10^9$ cells/L), and once weekly thereafter until improvement of NET formation was seen. All parents provided informed signed consent in accordance with the Declaration of Helsinki. This study was approved by Institutional Review Board of Sourasky Medical Center Tel Aviv (IRB protocol 920130015).

Control Population

Fifty healthy volunteers (excluding pregnant woman and individuals with active infection or known immunodeficiency) were recruited to establish reference ranges of NE and MPO activity assays. All individuals provided informed consent in accordance with the Declaration of Helsinki.

Materials

Phosphate-buffered saline (PBS) was obtained from Biological Industries (Kibbutz Beit-Haemek, Israel). Ethylene diamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), glucose, phorbol 12-myristate 13-acetate (PMA), and Triton X-100 were all purchased from Sigma-Aldrich (St Louis, MO). Poly-L-lysine solution (.01%) and buffered 4% formaldehyde solution were acquired from Merck (Kenilworth, NJ).

Whole Blood Collection and Neutrophil Isolation

Between 2 and 5 mL of blood was obtained in plastic EDTA tubes. Neutrophils were isolated using the EasySep Direct Human Neutrophil Isolation Kit

Table 1
Patient Demographics

Patient	Age, yr	Background Disease	Sex	Type of HSCT	Source of SC	Donor	$CD34^+$ Cells/kg	Day of Neutrophil Engraftment	Day of Platelet Engraftment	Conditioning Regimen
1	2.75	Epidermolysis bullosa	F	Allogeneic	BM	MSD	11.0	6	No platelet transfusion	Cyclophosphamide 50 mg/kg; fludarabine 200 mg/m^2; ATG (horse) 90 mg/kg; TBI 300 cGy
2	3.33	Epidermolysis bullosa	F	Allogeneic	BM	MRD	8.0	11	No platelet transfusion	Cyclophosphamide 50 mg/kg; fludarabine 200 mg/m^2; ATG (horse) 90 mg/kg; TBI 300 cGy
3	4.75	Rhabdomyo-sarcoma	M	Allogeneic	BM	Haplo	10.0	17	23	Fludarabine 150 mg/m^2; melphalan 100 mg/m^2; cyclophosphamide 30 mg/kg; TBI 200 cGy
4	4.83	Acute lymphoblastic leukemia	F	Allogeneic	PB	MUD	5.0	17	25	VP16 60 mg/kg; TBI 1200 cGy; ATG 45 mg/kg
5	6.83	Acute lymphoblastic leukemia	F	Allogeneic	BM	MRD	9.0	17	27	Cyclophosphamide 120 mg/kg; TBI 1200 cGy
6	18.00	Acute myelogenous leukemia	F	Allogeneic	PB	MUD	5.8	18	24	Busulfan 12.8 mg/kg; fludarabine 160 mg/m^2; ATG Fresenius 20 mg/kg
7	3.50	Desmoplastic small round cell tumor	M	Autologous	PB	NA	10.3	10	18	Treosulfan 36 g/m^2; melphalan 140 mg/m^2
8	8.25	Ewing sarcoma	M	Autologous	PB	NA	5.9	9	11	Treosulfan 36 g/m^2; melphalan 140 mg/m^2
9	14.42	Ewing sarcoma	M	Autologous	PB	NA	3.9	10	29	Busulfan 12.6 mg/kg; melphalan 140 mg/m^2
10	16.42	Ewing sarcoma	M	Autologous	PB	NA	4.6	10	11	Busulfan 18.3 mg/kg; melphalan 140 mg/m^2
11	22.00	Ewing sarcoma	M	Autologous	PB	NA	4.0	10	11	Busulfan 17.4 mg/kg; melphalan 140 mg/m^2

ST indicates stem cells; M, male; F female; PB, peripheral blood; BM, bone marrow; MSD, matched sibling donor; MRD, matched related donor; Haplo, haploidentical; MUD, matched unrelated donor; NA, not applicable; ATG, antithymocyte globulin; TBI, total body irradiation

(Stemcell Technologies, Vancouver, BC, Canada) by immunomagnetic negative selection or by Ficoll-Histopaque gradient centrifugation (Sigma-Aldrich) according to the manufacturer's instructions. The number of isolated neutrophils was measured with Coulter Ac.T diff2 (Beckman Coulter, Brea, CA), and the final concentration was adjusted to 10^7/mL in neutrophil medium, PBS containing 1% glucose, and 1% BSA.

