# Sensitivity and specificity of passive immune-basophil activation test to detect allergic transfusion reactions

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**BACKGROUND:** The basophil activation test (BAT), performed with patient blood samples and supernatants from transfused blood, was developed to elucidate the mechanistic relationship between transfusion and the resultant allergic transfusion reactions (ATRs). This test cannot be performed on myelosuppressed patients and neonates because of the absence of basophils. Therefore, we devised the passive immune basophil activation test (pi-BAT) using patients' plasma and residual transfused blood as sources of immunoglobulin E and allergen, respectively, and the basophils of healthy volunteers served as a source of the responder cells. The sensitivity and specificity of the pi-BAT, however, remained largely unknown.

**STUDY DESIGN AND METHODS:** In this study, the pi-BAT was performed on 31 patients with nonhemolytic transfusion reactions including nine non-ATR and 22 ATR (12 mild and 10 moderate-to-severe) cases to examine its sensitivity and specificity.

**RESULTS:** Nine of the 10 cases with moderate-to-severe ATR tested positive, whereas all the non-ATR cases negative, strongly indicating immunoglobulin E and allergens are involved in the pathogenesis underlying the blood transfusion-triggered adverse effects.

**CONCLUSION:** Thus, we propose that pi-BAT can be used to detect moderate-to-severe ATRs and their underlying mechanisms.

Blood transfusion provides tremendous clinical benefits and can sometimes be lifesaving. However, it carries considerable risks. To reduce the risks to transfusion recipients, such as viral and bacterial infections, hemolytic transfusion reactions, and transfusion-related lung injury, several safety measures and look-back studies have been implemented based on the concrete causal relationship between the adverse reactions (ARs) and transfusion.[1–7] The AR incidence rate in Japan to red blood cells (RBCs), fresh-frozen plasma, and platelet concentrates per transfused unit has been estimated as 0.6%, 1.3%, and 3.8%, respectively.[8] RBC ARs were symptomatically either allergic transfusion reactions (ATRs) (44.7%) or fever (33.3%), whereas most fresh frozen plasma ARs and platelet concentrate ARs were ATRs (75.8% and 78.1%, respectively). Most ATRs are mild, but some serious ones have also occurred.[9] However, safety measures have not been well established. ATRs have not been studied well until recently, nor has their standard assay system been established.

The basophil activation test (BAT), first developed as a simple blood test to diagnose allergic diseases,[10–12] has recently been performed in transfusion medicine to investigate the causative relationship between transfusion and ATRs and

**ABBREVIATIONS:** ARs = adverse reactions; ATRs = allergic transfusion reactions; BAT = basophil activation test; NHTR = nonhemolytic transfusion reaction; PBMCs = peripheral blood mononuclear cells; PBS = phosphate-buffered saline; PE = phycoerythrin; pi-BAT = passive immune basophil activation test; ROC = receiver operating characteristic.

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their underlying mechanisms.[9,13] However, several patients with ATRs have been diagnosed with hematologic malignancies. The primary diseases and/or treatments for these patients may cause myelosuppression, which increases the difficulty of obtaining a sufficient number of basophils for the BAT. To overcome this drawback,[14] a pilot study recently reported that even basophils from healthy volunteers, whose own receptor-bound immunoglobulin E (IgE) was replaced with IgE in patients with ATRs, were activated by the supernatants of the corresponding transfused blood components. This system was known as passive immune BAT (pi-BAT), and the possibility of its application was suggested in the field of transfusion medicine to assess the causal relationship even in patients under myelosuppression.[14] In this study, a detailed evaluation of the sensitivity and specificity of the pi-BAT is performed.

## METHODS

### Clinical samples

Among the patients' serum/plasma samples and the relevant residual blood of nonhemolytic transfusion reaction (NHTR), 22 were diagnosed with ATR and nine with non-ATR. The detailed information on these patients is summarized in Table 1.

### Reagents

Purified mouse anti-human IgE, fluorescein isothiocyanate–conjugated mouse anti-HLA-DR, and phycoerythrin (PE)-conjugated mouse anti-CD203c monoclonal antibodies were purchased from Beckman Coulter. PE-Cy5-conjugated mouse anti-CD123 monoclonal antibody and a cell lysing solution (FACS lysing solution) were purchased from BD Biosciences. Hydroxyethyl starch (HES40) was purchased from Nipro. Heparin sodium was purchased from Mochida Pharmaceutical Co. Ltd. $CaCl_2$ was purchased from Nacalai Tesque (Kyoto, Japan).

