Increased Exhaled Nitric Oxide Following Autologous Peripheral Hematopoietic Stem-Cell Transplantation*

A Potential Marker of Idiopathic Pneumonia Syndrome

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Background: Increased production of nitric oxide (NO) and oxidative stress following bone marrow transplantation may play a role in the pathogenesis of idiopathic pneumonia syndrome (IPS). We hypothesize that patients who received high-dose chemotherapy followed by autologous peripheral hematopoietic stem-cell transplantation (APHSCT) have increased exhaled NO.

Method: We measured exhaled lower respiratory tract NO concentration with a chemiluminescent NO analyzer during a slow vital capacity maneuver against a positive pressure of 16 cm H2O at an expiratory flow rate of 50 mL/s in 20 female patients who received high-dose chemotherapy (cyclophosphamide, carmustine, and cisplatin) followed by APHSCT for the treatment of stage III or IV breast carcinoma. Pulmonary function tests were performed, and exhaled NO measurements and clinical and laboratory data were obtained before transplantation and at every 6-week visit after transplantation for 24 weeks.

Results: All study patients had evidence of IPS with dyspnea and reduction in diffusion capacity of the lung for carbon monoxide (DLco). Lower respiratory tract exhaled NO was significantly higher after APHSCT and during the 6 months of follow-up. Mean (± SD) exhaled NO increased from (mean ± SD) 12.54 ± 1.32 parts per billion (ppb) before APHSCT to 21.26 ± 1.94 ppb at 6 weeks (p = 0.099), 21.26 ± 1.94 ppb (p = 0.006) at 12 weeks, 24.62 ± 2.55 ppb (p = 0.012) at 18 weeks, and 25.28 ± 3.31 ppb (p = 0.013) at 24 weeks (all p values were compared to baseline). There was a strong negative correlation between DLco and exhaled NO (regression coefficient -0.60, p = 0.01).

Conclusion: Lower respiratory tract concentration of exhaled NO is significantly increased following APHSCT and correlates with reduction in DLco. Increase in lower respiratory tract concentration of NO is a potential marker of IPS.

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Key words: bone marrow transplantation; exhaled nitric oxide; idiopathic pneumonia syndrome; nitric oxide; peripheral hematopoietic stem-cell transplantation; pulmonary complications; pulmonary drug toxicity

Abbreviations: APHSCT = autologous peripheral hematopoietic stem-cell transplantation; BMT = bone marrow transplantation; DLco = diffusion capacity of the lung for carbon monoxide; Hb = hemoglobin; IPS = idiopathic pneumonia syndrome; MCP = monocyte chemoattractant protein; NO = nitric oxide; NOS = nitric oxide synthase; NYHA = New York Heart Association; PFT = pulmonary function test; ppb = parts per billion

Pulmonary complications are a major cause of morbidity and mortality following bone marrow transplantation (BMT) or peripheral hematopoietic stem-cell transplantation.1 Idiopathic pneumonia syndrome (IPS) is a noninfectious diffuse and severe lung injury that commonly occurs following allogeneic BMT, and may be related to older age, cond-

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tions of exhaled NO will increase compared to baseline and be associated with clinical and physiologic findings of diffuse lung injury following transplantation.

**Materials and Methods**

The aim of this pilot study was achieved by measuring the lower respiratory tract NO concentration as derived from exhaled-breath analysis in a homogeneous group of 20 female patients with advanced breast cancer who were scheduled for high-dose chemotherapy followed by APHSCT. The study was approved by the Institutional Review Board of Wayne State University (Detroit, MI), and informed written consent was obtained from all subjects. Inclusion criteria were as follows: (1) stage III and IV breast cancer patients 18 to 70 years of age undergoing APHSCT, and (2) the ability to adequately perform exhaled NO and PFT maneuvers. Exclusion criteria included the following: (1) smoking within the last 6 months, (2) respiratory tract infection within 4 weeks, (3) intervention illness or acute exacerbation of underlying disease within 4 weeks, (4) use of supplemental L-arginine, nitrates, or phosphodiesterase inhibitors, and (5) history of hyperreactive airways.

The APHSCT protocol employed in our hospital has been previously described in detail.11,12 All patients had high-risk primary or metastatic breast cancer. Before high-dose chemotherapy, peripheral hematopoietic stem cells were mobilized with filgrastim or filgrastim plus chemotherapy, washed, frozen, and stored until use. High-dose chemotherapy consisted of IV cyclophosphamide (1,875 mg/m²/d) as a 1-h infusion on days − 6, − 5, and − 4, IV cisplatin (55 mg/m²/d) as a continuous infusion on days − 6, − 5, and − 4, and IV Carmustine (600 mg/m²) as a 2-h infusion on day − 3. Chemotherapy was administered along with fluids via an indwelling central venous catheter.