Neutrophil Elastase Enzymatic Activity

A total of 10^5 neutrophils were lysed in .2% Triton X-100 containing PBS solution and then incubated with 500 μM chromogenic peptide elastase substrate (Calbiochem, San Diego, CA) for 90 minutes at 37 °C. Enzymatic activity was measured with an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA) at 415 nm. A calibration curve was set up using various amounts of purified NE (Athens Research & Technology, Athens, GA). A specific amount of purified NE served as a positive control, and a specific NE inhibitor IV (Calbiochem) together with the purified enzyme were served as a negative control each time.

Myeloperoxidase Enzymatic Activity

A total of 10^5 neutrophils were lysed in .2% Triton X-100 containing PBS solution and then incubated with .1 mg/mL O-phenylenediamine (Sigma-Aldrich) and 1 mM H_2O_2 (Sigma-Aldrich) for 20 minutes at room temperature. Enzymatic activity was measured with an iMark Microplate Absorbance Reader (Bio-Rad) at 450 nm. A calibration curve was set up using various amounts of purified MPO (Athens Research & Technology). A specific amount of purified MPO served as a positive control, and 4-aminobenzoic acid hydrazide (Cayman Chemical, Ann Arbor, MI), a specific MPO inhibitor, together with the purified enzyme served as a negative control each time.

NET Induction and Immunostaining

A total of 2×10^5 neutrophils were seeded on coverslips coated with poly-L-lysine and activated by 100 nM PMA for 3 hours at 37 °C and then fixed with 4% formaldehyde solution. Neutrophils were labeled with Sytox Green (Thermo Fischer Scientific, Waltham, MA) and Hoechst 33342 (Sigma-Aldrich) nuclear acid stains. Imaging was performed on an LSM700 laser scanning confocal fluorescence microscope (Carl Zeiss, Tuttlingen, Germany). NET formation was counted manually by checking the morphology of the stained DNA. An intact nucleus with compact DNA was stained with both nuclear dyes and indicated a non-NETotic cell, whereas diffuse DNA stained only with Sytox Green indicated NETotic cells. Between 50 and 100 cells were counted in each sample.

Statistical Analysis

The data are presented as mean ± standard error of the mean. Statistical differences were determined with ANOVA with a Bonferroni post hoc test or Student's *t* test using Prism software (GraphPad Software, La Jolla, CA). Linear regression was used to calculate correlation data. Statistical significance was determined at $P < .05$. Reference ranges were measured and calculated from measurements of 50 healthy volunteers using the quartile method.

RESULTS

Reference Values for NE and MPO Activity

Twenty-one males and 29 females (median age, 36 years) volunteered to provide blood samples for analysis. The enzymatic activity of NE ranged between 3.3 and 12.4 mU (mean. 7.5 ± .4 mU) (Figure 1A), and the reference range of MPO was .53 to 4.06 U (mean, 2.17 ± .4 U) (Figure 1B), all normalized to 10^5 neutrophils. Enzymatic activities were higher in the males compared with the females (Figure 1C and D), but the difference was significant only for MPO ($P = .062$ for NE and $P = .042$ for MPO). There was no correlation between the activities of the 2 enzymes ($R^2 = .0799$) (Figure 1E).

Recovery Dynamics of NET Formation following HSCT

Eleven consecutive patients (5 girls and 6 boys; median age, 6.8 years; range, 2 to 22 years) were enrolled into the study. Patient characteristics are summarized in Table 1. The median duration of follow-up was 30 weeks (range, 5 to 69 weeks). Neutrophil engraftment occurred at a median of 10 days. Eight patients (73%) developed fever during neutropenia, and 3 patients (27%) had documented bacterial infection; all underwent allogeneic HSCT (Table 2). Bacterial infection after engraftment occurred in one-third of the patients undergoing allogeneic HSCT but in none of the patients undergoing

