

## Post-transplant events

# A noninvasive oral rinse assay to monitor engraftment, neutrophil tissue delivery and susceptibility to infection following HSCT in pediatric patients

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### Summary:

The time interval between neutrophil tissue delivery and blood confirmed engraftment following hematopoietic stem cell transplantation (HSCT) may serve as an indicator of patient susceptibility to infection. Using an oral rinse protocol, we studied neutrophil tissue delivery kinetics and its relationship to clinical parameters post-HSCT in 29 pediatric patients. Oral neutrophil counts were compared to circulating neutrophil levels, oral mucositis scores and infection-related febrile episodes after engraftment. Blood engraftment (BE) is currently defined by a blood neutrophil count of  $\geq 0.5 \times 10^9/l$ . We defined oral engraftment (OE) as the day neutrophils returned in the mouth post-HSCT ( $\geq 0.25 \times 10^4/ml$  oral neutrophils in the rinse sample). We found that neutrophils reappeared  $6.3 \pm 3.9$  s.d. days earlier in the mouth than in the circulation enabling us to identify successful engraftment almost 1 week sooner than using blood count values alone. Furthermore, the time-span between OE and BE was inversely related to the number of infection-related febrile episodes post-BE. We conclude that monitoring the timing of neutrophil tissue delivery through a rapid oral rinse may yield important insights into the biology of neutrophil recovery during and after engraftment and the factors associated with neutrophil tissue recruitment.

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there are currently no noninvasive methods available to assess accurately this important end point. Blood counts are obtained routinely from HSCT patients treated with intense myelosuppressive conditioning regimens to monitor engraftment and the onset and resolution of neutropenia. However, some HSCT patients present with an ANC that suggests engraftment, but continue to experience life threatening, prolonged or recurrent infection.<sup>5</sup> Clearly, ANC measures the return of the neutrophils to the circulation but does not provide any information on neutrophil function and specifically, their ability to reach peripheral sites of microbial challenge.<sup>6</sup> Understanding the timing of neutrophil recovery kinetics following HSCT may allow for insights into neutrophil and engraftment biology.

We have validated a noninvasive oral rinse assay to potentially help in the management of pediatric patients undergoing HSCT. The human mouth has a constant bacterial presence that is kept under control in part by a constant influx of neutrophils from surrounding periodontal tissues.<sup>7</sup> The oral rinse assay enables a noninvasive assessment of neutrophil tissue delivery by measuring the level of neutrophils in oral tissues in patients recovering from HSCT. We outline here the insights gleaned from the noninvasive oral rinse assay used to assess neutrophil recovery in pediatric patients undergoing HSCT. We describe here how this model may allow us to begin to understand the factors involved in neutrophil engraftment, neutrophil tissue delivery biology and susceptibility to infection in patients with neutrophil-related disorders.

### Methods

#### Study subjects

A total of 29 pediatric HSCT patients (20 males, nine females) between 7 and 19 years of age were enrolled in this study at the Hospital for Sick Children (HSC) in Toronto, Canada, following procurement of informed consent. This protocol was approved by the institution's Research Ethics Board. The patients were treated with preparatory regimens tailored to their basic disease. All patients received myeloablative preparative therapy excluding one patient initially diagnosed with Diamond Blackfan anemia who received a reduced intensity chemotherapy regimen. Patients initially diagnosed with Fanconi anemia were treated with a fludarabine-based protocol. Methotrexate

Myeloid engraftment following hematopoietic stem cell transplantation (HSCT) has conventionally been defined as the first of three consecutive days the patient presents an absolute neutrophil count (ANC) of  $0.5 \times 10^9/l$  or more.<sup>1–4</sup> Although it would be ideal to be able to monitor routinely the return of white blood cells to tissues following HSCT,

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and cyclosporine were administered to the patients for graft-versus-host disease (GVHD) prophylaxis. The indication for HSCT included non-malignant marrow failure (idiopathic aplastic anemia, Fanconi anemia, Diamond Blackfan anemia, thalassemia major, myelodysplastic syndrome) and leukemia (acute lymphoblastic (ALL) and nonlymphoblastic leukemia (ANLL), acute myelogenous leukemia (AML)) primarily treated with allogeneic bone marrow transplants, lymphoma (Hodgkin's (HL) and non-Hodgkin's lymphoma (NHL)) and solid tumors (neuroblastoma, Wilm's tumor and Ewing's sarcoma) treated with autologous peripheral blood stem cell transfusions. Among the marrow failure patients, Patients 18 and 24 were treated with allogeneic peripheral blood stem cell infusions, and Patient 20 received cord blood cells (Table 1). Patients were recruited at the pre-transplant assessment, 1–2 weeks prior to their HSCT.