### Passive immune BAT (pi-BAT)

Replacement of IgE bound on basophils was performed with the following two-step procedure, as reported elsewhere[14] (Fig. 1A): Briefly, HES40 was used to separate peripheral blood mononuclear cells (PBMCs) according to a standard protocol. To remove receptor-bound IgE, PBMCs ($5 \times 10^6$ cells/well) were suspended in 1 mL of lactic acid solution (13.4 mM lactate, 140 mM NaCl, and 5 mM KCl) and incubated for 5 minutes on ice. To stop the

**TABLE 1. Information on the clinical samples**

| | Patient | | | NHTRs | | |
|---|---|---|---|---|---|---|
| Case no. | Age | Sex | Primary disease | Elicited symptoms | Severity | pi- BAT |
| N1 | 58 | F | Chronic lymphocytic leukemia | Hypoxemia | Non-ATR | – |
| N2 | 77 | M | Myelodysplastic syndrome | Shaking chills | Non-ATR | – |
| N3 | 55 | F | Acute myeloid leukemia | Cough | Non-ATR | – |
| N4 | 80 | M | Myelodysplastic syndrome | Fever | Non-ATR | – |
| N5 | 60 | F | Acute myeloid leukemia | Fever | Non-ATR | – |
| N6 | 48 | F | Myelodysplastic syndrome | Fever | Non-ATR | – |
| N7 | 31 | M | Acute myeloid leukemia | Dyspea | Non-ATR | – |
| N8 | 74 | M | Small lymphocytic lymphoma | Fever | Non-ATR | – |
| N9 | 65 | F | Systemic lupus erythematosus | Fever | Non-ATR | – |
| A1 | 64 | F | Angioimmunoblastic T-cell lymphoma | Urticaria (partially) | Mild ATR | – |
| A2 | 49 | M | Acute myeloid leukemia | Urticaria (partially) | Mild ATR | + |
| A3 | 55 | F | Acute myeloid leukemia | Urticaria (partially) | Mild ATR | – |
| A4 | 12 | M | Pelvic fracture | Urticaria (partially) | Mild ATR | + |
| A5 | 81 | F | Aortic regurgitation | Urticaria (partially) | Mild ATR | – |
| A6 | 55 | F | Myelodysplastic syndrome | Urticaria (partially) | Mild ATR | – |
| A7 | 31 | M | Myelodysplastic syndrome | Itch sensation (self-control) | Mild ATR | – |
| A8 | 67 | M | Myelodysplastic syndrome | Erythema (partially) | Mild ATR | – |
| A9 | 80 | M | Myelodysplastic syndrome | Erythema (partially) | Mild ATR | – |
| A10 | 31 | M | Acute myeloid leukemia | Wheal (partially) | Mild ATR | – |
| A11 | 2 | F | Thrombotic thrombocytopenic purpura | Urticaria (partially) | Mild ATR | – |
| A12 | 55 | F | Acute myeloid leukemia | Urticaria (partially) | Mild ATR | + |
| A13 | 84 | F | Aplastic anemia | Urticaria (systemic) | Moderate ATR | + |
| A14 | 65 | F | Acute myeloid leukemia | Urticarial (systemic), itch | Moderate ATR | + |
| A15 | 47 | F | Acute myeloid leukemia | Wheal (systemic) | Moderate ATR | + |
| A16 | 24 | M | Gray zone lymphoma | Urticarial (systemic) | Moderate ATR | + |
| A17 | 85 | F | Myelodysplastic syndrome | Anaphylaxis | Severe ATR | + |
| A18* | 53 | M | Acute lymphoid leukemia | Anaphylaxis | Severe ATR | + |
| A19* | 54 | F | Acute mixed leukemia | Anaphylaxis | Severe ATR | + |
| A20 | 0 | F | Anemia | Anaphylaxic shock | Severe ATR | + |
| A21 | 28 | F | Ectopic pregnancy | Anaphylaxic shock | Severe ATR | + |
| A22 | 84 | F | Myelodysplastic syndrome | Anaphylaxis | Severe ATR | – |

* These data were previously reported.[14]