Table 2
Infectious Episodes and Treatment

Patient	Infectious Event	Time of Infection	Neutrophils at Time of Infection, $\times 10^3/\mu$L	Treatment
1	*Staphylococcus aureus* bacteremia	Day -1	2.4	i.v. orbenil
	CMV reactivation	Day +18	2.2	Ganciclovir
2	MSSA bacteremia	Day -2	6.4	i.v. orbenil + rifampin
	Fever	Day +76	.4	i.v. Tazocin + amikacin
	Klebsiella pneumoniae	Day +112	2.9	i.v. ceftriaxone
3	Fever and neutropenia	Day +4	0	i.v. Tazocin + amikacin
	Fever and perianal mucositis	Day +12	0	i.v. Tazocin +amikacin
	CMV reactivation	Day +22	3.4	i.v. foscarnate
	CMV reactivation	Day +61	1.3	i.v. foscarnate
	EBV reactivation	Day +111	2.1	i.v. MabThera
4	Fever and neutropenia with *Pseudomonas* bacteremia	Day +2	0	i.v. meropenem + amikacin; later Tazocin + amikacin
	CMV reactivation	Day 0	.1	i.v. foscarnate
	Bacteroides fragilis bacteremia	Day +12	0	i.v. Flagyl
	Staphylococcus aureus bacteremia	Day +32	7.8	i.v. vancomycin
	Enterococcus faecalis urinary tract infection	Day +32	7.8	i.v. meropenem
5	Fever and neutropenia	Day +6	0	i.v. Tazocin + amikacin
	Fever	Day +35	3.4	i.v. Tazocin + amikacin
6	Fever and neutropenia	Day +9	0	i.v. Tazocin, amikacin, ciprofloxacin, + anidulafungin
	BK virus hemorrhagic cystitis	Day + 16	0	i.v. cidofovir
7	Fever	Day -1	3.5	i.v. tazocin, amikacin, + vancomycin
	Fever	Day +7	0	i.v. Tazocin + amikacin
8	Colitis, diarrhea	Day +5	0	i.v. Tazocin, amikacin, + Flagyl
9	Fever and neutropenia; mucositis	Day +6	0	i.v. Tazocin + amikacin
10	Fever and neutropenia	Day +5	.3	i.v. Tazocin + amikacin
	Fever	Day +9	.2	i.v. Tazocin + amikacin
	Hidradenitis	Day +23	3.5	i.v. Augmentin
11	Fever and neutropenia; mucositis	Day +5	0	i.v. Tazocin + amikacin

CMV indicates cytomegalovirus; MSSA, methicillin-susceptible *Staphylococcus aureus*; EBV, Epstein-Barr virus.

autologous HSCT (Table 2). All 11 patients demonstrated significantly reduced NE activity (.6 to 2.1 mU) by the first 3 weeks following neutrophil engraftment compared with later time points ($P < .05$) (Figure 2A). NE activity reached the lower normal reference range at 4 weeks postengraftment. NE activity increased throughout the entire monitoring period, with a significant difference between each time point ($P < .0001$). MPO activity was above the lower normal reference range at all time points (Figure 2B).

The patients' percentage of NETosis following PMA activation was compared to that of the controls (Figure 2C). Similar to NE activity, NETosis was negligible for 2 weeks postengraftment (Figure 2C and 2D) and became evident at the third week after engraftment. At 4 weeks postengraftment, when NE activity reached the lower normal reference range, mean NETosis was 36.7 $\pm$ 6.3%, compared with 65.6 $\pm$ 3.2% for the controls ($P = .005$). As with NE activity, NET formation increased over time, reaching an average of 42.1 $\pm$ 4.8% by 30 weeks following HSCT (Figure 2C and D). NE correlated linearly to NETosis with an R^2value of .979 (Figure 3A), whereas the linear correlation with MPO had an R^2value of .709 (Figure 3B). Platelet number also increased with time and showed linear correlation with NETosis, with an R^2value of .841 (Figure 3C).

NETosis was significantly lower following allogeneic HSCT compared with autologous HSCT ($P = .006$; Figure 3D), and it was significantly lower in patients with bacteremia compared with those without bacteremia ($P = .005$; Figure 3E). Male patients had significantly higher NET formation capacity than female patients ($P = .02$; Figure 3F). NE and MPO values did not differ significantly after allogeneic HSCT compared with after autologous HSCT (P = .219 and .063, respectively).