After receiving high-dose chemotherapy, the patients were discharged from the hospital and then were seen daily in the outpatient transplantation clinic. On day 0, peripheral hematopoietic stem cells were infused IV into patients via the central vascular catheter. Filgrastim was administered IV at a dosage of 5 μg/kg/d from day 0 until the absolute neutrophil cell count was ≥ 5 × 10⁹/L for at least 3 days.

All patients were evaluated at baseline prior to high-dose chemotherapy and APHSCT, and every 6 weeks following transplant for 6 months. The evaluation on each visit included a complete history and physical examination, New York Heart Association (NYHA) dyspnea score, CBC count, standard posteroanterior and lateral chest radiographs, PFTs, and measurement of exhaled NO.

The severity of dyspnea was categorized according to the NYHA classification. Patients were considered to have NYHA class I if they had no dyspnea, and NYHA class II-IV if dyspneic. PFTs were performed according to American Thoracic Society guidelines (Vmax 22, SensorMedics; Yorba Linda, CA).13,14 Predicted values were taken from Crapo and coworkers15 and Miller and coworkers.16 DLCO values were corrected for hemoglobin concentrations (Hb) as follows: corrected DLCO = measured DLCO × [10.22 + (Hb/1.7(Hb))].

**Exhaled NO Measurement**

The standardized procedure recommended by the American Thoracic Society17 for the online measurement of plateau exhaled NO was followed. Exhaled NO was measured online with a chemiluminescent analyzer (model 280 NOA: Sievers; Boulder, CO) with a low detection threshold (sensitivity < 1 parts per billion [ppb]), a fast response time [0 to 90% response time of 200 ms], and a sample rate of 250 mL/min). Ambient NO was also recorded at the time measuring on-line exhaled NO. The analyzer was calibrated daily with certified, commercially prepared NO gas (45 ppm) and zero gas generated by passage of air through an NO scrubbing filter. The subject would rest seated for 10 min before the measurement circuit was placed. The measurement circuit consisted of a mouthpiece with two one-way valves allowing inspiration from room air and exhalation into the circuit. A resistor valve with known flow rates at various pressures was placed in the circuit. A manometer in the circuit constantly monitored pressure, graphically allowing the subject to target a certain pressure, thereby maintaining a constant flow. Subjects performed slow exhalations from a full inspiration against certain pressures of 11 to 16 cm H₂O to generate flow rate of 50 mL/s. The NO concentration was recorded from the plateau value (NO plateau) of at least 3 s, during which the desired pressure was maintained to within 10% and NO concentration varied < 10% or 1 ppb, whichever was greater.17 Ambient NO levels were always < 20 ppb, which have been shown not to affect NO plateau reading.17 Repeated exhalations to give three values of

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NO plateau that vary < 10% for each flow rate were performed, and the mean of the three measurements was obtained.

During the study period, baseline exhaled NO, PFTs, and clinical signs and symptoms were compared with follow-up visits. Patients presenting with dyspnea, dry cough, and reduction in DLCO (corrected DLCO ≥ 20% reduction from baseline), with or without pulmonary infiltrates, and no signs of infection were presumed to have IPS, and were treated with corticosteroids (prednisone, 60 mg/d tapered over 10 weeks). PFTs were followed up periodically, and the corticosteroid dose was increased again if there was worsening symptoms or a reduction in DLCO.

The statistical analyses were performed using the random-effects, linear-models Holm method to adjust for multiple comparisons. Exhaled NO and DLCO were assessed for symmetry graphically, and a ladder of powers was used to find an appropriate transformation when gross asymmetry was detected. Only the exhaled NO values required transformation, and a log transformation was applied to reduce the skewness of the distribution. NYHA dyspnea scores ≥ 2 were combined into a single category because the data are quite sparse in the higher categories. A random-effects logistic model was used to evaluate changes over time beginning with the first posttransplant visit. Exact McNemar tests were used to compare changes from baseline.