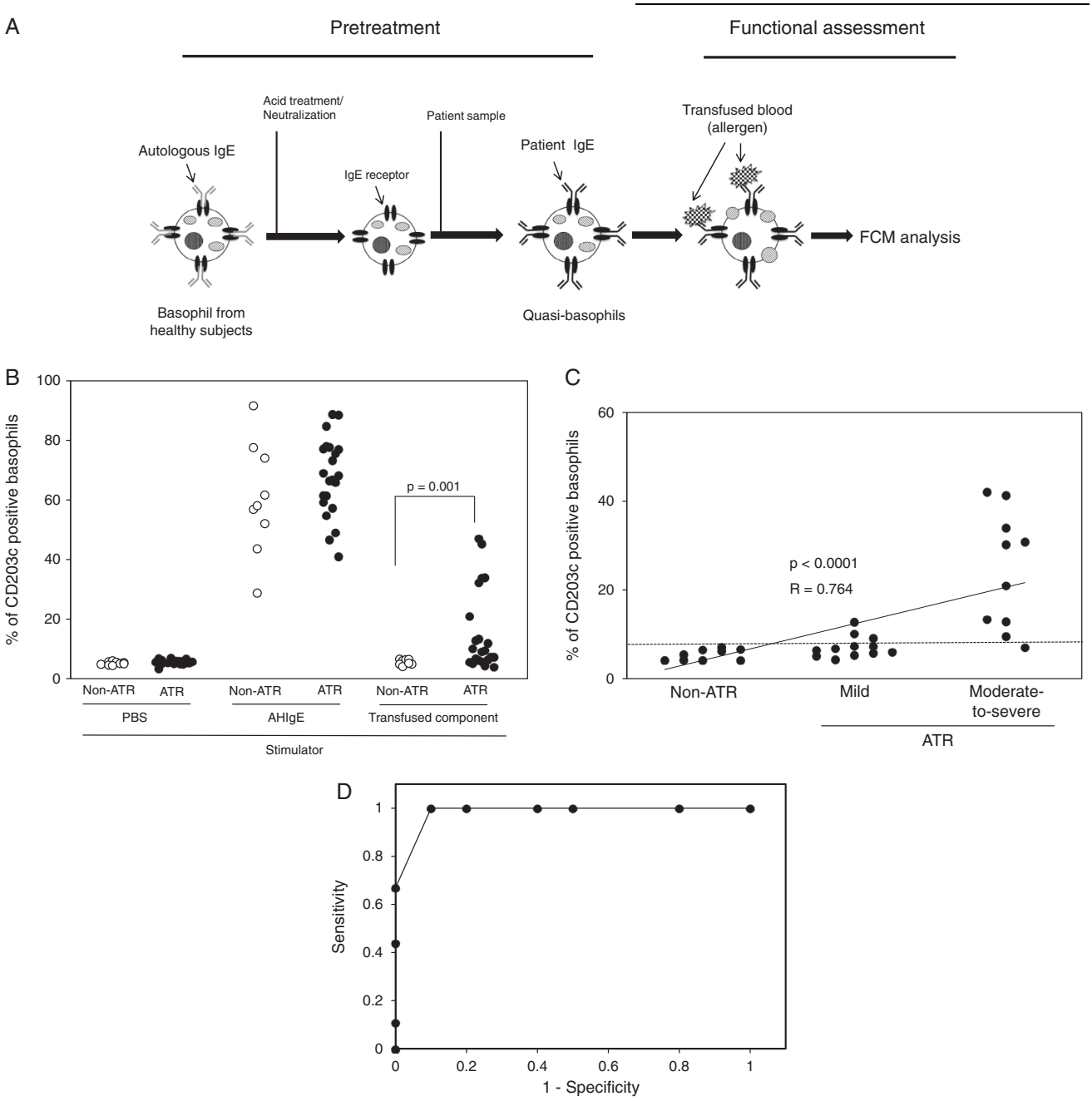


**Fig. 1. Verification of the pi-BAT by use of clinical samples. (A) The pi-BAT is configured by use of the following two processes: the pretreatment and functional assessment process. In the pretreatment process, IgE from its own basophils was replaced with that of patients who underwent acid treatment, and the basophils were subsequently treated with patient plasma or serum. In the functional assessment process, the quasi-basophils were incubated with the residual transfused blood component and expression of CD203c, a marker for basophil activation, was analyzed via flow cytometry. (B) The quasi-basophils were stimulated with PBS as a negative control (1/10 vol), and a monoclonal antibody against human IgE (AHIgE), known as a strong basophil stimulator (5 μg/mL) as the positive control, or the residual transfused blood components (1/10 vol). Open circle (○) shows the quasi-basophils in their own IgE surface as replaced with the non-ATR patient IgE (n = 9), and closed circle (•) shows that those were replaced with ATR patient IgE (n = 22). The Mann–Whitney U test was performed to determine statistical significance. A p value of <0.01 was considered statistically significant. (C) The clinical samples were classified into three groups: non-ATR, mild ATR, and moderate-to-severe ATR groups. The percentage of CD203c-positive quasi-basophils, stimulated with the residual transfused blood components, was represented in each of the three groups. The percentage of CD203c-positive quasi-basophils exceeds 7.2% (solid line), categorized as the pi-BAT positive. The Spearman correlation test was used to distinguish the significant correlation. A p value of <0.05 was considered as correction coefficient. (D) The ROC curve was drawn by calculating the sensitivity and specificity of the percentage of CD203c-positive quasi-basophils of moderate-to-severe ATR samples and non-ATR samples. The cutoff point on the ROC curve closest to the Youden index that was calculated as the maximum of (sensitivity + specificity – 1) were calculated.**

reaction, the PBMC suspension was neutralized by adding 1 mL of human serum albumin solution (HAS) (RPMI-1640 medium, pH 8.0, supplemented with 0.5% human serum albumin (HSA) and 12 mM Tris–HCl). The cells were centrifuged at 1200 × *g* for 5 minutes at 4°C. After washing with phosphate-buffered saline (PBS), the cell pellet was resuspended in 0.5 mL OF plasma supplemented with heparin sodium (10 U/mL) and 10 mM $CaCl_2$ and incubated for 60 minutes at 37°C. The plasma used was derived from blood samples of healthy laboratory personnel or 31 patients with NHTRs. Basophils in the cell