DISCUSSION

In the setting of HSCT, where infections pose a threat to the patient's life, the period of time in which the patient is most vulnerable to infections must be realized. The results of this study demonstrated that neutrophils have impaired NETosis in the early weeks of engraftment following HSCT, thereby rendering the patient at risk for bacterial infections. Importantly, patients who underwent allogeneic HSCT had significantly lower NETosis compared with those who underwent autologous HSCT, supporting the higher incidence of severe infections in allogeneic HSCT [11]. Although most of infections occurred during the period of neutropenia before engraftment, 33% of the patients who underwent allogeneic HSCT had severe bacterial infections while not neutropenic (days +112 and +32, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis*), compared with no patients in the autologous HSCT setting. The only infection occurring after engraftment (on day +23) that necessitated antibiotic treatment in the autologous HSCT setting was uncomplicated hidradenitis, without documented bacteremia. This might be explained by the lower NETosis in the allogeneic HSCT recipients. We also demonstrated a linear correlation between NE activity and NET formation capacity, but not between MPO activity and NET formation capability, suggesting that NE activity can serve

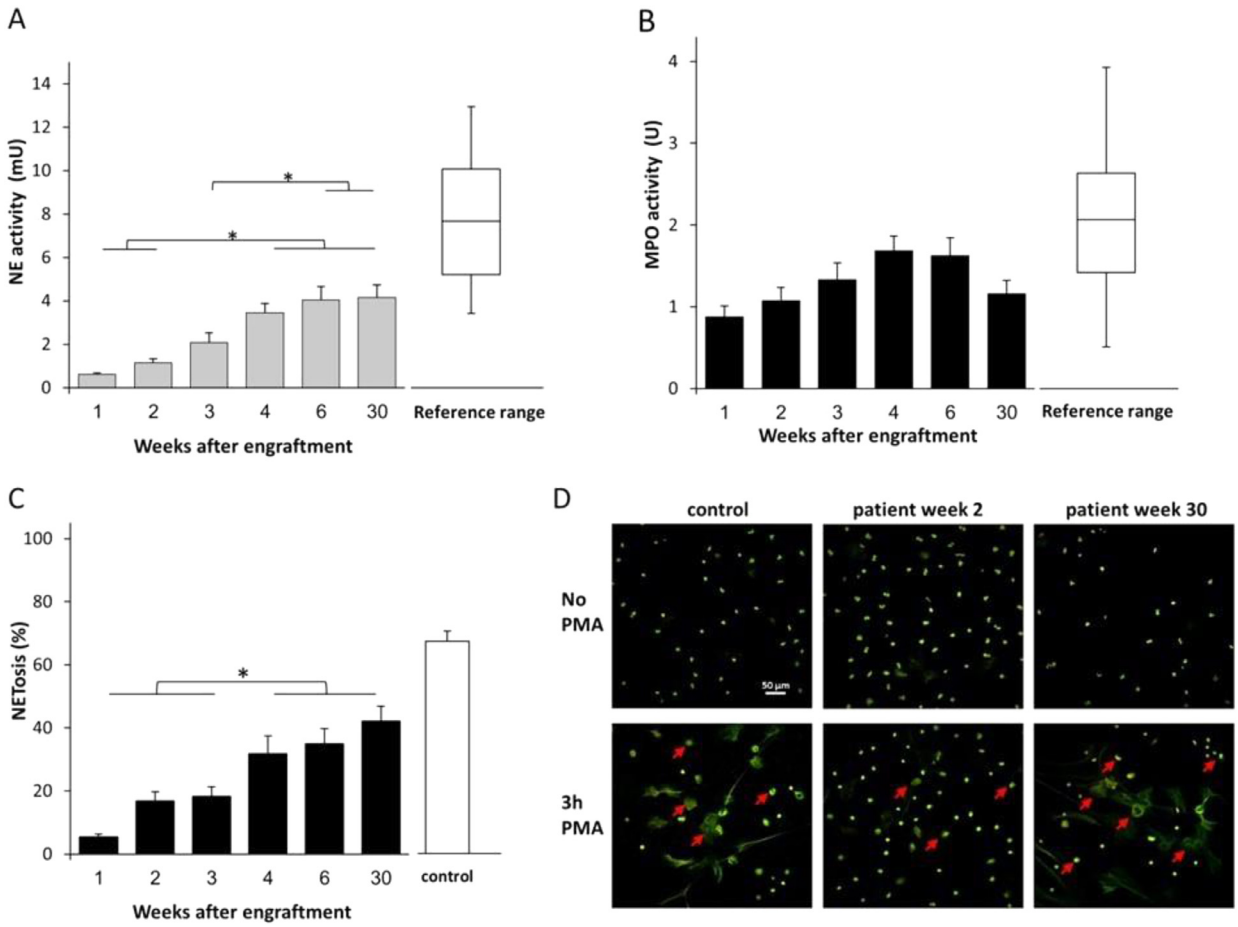

Figure 2. NE and MPO activity and NET formation following HSCT. (A) Average of NE activity. (B) Average of MPO activity. (C) Average of NET formation (n = 11; $n_{control}$ = 10) during 30 weeks after HSCT. (D) Representative images from a healthy control and from a patient following HSCT at week 2 and at week 30. Negative controls are without PMA activation compared to the PMA-activated samples. Red arrows represent cells showing NETosis.

as a surrogate marker for NET formation. Moreover, because there are no published data on NE and MPO activities in healthy individuals, we quantified the activities of these enzymes in 50 healthy adult volunteers to establish reference ranges and found that males tend to have higher MPO and NE activities compared with females.