**Results**

Twenty female APHSCT recipients with stage III or IV breast cancer were studied. The average age of the participants was 46 years; they were predominantly white, and most were nonsmokers (Table 1). Patients were assessed before APHSCT and every 6 weeks afterwards for 24 weeks. NYHA dyspnea score, chest radiographs, laboratory data, PFTs, and exhaled NO measurements were recorded on each visit. All 20 patients were evaluated in the first two post-APHSCT visits; however, only 17 of the participants were evaluable on the third visit and only 13 participants on the fourth visit. One patient left the country, one died of respiratory failure, and the rest refused to continue the study. There were no differences in demographic, clinical, or baseline PFT characteristics between those who dropped out and those who completed the study.

All patients were free of dyspnea before APHSCT. The proportion of individuals with dyspnea was 30% at the first visit, increased to 85% at the second visit, and appeared to stabilize at 88% on the third visit and 77% on the fourth visit (Table 2). Only two patients never developed dyspnea, while the majority of patients continued to have dyspnea on subsequent visits. In only four instances did the dyspnea resolve.

The PFT results were within normal limits for all patients at baseline. There were no significant differences in FEV_1, FVC, FEV_1/FVC, and total lung capacity between baseline follow-up visits following transplantation (data not shown). However, DLCO did decrease significantly after APHSCT when compared to baseline (Table 3). On average, DLCO reached a nadir of 59 ± 3.69% at 24 weeks.

Exhaled NO measurements before APHSCT were compared with posttransplant values (Table 4). Pre-APHSCT mean exhaled NO was 12.54 ± 1.32 ppb. Posttransplant exhaled NO increased to a mean of 21.62 ± 1.94 ppb, 24.62 ± 2.55 ppb, 24.84 ± 2.71 ppb, and 25.28 ± 3.31 ppb on first, second, third, and fourth visits, respectively. All values were significantly higher than baseline; however, there were no significant differences between the successive visits after transplantation.

To determine the association between the reduction in DLCO and increase in exhaled NO following transplantation, a generalized estimating equation model with Gaussian family, identity link, unstructured correlation, and robust SEs was used, account-

### Table 1—Baseline Clinical Characteristics of Patients Undergoing APHSCT*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>46 ± 8.5</td>
</tr>
<tr>
<td>Female</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>15 (75)</td>
</tr>
<tr>
<td>Black</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Remote history of smoking</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Stage of breast cancer</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>7 (35)</td>
</tr>
<tr>
<td>IV</td>
<td>13 (65)</td>
</tr>
<tr>
<td>Previous history of radiation</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Previous pulmonary disease</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Pulmonary metastasis</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Baseline PFTs, results % predicted DLCO, corrected</td>
<td>89.6 ± 13.5</td>
</tr>
<tr>
<td>Total lung capacity</td>
<td>103.5 ± 16.7</td>
</tr>
<tr>
<td>FEV₁</td>
<td>99.3 ± 16.8</td>
</tr>
<tr>
<td>FEV₁/FVC ratio</td>
<td>77.9 ± 5.3</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD or No. (%).

### Table 2—NYHA Dyspnea Score for Patients Before and After APHSCT (Every 6 Weeks for 24 Weeks)

<table>
<thead>
<tr>
<th>Visit</th>
<th>Patients, No.</th>
<th>Patients With Dyspnea, No. (%</th>
<th>p Value, Differences Compared to Baseline*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before APHSCT</td>
<td>20</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>6 (30)</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>17 (85)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>15 (88)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>10 (77)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*McNemar test corrected for multiple comparisons. Prevalence of dyspnea at post-APHSCT visits 2, 3, and 4 is significantly greater than prevalence at post-APHSCT visit 1 (p = 0.02). There are no statistically significant differences in prevalence of dyspnea between post-APHSCT visits 2, 3, and 4 (p > 0.25).
ing for within-individual correlations. The regression coefficient estimated from this model was -0.60 (SE 0.24), indicating a significant (p = 0.01) negative association between DLCO and exhaled NO (Fig 1).

All patients required corticosteroid therapy following APHSCT for IPS. This determination was based on the clinical picture and reduction in DLCO as defined above. During the first 12 weeks following transplantation, 18 patients were started on prednisone; 2 patients were started on prednisone after 16 weeks. During the study period, none of the patients acquired a serious infection. All patients were cared for on an outpatient basis, and none had clinical or physiologic features of ARDS. Two patients were readmitted to the hospital during the study period: one patient for deep venous thrombosis of the lower extremity, and the other patient died on the third hospital day with acute respiratory failure 18 weeks following transplantation. The respiratory failure was presumed to be due to drug-induced pulmonary toxicity. A postmortem examination was not performed. Her DLCO prior to hospital admission was 43% of predicted and exhaled NO was 55 ppb.