### Cell collection

The major component of this project was the use of a brief oral rinse to collect neutrophils. Patients at the HSC Bone

Marrow Transplant Unit rinse daily with a sodium bicarbonate rinse to aid in oral cleansing. In order to minimize patient discomfort, we had the patients rinse with 10 ml of the same bicarbonate solution for 30 s. This assay has been validated and used previously as described.<sup>8,9</sup> The rinse samples were immediately collected and transported to the laboratory where the cells underwent quantification and analysis.

### Sample collection frequency

Oral rinses were collected prior to beginning conditioning therapy. This served as a baseline measure of neutrophil levels and also allowed patients to gain familiarity with the rinse procedure. Washings were then collected at approximately the same time in the mornings and every 1–2 days.

Some patients refused to perform oral care due to nausea or when their oral mucositis was most severe. A pause in the collection of samples during this phase did not adversely affect the study since oral neutrophil levels were followed longitudinally, and a gap of 1–3 rinses did not

**Table 1** Patient characteristics and findings

Patient	Diagnosis	BE day	OE day	BE-OE	Fevers post-BE	Mucositis score day OE-2	Mucositis score day OE+2
<i>Leukemia group means (n = 11)</i>							
		<i>15.6</i>	<i>9.9</i>	<i>5.7 ± 4.0 s.d.</i>	<i>1.27</i>	<i>2.4</i>	<i>1.6</i>
1	ALL	26	13	13	0	NA	NA
2	AML	20	9	11	0	3	2
3	ALL	19	9	10	0	1	2
4	ALL	16	9	7	0	2	1
5	ANLL	13	9	4	1	1	3
6	AML	15	11	4	4	4	1
7	ALL	11	8	3	1	NA	NA
8	ALL	15	12	3	3	NA	NA
9	ALL	15	13	2	4	3	1
10	ALL	10	9	1	1	NA	NA
11	ALL	12	7	5	0	3	1
<i>Lymphoma group means (n = 6)</i>							
		<i>9.5</i>	<i>5.7</i>	<i>3.8 ± 1.2 s.d.</i>	<i>0</i>	<i>1.5</i>	<i>1</i>
12	HL	10	5	5	0	1	0
13	HL	10	5	5	0	2	1
14	HL	8	4	4	0	1	2
15	HL	10	6	4	0	NA	NA
16	HL	9	6	3	0	NA	NA
17	NHL	10	8	2	0	2	1
<i>Nonmalignant group means (n = 8)</i>							
		<i>20.3</i>	<i>10.7</i>	<i>9.6 ± 4.5 s.d.</i>	<i>0.14</i>	<i>2.3</i>	<i>1.5</i>
18	Fanconi anemia	—	—	—	—	NA	NA
19	Idiopathic MDS	26	9	17	0	3	2
20	Fanconi anemia	22	9	13	0	3	2
21	Aplastic anemia	21	11	10	0	NA	NA
22	Aplastic anemia	23	14	9	0	0	1
23	Aplastic anemia	23	14	9	0	NA	NA
24	Diam.Black.anemia	12	6	6	0	NA	NA
25	Thalassemia	15	12	3	1	3	1
<i>Solid tumors group means (n = 4)</i>							
		<i>9</i>	<i>6.5</i>	<i>5.5 ± 1.7 s.d.</i>	<i>0.25</i>	<i>2.3</i>	<i>1.5</i>
25	Neuroblastoma	12	5	7	0	2	1
26	Neuroblastoma	9	3	6	0	2	1
27	Ewing's sarcoma	10	7	3	1	1	1
28	Wilm's tumor	5	11	6	0	4	3
<b>Total means (n = 28)</b>							
		<b>14.5</b>	<b>8.7</b>	<b>6.3 ± 3.9 s.d.</b>	<b>0.57</b>	<b>2.2</b>	<b>1.4</b>