Recent evidence has suggested impaired NETosis following HSCT, but no data were available on the longitudinal recovery of NETs formation over time. Domingo-Gonzalez et al [5] found deficient NET formation in 12 adult patients undergoing allogeneic HSCT, but no information on the timing of post-transplantation NETs analysis was provided. Glenn et al [6] studied NETs formation during and after HSCT in 6 pediatric patients at 3 time points ranging between 14-199 days (pre-transplantation, pre-engraftment, and post-engraftment) and found decreased NETosis compared to controls at all time points, but there was no documentation of improvement in of NETosis over time. Kent et al [7] also analyzed neutrophil function in children following HSCT and demonstrated a significantly decreased NE release, which normalized by 6 months following allogeneic HSCT. This corresponds to our finding of low NE enzymatic activity and impaired NETosis in the same setting. Our results of a significant correlation between reduced NE activity and the inability to form NETs also corroborates with the mechanism described by Papayannopoulos et al [12], in which NE plays a key role in NETosis.

The emerging question is what causes the impairment of NE activity resulting in decreased NETosis post-HSCT. Studies performed in neonates, in whom NET formation is reportedly weaker [13,14], demonstrated a family of endogenous inhibitors [15]. The family, termed nNIF-related peptides (NRPs) after the first identified peptide (NET-inhibitory factor; nNIF), also consists of cancer-associated SCM recognition, immune defense suppression by serine protease protection peptide (CRISPP), and a 44-amino acid carboxy terminus cleavage fragment of α1-antitrypsin (A1AT), A1ATm358 [15]. nNIF, as well as CRISPP, were found to inhibit NETosis via repression of PAD4 activity and nuclear histone citrullination. Nevertheless, NRPs did not directly inhibit NE activity in the in vitro assays. Thus, it is plausible that the NE down-regulation seen in our study stems from a parallel pathway for inhibition of NETosis. However, the interplay between NE and PAD4 activation and their role in NETosis mechanism merits further study.

There are several ways to detect and quantity NETosis. Florescence microscopy is the widely accepted method for visualizing and quantifying of NETs [16]. However, this method is subjective and time-consuming and yields low numbers of analyzed polymorphonuclear cells per sample. Interest has increased in identifying NETs using flow cytometry techniques [17,18], but these assays remain mostly premature and, in our experience, suffer from technical difficulties. On the other hand, the NE activity assay is simple, quick,