Table 3—DLCO Before and After APHSCT (Every 6 Weeks for 24 Weeks)

<table>
<thead>
<tr>
<th>Visit</th>
<th>Patients, No.</th>
<th>DLCO % Predicted, Mean (SD)</th>
<th>p Value, Differences Compared to Baseline*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before APHSCT</td>
<td>20</td>
<td>89.6 (3.0)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>71.7 (3.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>66.9 (3.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>60.1 (3.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>59.0 (3.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Compared to baseline and corrected for multiple comparisons. DLCO at visits 3 and 4 are significantly less than DLCO at visit 1 (p < 0.02 for each comparison), while the difference between visits 1 and 2 is not statistically significant (p = 0.19). None of the other differences in DLCO between visits are statistically significant (p > 0.15).

Table 4—Exhaled NO Measurements Before and After APHSCT (Every 6 Weeks for 24 Weeks)

<table>
<thead>
<tr>
<th>Visit</th>
<th>Patients, No.</th>
<th>Exhaled NO (ppb) Predicted, Mean (SD)</th>
<th>p Value, Differences Compared to Baseline*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before APHSCT</td>
<td>20</td>
<td>12.5 (1.3)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>21.3 (1.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>24.6 (2.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>24.8 (2.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>25.3 (3.3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*None of the other differences in exhaled NO between visits are statistically significant (all p > 0.20).

Discussion

The main findings of this pilot study are as follows: (1) patients who received high-dose chemotherapy followed by APHSCT acquired dyspnea with significant decrease in DLCO, consistent with IPS; (2) single-breath exhaled NO concentrations, at a relatively low expiratory flow rate of 50 mL/s, were increased following APHSCT and remained elevated during the study period; and (3) lower respiratory tract concentrations of exhaled NO were inversely correlated with DLCO. The finding of increased lower respiratory tract NO during clinically manifest lung injury suggests that exhaled NO may be a potential marker of IPS following APHSCT. Further studies are needed to determine whether NO plays a role in the pathogenesis of this condition. To our knowledge, this is the first report studying exhaled NO in patients after APHSCT.

NO is synthesized from L-arginine using NO synthase (NOS) as an enzyme. There are three types of NOS: type 1 (neuronal), type 2 (inducible), and type 3 (endothelial). Type 1 and type 3 are together called constitutive NOS.18 NO is produced through the entire length of airway. The normal lower respiratory tract NO levels are estimated to be 1 to 9 ppb. The upper respiratory tract (200 to 1,000 ppb) and sinuses (1,000 to 30,000 ppb) are other significant sources of NO.18–22 Blocking NOS by inhaled amino guanidine is shown to reduce exhaled NO by 40 to 70%, while blocking NOS by systemic N(G)-monomethyl-L-arginine did not result in significant reduction in exhaled NO.23 This fact highlights the minimal role of endothelial NO source for the exhaled NO. Direct sampling via fiberoptic bronchoscopy reveals that the concentration of the exhaled NO is similar at the mouth and in the trachea and main bronchi.24,25 Moreover, the exhaled NO measurement is shown to be independent of age, sex, lung functions, and diurnal variations, and is highly reproducible.19,26

The two important steps in measuring exhaled NO are excluding the nasopharyngeal source and recording the plateau reading of exhaled NO at constant flow rates.17 Exhaled NO at any flow rate reflects both the alveolar and the conducting airway source. Measuring exhaled NO levels at various flow rates and plotting them as NO output in nanoliters per second on y-axis and flow rates in milliliters per second in x-axis can help in partitioning alveolar from the conducting airway source of exhaled NO as described by Girgis et al27 and Tsoukias and George.28

Two previous reports studied the concentration of exhaled NO in patients with interstitial lung disease (hypersensitivity pneumonitis29 and scleroderma27),
and both studies showed that the increased concentration of exhaled NO is primarily due to an alveolar component, raising the possibility that NO plays a role in the pathogenesis of these conditions. Our report is the first to show increased concentration of exhaled NO in parenchymal lung injury following BMT. However, the partition of the two components (alveolar vs conducting airways) was not attempted in this pilot study, and may need to be determined in future studies.