Table representing the day of blood neutrophil engraftment (BE), the day of oral neutrophil engraftment (OE), the difference between them (BE-OE), the number of infection-related febrile episodes after BE, and the mucositis scores taken 2 days before and 2 days after OE. In addition to the overall means, we show specific means for sub samples of diseases (in italics) (leukemia, lymphoma, nonmalignant marrow failure and solid tumor patients). NA – patients never developed mucositis. Patient 18 never engrafted.

dramatically affect our ability to interpret the results. Patient oral neutrophils were monitored over the duration of their stay in the ward.

### Cell analysis

Oral rinses underwent centrifugation at 2500 rpm for 15 min at 21°C (Hettich Rotina 35R, Albertville, MN, USA) within 1 h of sampling. The cell pellets were resuspended in 1.0 ml of Hanks' balanced salt solution without phenol red and 2.0 µg/ml of acridine orange (3,6-bis[*N,N*-dimethylamino]acridium chloride hemi[zinc chloride salt], Sigma, St Louis, MO, USA), which gives a very characteristic fluorescent staining pattern in neutrophils facilitating manual counting. The suspension was then shielded from light at 37°C for 15 min. The cells were resuspended before transferring 10 µl onto a hemacytometer (Reichert, Buffalo, NY, USA) for visual examination using a fluorescence microscope (Nikon, Mississauga, ON, Canada). Neutrophils were easily differentiated from the abundant epithelial cells by their characteristic multilobulated nuclei and shape (Figure 1). Oral neutrophil (ON) counts were obtained from four fields on the hemacytometer (noted as ON/4) and the concentration of these cells in the original patient rinse sample was calculated (expressed as ON/ml). FITC-conjugated mouse anti-human CD3 antibodies (Cedarlane Labs Ltd., Toronto, ON, Canada) were used to identify T lymphocytes in the oral rinse samples of Patients 9 and 18 (data not shown).

### Blood neutrophil counts

Peripheral blood neutrophil counts are routinely analyzed on Mondays, Wednesdays and Fridays at the HSC Department of Pediatric Laboratory Medicine and at times when the total white blood cell count was equal to or greater than  $0.5 \times 10^9/l$ . They are determined by a Coulter

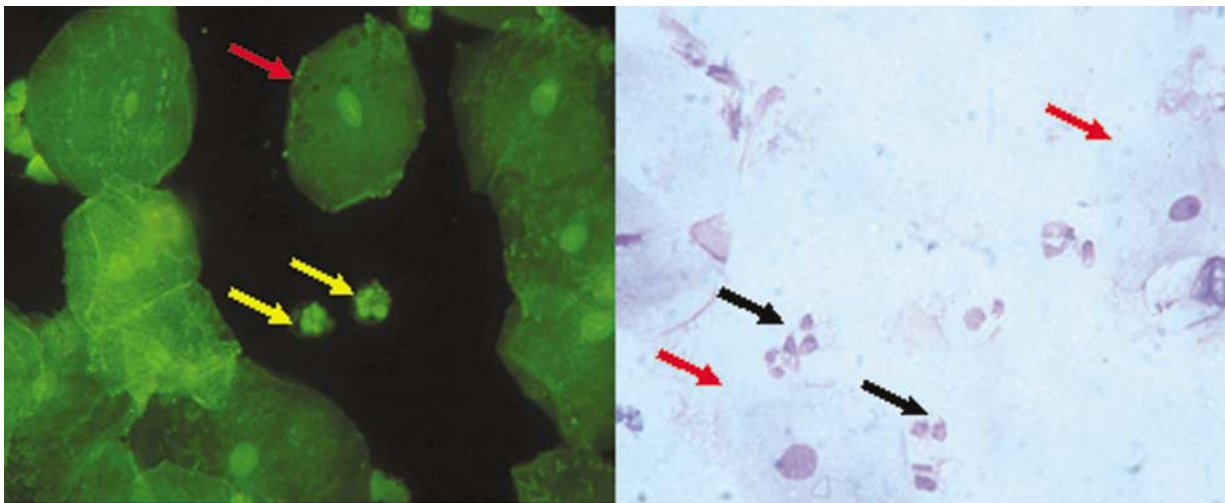
particle counter (Beckman Coulter Inc., Fullerton, CA, USA). Blood smears are also prepared daily and stained using an automated method with Wright–Giemsa. When deemed necessary for data analysis, ANC values for samples collected on Tuesdays, Thursdays and weekends were calculated by a single observer (CC) from leukocyte differentials and total white blood cell counts. At times where severe leukopenia did not permit the measurement of an ANC, we assumed it to be  $\leq 0.1 \times 10^9/l$  to facilitate analysis.