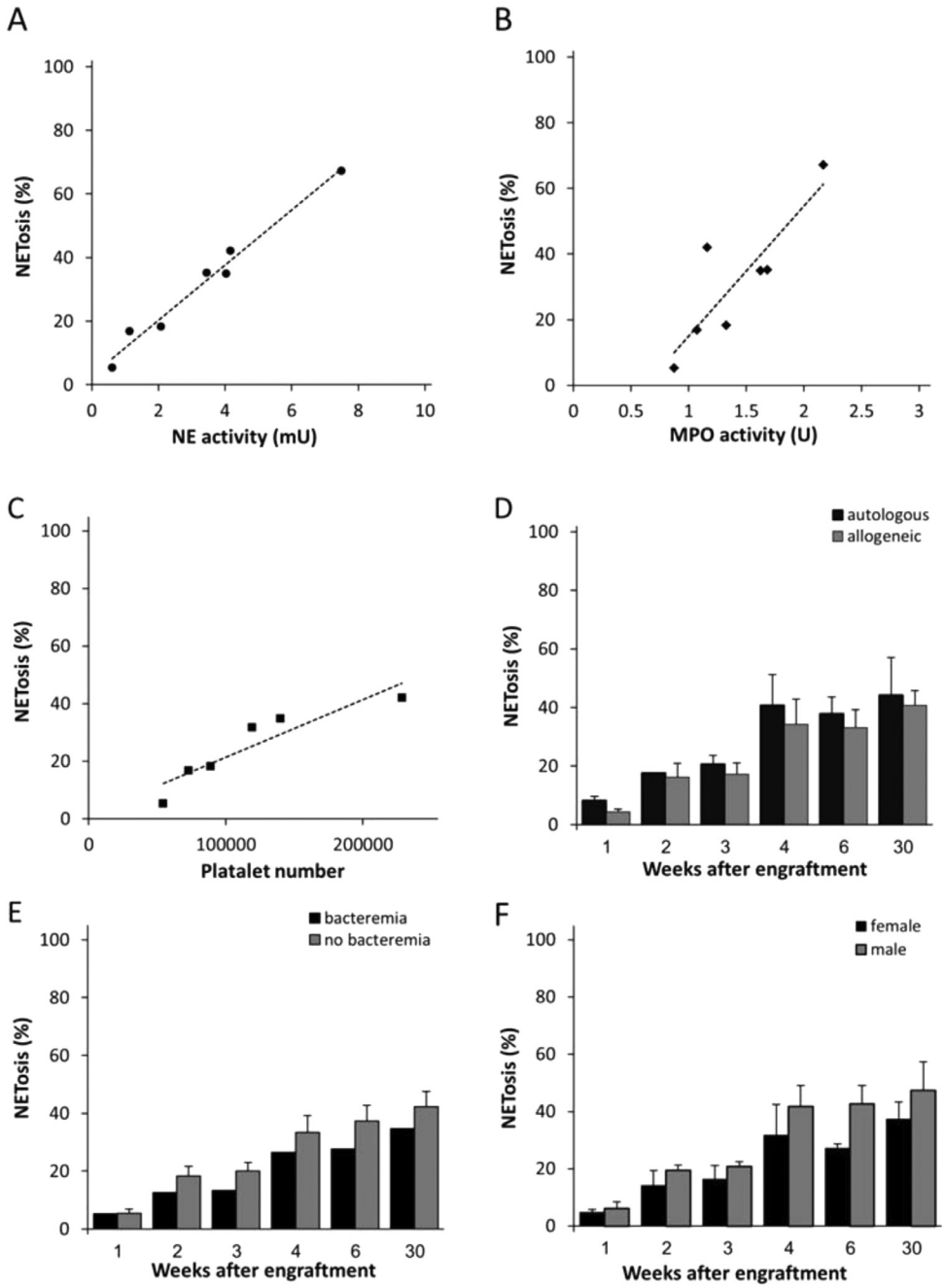

Figure 3. Correlation between enzymatic activities of NE and MPO and platelet number with NETosis; course of NETosis in different patient groups. (A) Linear correlation between NE activity and NET formation (R^2 = .979). (B) Linear correlation between MPO activity and NET formation (R^2 = .709). (C) Linear correlation between platelet number and NET formation (R^2 = .841). (D) NETosis in allogeneic HSCT recipients and autologous HSCT recipients. (E) NETosis in patients with bacteremia and those without bacteremia. (F) NETosis in female and male patients.

readily reproducible, and cheap. If the linear correlation between NE activity and NETosis can be further validated in another patient cohort, this assay could be used to estimate NETosis capacity in the clinical setting. Interestingly, the normal values of NE and MPO activities in the healthy volunteers were higher in the males. This might be explained by the known influence of sex hormones on neutrophil functions, such as chemotaxis, and adds a layer of complexity to the scarce data on the aspect of sexual dimorphism of neutrophil biology [19].

Limitations of this work include the relatively small number of children in the study group. In addition, the patients and diseases were heterogeneous, with one-half involving allogeneic HSCT and the other half involving autologous HSCT. NETosis and enzymatic activities were assessed only after transplantation, and not before conditioning. Nevertheless, the pattern of increasing NETosis and NE activity over time was identical in all patients irrespective of treatment regimen. A larger cohort is needed to delineate the exact contribution of impaired NETosis to the risk of bacterial infection and to correctly predict impaired NETosis by measuring NE activity.

The process of NET impairment and recovery post-HSCT may serve as a human model enabling exploration of the underlying mechanisms responsible for NET formation and

especially the role of NE. Understanding the mechanism of NET recovery post-transplantation might have important therapeutic implications, leading to the development of specific drugs that could improve NET formation, infection control, and post-transplantation morbidity and mortality.

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