pellet whose receptor-bound IgE was replaced by third-party IgE were defined as "quasi-basophils." PBMCs containing quasi-basophils ($2.5 \times 10^5$ cells/well) were incubated with 5 μL of supernatant from residual blood of the 31 patients with NHTR at 37°C for 30 minutes. These mixtures were then washed twice with PBS/bovine serum albumin/ethylenediaminetetraacetic acid. The samples were incubated at 4°C for 15 minutes with 10 μL of each of the following monoclonal antibodies: fluorescein isothiocyanate–conjugated anti-HLA-DR, PE-conjugated anti-CD203c, and PE-Cy5-conjugated anti-CD123. After washing with PBS/bovine serum albumin/ethylenediaminetetraacetic acid, the remaining RBCs were lysed at room temperature for 5 minutes by adding 200 μL FACS lysing solution, and the cells were subjected to flow cytometry. $HLA\text{-}DR^-$ and $CD123^+$ cells in the forward scatter versus side scatter blast cell window were identified as basophils. CD203c was used as a marker to identify activated basophils. The cursor was adjusted on the CD203c histograms at the point where 5% of the basophils were CD203c-positive without stimulation to calculate the percentage of CD203c-positive basophils. The receiver operating characteristic (ROC) curve was constructed by calculating the sensitivity and specificity under various cutoff levels using non-ATR samples and moderate-to-severe ATR samples as negative and positive controls, respectively. To determine the optimal cutoff point for the pi-BAT, we selected the point on the ROC curve closest to the Youden index that was calculated as the maximum of (sensitivity + specificity −1).

## RESULTS

### Activation ability of quasi-basophils composed of third-party basophils bearing patient IgE

The response of quasi-basophils bearing patient IgE was firstly checked with PBS as a negative control and then with an anti-human IgE monoclonal antibody as a positive control. As shown in Fig. 1B (left, middle), the background responses were quite low, whereas the maximum responses were significantly high, whether the IgE molecules were derived from patients with or without ATR.

### Comparison of the activation ability of quasi-basophils in the pi-BAT between patients with and without ATR

Subsequently, quasi-basophils were stimulated with supernatants of the corresponding residual blood components in the pi-BAT. As shown in Fig. 1B (right), the activation responses of quasi-basophils were significantly higher when the IgE of patients with ATR were stimulated with the corresponding residual ATR supernatants (ATR group) than when the IgE of those with non-ATR with the non-ATR supernatants (non-ATR group; $p = 0.001$).