### Clinical parameters and mucositis scoring

Longitudinal monitoring of oral neutrophil levels was compared to circulating neutrophil levels and clinical outcome measures including oral mucositis severity score and febrile episodes monitored by daily maximum body temperatures. Mucositis was graded using the Sonis Mucositis Scoring System.<sup>10</sup> Scoring was performed by the same individual for the entire study period (CC). Infection susceptibility was determined by the number of febrile episodes ( $\geq 38^\circ\text{C}$ ) following blood confirmed engraftment caused by blood culture verified infection. A febrile episode begins with a temperature  $\geq 38^\circ\text{C}$  and ends when the temperature falls below this value for more than a day.

### Statistical analysis

Data included observations made on patients following HSCT and only on patients in whom engraftment occurred. The outcomes of primary interest were: The difference in the timing of engraftment as defined by oral neutrophil concentrations (ON) and blood neutrophil concentrations (BN), the number of infection-related febrile episodes after blood neutrophil engraftment (BE) and mucositis scores 2 days before and after oral neutrophil engraftment (OE). Data are summarized as mean  $\pm$  s.d. To analyse the data



**Figure 1** Fluorescent and light micrographs of oral rinse samples primarily consisting of neutrophils (yellow and black arrows) and epithelial cells (red arrow). Original magnification  $\times 40$  for all panels. (Left) Oral rinse sample stained with 3,6-bis[*N,N*-dimethylamino]acridium chloride hemi[zinc chloride salt], Sigma, St Louis, MO, USA) and viewed using fluorescent microscopy. (Right) Cytospin preparation of an oral rinse sample stained using the Wright–Giemsa method. Neutrophils are easily differentiated from the abundant epithelial cells by their characteristic multilobulated nuclei, shape and size.

statistically, Student's *t*-tests were used and differences were considered significant at  $P \leq 0.05$ .

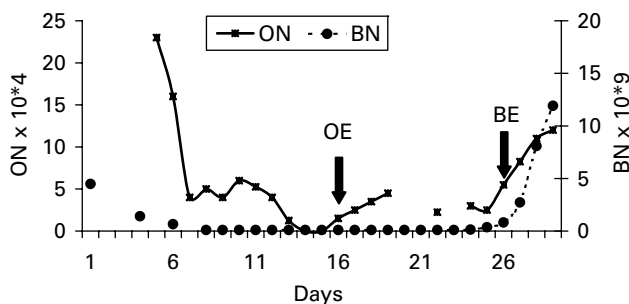
## Results

### Monitoring engraftment with oral neutrophil levels

As expected, all our subjects became severely neutropenic with both ON and BN being undetectable following the intense conditioning regimen. Using a 30 s oral rinse, we were able to monitor neutrophil tissue delivery to the oral cavity and to correlate it with the kinetics of neutrophil return to the circulation (Figure 2). OE was defined as  $\geq 0.25 \times 10^4/\text{ml}$  ON per rinse, which corresponds to visualization of at least one ON per four hemacytometer fields. Every patient ( $n = 28$ ) who presented with  $0.25 \times 10^4/\text{ml}$  ON per rinse for two consecutive days post-HSCT engrafted as per the conventional definition of engraftment. For the 28 patients with neutrophils recovered in the oral cavity post-HSCT, they were detected in the mouth  $6.25 \pm 3.92$  s.d. days earlier than in the blood (ANC  $0.5 \times 10^9/\text{l}$ ). The mean number of days between OE and BE was significantly greater than 0 for all patients ( $t = 8.4331$ , 27 d.f.,  $P \ll 0.0001$ ). This was also true when the patients were sorted by initial diagnosis: 11 leukemia patients ( $t = 4.7760$ , 10 d.f.,  $P = 0.0004$ ); six lymphoma patients ( $t = 8.0319$ , 5 d.f.,  $P = 0.0002$ ) and the seven non-malignant marrow failure patients ( $t = 5.5769$ , 6 d.f.,  $P = 0.0007$ ) (Table 1). In Patient 18, neutrophils were never detected in the oral rinses, and she never engrafted following three separate stem cell transfusions. However, on days 5 and 15 following her second transfusion, we detected T lymphocytes, which normally are not present in our oral rinse samples. T lymphocytes were also identified in Patient 9 prior to his diagnosis of GVHD. In this patient, although ON were detected previous to BE, they were outnumbered by T lymphocytes from day 36 post-HSCT onward. Patient 9 passed away from complications related to severe GVHD.