Most probable reasons for increased exhaled NO in our study are either increased NO production or decreased NO diffusion. Increased NO production in the absence of L-arginine supplementation may be due to increase in inducible NOS (due to chemotherapeutic agent-related oxidative stress that mediate induction of inducible NOS messenger RNA) or increased production from inflammatory cells involved in IPS.30–33

The increase in exhaled NO in our study supports the findings of Haddad and co-workers,10 who studied a mouse model of IPS following BMT. They showed that alveolar macrophages, after being stimulated by allogeneic T cells, express higher NOS levels and thus produce more NO. Cylophosphamide in their model stimulated superoxide production by the alveolar macrophage. They concluded that the resultant higher NO and superoxides levels might have lead to production of the peroxynitrates and nitrotyrosines, which mediated lung damage. In another mouse model developed by Shanker et al34 and in the murine model developed by Panoskaltsis-Mortari et al35 for IPS, macrophage activation was dependent on allogeneic T cells. In the absence of allogeneic T cells in our patient population, it is intriguing to explore alternate mechanisms for the higher NO levels shown in our study.

The lack of allogeneic T cells in APHSCT makes it reasonable to postulate that the reason for increased exhaled NO in our patients may be the result of pharmacotoxicity of the chemotherapeutic regimen used. Cytotoxic drugs not only offset the oxidant-antioxidant balance, but also cause activation of the pulmonary alveolar macrophages and pulmonary epithelial cells to produce pro-inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin-6, and others as shown in studies by Bhalla and Folz.9 Moreover, NO is shown to modulate MCP-1 expression in the endothelial cells under cyclic strain.36 There appears to be a vicious cycle of proinflammatory and antioxidant mechanisms associated with lung damage. It is possible that increased exhaled NO in our study reflects a role of NO in the propagation and modulation of the inflammatory (by modulating MCP-1 activity)36 and oxidant (by supplying substrate for peroxynitrates)37,38 lung damage induced by chemotherapeutic agents.

FIGURE 1. The plot of mean exhaled NO vs DLco before and after transplantation. The error bars represent SD. The inverse relation was significant in the 12th, 18th, and 24th weeks after transplantation.
The biological role of increased exhaled NO as shown in our study is yet to be elucidated. It may be proinflammatory and damaging through peroxynitrite or it may be anti-inflammatory, preventing platelet aggregation and leukocyte adhesion. In our study, we found no relationship between corticosteroid initiation and exhaled NO levels, suggesting a possible noninflammatory source for the origin of increased exhaled NO. The fact that increased exhaled NO correlated with decreased DLCO in our study may reflect that the decreased diffusion of NO from the alveolar space into pulmonary capillary blood is a reason for increased exhaled NO. Direct measurement of diffusion of NO from the alveolar space into pulmonary capillary blood, as described by Perillo and coworkers, was not done on our patients. The inverse relationship of increasing exhaled NO and decreasing DLCO in our patients is similar to the finding by Girgis et al in a study of patients with scleroderma lung disease. In that study, it was shown that there is a negative correlation between alveolar concentration of the exhaled NO and DLCO ($r = -0.66$, $p = 0.002$). There is a possibility, however, that decreased DLCO may be due to increased NO and Hb interaction (which combines much faster with hemoglobin than carbon monoxide), thus impairing DLCO.

Our study was performed on patients with advanced breast cancer undergoing high-dose chemotherapy followed by APHSCT. In this particular population, the incidence of IPS is reported to be as high as 72%, and is the major pulmonary complication after transplantation. Although this procedure is currently restricted to a specific group of patients with advanced breast cancer, APHSCT is increasingly employed in the treatment of a variety of malignant and nonmalignant conditions. Moreover, carmustine and cyclophosphamide remain a cornerstone of many of these protocols. The findings of this study are significant in shedding light on potential mechanisms leading to IPS following BMT, and suggest that exhaled NO may be a noninvasive marker of this condition. The findings of this preliminary report need to be confirmed in patients undergoing allogeneic BMT or APHSCT for diseases other than breast cancer. In addition, further studies are necessary to determine whether the increased exhaled NO is directly related to high-dose chemotherapy or is affected by APHSCT. Furthermore, examining bronchoalveolar fluid for NO products and alveolar cellular composition will advance our understanding of the role of NO in the pathogenesis of IPS following BMT.

In summary, our pilot study showed that exhaled NO levels increase significantly following high-dose chemotherapy and APHSCT. The increase in exhaled NO was statistically significant when compared to baseline values, and correlated positively with the worsening dyspnea score and decreasing DLCO measurement. The diagnostic and therapeutic implications of these findings should be explored further.

REFERENCES