### Infection and timing of oral engraftment

As expected, all our patients became febrile at least once during their treatment due to compromised host defenses.

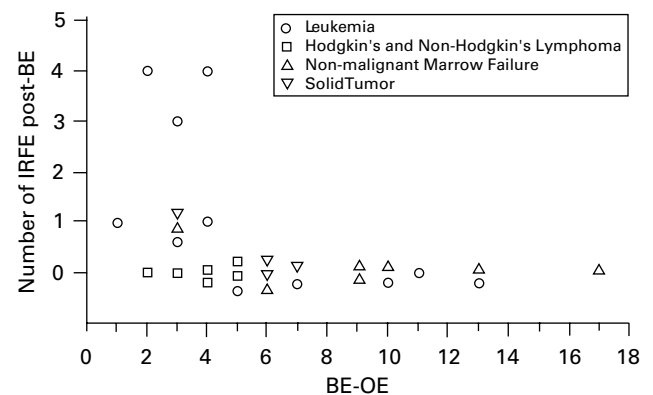


**Figure 2** Sample plot depicting ON and BN counts over time in a pediatric leukemia patient (Patient 3) during HSCT treatment. Days of OE (ON =  $0.25 \times 10^4/\text{oral rinse}$ ) and BE (ANC =  $0.5 \times 10^9/\text{l}$ ) are shown. In this patient, HSCT occurred on day 7, OE on day 16 and BE on day 26. We predicted engraftment 10 days before ANC rose to  $0.5 \times 10^9/\text{l}$ .

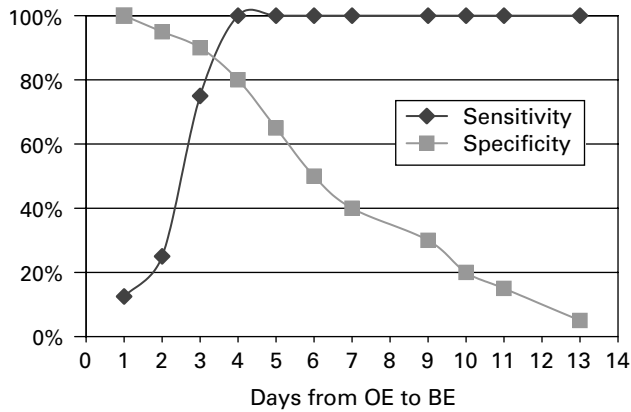
We examined the changes in ON and BN on days before and after the first fever spike and on days before and after resolution of febrile neutropenia. There was no evidence of a relationship between either of these sets of variables in our patient population (data not shown). We then studied the relationship involving the time interval between OE and BE (BE-OE) and the number of fever episodes caused by infection after BE (Figure 3). Consecutive febrile episodes are differentiated by the microbiological profile determined from blood cultures. The mean number of days between OE and BE for the 20 patients with no infection-related fever episodes post-BE compared to the mean of the eight patients with one or more infection-related fever episodes post-BE was significantly higher ( $t = 5.0900$ , d.f. = 24,  $P = 0.00002$ ) (Table 1). Excluding the lymphoma patients (none of whom developed infections post-BE) from this data set revealed an even higher level of statistical significance ( $t = 6.4704$ , d.f. = 16,  $P = 0.000004$ ). The lymphoma patients were all treated with less intense preparatory regimens and peripheral blood stem cell transfusions that contributed to faster engraftment and fewer or no post-HSCT complications. As a result, all of our lymphoma patients were discharged a few days following BE. In order to assess the possible significance of using the BE-OE value as a marker of susceptibility to infection, sensitivity and specificity values were evaluated for the BE-OE value as a predictor of post-BE fever and infection (Figure 4). All possible cutoff BE-OE days were explored and a cutoff value of four demonstrated the strongest screening power with a sensitivity and specificity of 100 and 80%, respectively.

### Oral mucositis and oral engraftment

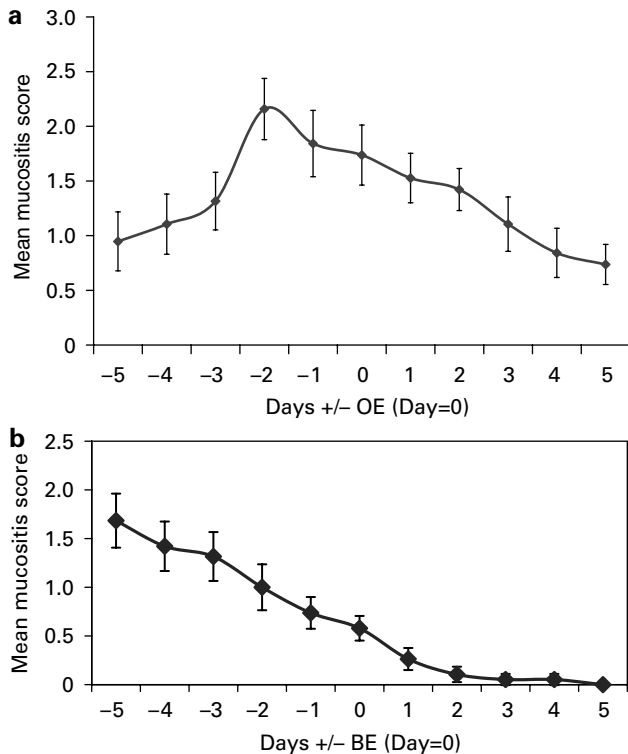
Onset of oral mucositis occurred in the pre-BE phase for 19 of the 29 subjects who developed symptoms of mucositis. The change in mucositis severity was examined for each patient over the 5 days before and after OE and also BE (Figure 5). To examine the relationship between oral



**Figure 3** Plot of post-BE infection-related fever episode (IRFE) frequency as a function of days between OE and BE. Symbols represent patient groups based on initial diagnosis. Longer time periods between OE and BE are associated with fewer fever episodes. Note that all subjects with one episode of fever or more post-BE presented with only 4 days or less between OE and BE.



**Figure 4** Plot showing sensitivity and specificity scores for all possible cutoff BE-OE values reported in this study to be used as possible markers of fever occurrence post-BE. A cutoff post-OE day of 4 can be used to suggest a patient's susceptibility to infection soon after HSCT and before BE.



**Figure 5** Plots showing mean mucositis score values 5 days before and after OE and BE for the 19 HSCT patients who developed oral mucositis. Although oral mucositis symptoms continued to improve on days following BE shown in panel b, it was the recovery of neutrophils in the oral cavity depicted in panel a that marked the beginning of the recovery phase.

mucositis scores and OE timing for each of the 19 patients with mucositis, the mucositis score at day OE + 2 days was subtracted from the score at OE - 2 days (Table 1). We chose OE - 2 days because, based on our patient data analysis, mucositis symptoms peaked by this time. Patients with mucositis symptoms that improved from OE - 2 to

OE + 2 would have a positive difference while patients with worsening mucositis symptoms would have negative differences. The average difference is a positive value of  $0.74 \pm 1.24$  s.d., and statistically significantly different from zero or no overall improvement ( $t = 2.5898$ , 18 d.f.,  $P = 0.0092$ ).

## Discussion

Past evidence has suggested that neutrophil delivery to tissues is a much more relevant marker of susceptibility to infection than circulating white blood cell counts.<sup>6</sup> Wright *et al*<sup>6</sup> demonstrated that acute neutropenia resulted in reduced oral neutrophil levels. Moreover, his group confirmed a direct correlation between oral and blood neutrophil levels in adults undergoing myelosuppressive chemotherapy, and found that the rise in oral neutrophil levels preceded detectable blood neutrophil increases.<sup>9</sup> Infections are common in HSCT patients, even in the face of 'protective' circulating neutrophil levels following BE.<sup>5</sup> Circulating neutrophil levels indicate that neutrophils may be present in tissues, but verifying and measuring neutrophil penetration into the tissues themselves gives us insight into the ability of these cells to provide adequate defense from infection and ameliorate symptoms of mucositis after transplant.

### Predicting engraftment

We illustrate here that monitoring oral neutrophil delivery following HSCT serves as an early predictor of successful engraftment. We demonstrated in 28 pediatric HSCT patients that neutrophils were detected on average 6 days earlier in the oral cavity than in the circulation. As can be seen in Table 1, there is a difference in the timing of oral and blood engraftment between the groups based on initial diagnosis. The type of transplant (allogeneic *vs* autologous) and its source (bone marrow *vs* peripheral blood) may explain the differences between the groups. For instance, oral and blood engraftment were achieved more rapidly in patients originally diagnosed with Hodgkin's or non-Hodgkin's lymphoma compared to patients treated for leukemia or nonmalignant bone marrow failures. Lymphoma patients are treated with less intense preparatory regimens and autologous peripheral blood stem cell infusions, both of which may contribute to faster engraftment. The nonmalignant bone marrow failure patient group, on average, engrafted much later than the leukemia patient group, despite receiving primarily the same treatment. In this case, a marrow stromal cell defect presenting in marrow failure patients may have been a factor contributing to late engraftment in this patient group.

### Predicting susceptibility to infection

We demonstrate here that the time span between oral and blood engraftment (BE-OE) is inversely related to the number of infection-related febrile episodes after blood engraftment, with strong sensitivity and specificity values using a BE-OE value of four as a cut-off point for

predicting susceptibility to infection after blood engraftment (Figures 3 and 4). This suggests that BE-OE can be used as a measure of susceptibility to infection post-HSCT. These results have raised questions regarding aspects of neutrophil and engraftment biology, which may be responsible for the differences observed between patients with high and low BE-OE values. For example, different polymorphisms in the surface adhesion molecules of neutrophils may be responsible for the varying degrees and rates of neutrophil tissue penetration following HSCT, thus affecting infection susceptibility. Another possible explanation for the BE-OE observation is individual differences in the inherent neutrophil tissue pool size. In other words, it may be that some individuals are genetically able to store or recruit more neutrophils into their tissue compartments. As a result, it would take longer for neutrophil levels to be recovered in their tissue compartments before allowing for an accumulation in the circulation. Consequently, these patients present with a longer time period between OE and BE and therefore a higher number of neutrophils in the tissues readily available to fend off infections. Patients presenting with shorter time intervals between OE and BE may have an inherently lower neutrophil storage capacity in their tissues resulting in an increased susceptibility to acute infection. Further investigations are required to identify and characterize the determining factors in neutrophil tissue delivery and pool size as they may ultimately contribute to improving patient management in many clinical situations dealing with neutropenic patient populations.

#### Oral mucositis

Our findings indicate that oral engraftment is a better predictor of oral mucositis symptom resolution than blood engraftment. Oral mucositis is a common complication of radiotherapy and chemotherapy regimens. The signs and symptoms of mucositis in HSCT patients are more severe because of the intense myeloablative preparatory regimens administered during their treatment. Moreover, loss of integrity of the oral mucosa results in a portal of entry for resident microflora, thereby predisposing patients to infection, especially during periods of severe neutropenia.<sup>11</sup> The return of neutrophils to the tissues may contribute to the improvement of oral mucositis symptoms (Figure 5a). Although oral mucositis symptoms continued to improve on days following blood engraftment (Figure 5b), it was the return of neutrophils to the oral cavity that marked the beginning of the recovery phase (Figure 5a).

#### Conclusion

We conclude that monitoring the timing of neutrophil tissue delivery through a rapid oral rinse yields insights into the biology of neutrophil recovery during and after engraftment and the factors associated with susceptibility to infection. Using this model, we will begin to identify the

biological determinants of neutrophil recovery following HSCT and the elements involved in neutrophil tissue recruitment.

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