Next, we attempted to verify the relevance between the quasi-basophil reactivity in the ATR group and the ATR severity. To classify ATR severity, we used a grade classification scale for evaluating symptoms of the immediate hypersensitivity-type reaction prepared by the Japanese Society of Allergology based on the Vetander's severity criteria.[16] These criteria are a modified form of the criteria previously developed by the World Health Organization according to medical conditions[17] (Table 2). Using the criteria described in Table 2, we classified

**TABLE 2. Classification of ATR into three stages of severity**

| Grade | Skin and mucous membrane | Digestive organ | Respiratory and cardiovascular | Neurological |
|---|---|---|---|---|
| 1 | • Erythema, urticaria, and wheal (partially)<br>• pruritus (self-control)<br>• Swelling (partially) | • Uncomfortability in interaoral and pharynx<br>• mild abdominal pain (self-control)<br>• Vomiting and diarrhea (single time) | • Cough, nasal discharge, nasal obstruction, sneezing (occasionally) | • Change in activity level, anxiety, tiredness |
| 2 | • Erythema, urticaria, and wheal (systemic)<br>• Pruritus (loss of self-control)<br>• Swelling (systemic) | • Sore throat<br>• Abdominal pain (self-control)<br>• Vomiting and diarrhea (multiple times) | • Cough (intermittently)<br>• Wheezing (auscultation), slight dyspnea<br>• Tachycardia (+15/min), achromasia | • Light headache, sleepiness, nightmare |
| 3 | • Any of the above | • Abdominal pain (loss of self-control)<br>• Repeated vomiting, fecal incontinence | • Barking cough<br>• Appreciable wheezing, dyspnea, cyanosis, dysphagia<br>• Hypotension*, severe bradycardia, evanescent cardiac arrest | • Disquiet, incontinence, unconsciousness |

* The hypotension is defined when systolic blood pressure dropped ≥30 mm Hg as compared with that before transfusion.

the severity of ATRs into three categories: mild, moderate, and severe. The mild group was designated as having only one of the clinical findings from Grade 1. The moderate group was designated as having multiple clinical findings from Grade 1 and/or only one clinical finding from Grade 2. The severe group was designated as having multiple clinical findings from Grade 2 and/or one or more findings from Grade 3. Since the numbers of cases with moderate and severe ATR (four and six, respectively) were limited, we combined them into one group (the moderate-to-severe ATR group) and compared the responses between the non-ATR, mild ATR, and moderate-to-severe ATR groups. The rate of CD203c-positive basophils was found to be strongly correlated with ATR severity (R = 0.746; Fig. 1C).

### Sensitivity and specificity of the pi-BAT

Due to the wide distribution of basophil activation in the pi-BAT of the moderate to severe ATR group (7.1-43.2), it was practically, except for one sample, distinguished from that of the non-ATR group (4.2-7.2). This indicates that both the sensitivity and specificity of the pi-BAT were high enough to distinguish the causal relationship between moderate-to-severe ATR and transfusion; the cutoff point, sensitivity, and specificity of the pi-BAT performed on moderate-to-severe ATR cases were 7.2, 0.9, and 1.0, respectively, when the ROC curve analysis was used (Fig. 1D). When this optimal cutoff point was adapted, the sensitivity to detect mild ATRs turned to be 0.25.

## DISCUSSION

This study showed high sensitivity and specificity of the pi-BAT in detecting moderate-to-severe ATRs. The reason why one moderate-to-severe ATR case showed negative remains unclear; however, three possibilities were assumed. First, the patient basophils were somewhat primed, whereas our quasi-basophils were not.[18] Second, the ATR occurred via the allergen/IgE-independent pathway. Third, it was not associated with transfusion. In contrast to moderate-to-severe ATRs, we calculated the sensitivity for mild ATRs as 0.25 by applying the cutoff point determined using non-ATR samples and moderate-to-severe ATR samples as negative and positive control samples, respectively. There is a possibility that pi-BAT cannot detect the relatively low level of activation of quasi-basophils in mild ATR cases. Considering that moderate-to-severe ATR cases are more important for determining the causative relationship between transfusion and ATR as well as further determining the appropriate blood source for the next round of transfusion, the pi-BAT is a very useful tool for diagnoses of moderate-to-severe ATR at the very least. Using samples from non-ATR, mild ATR, and moderate-to-severe ATR cases, we calculated the negative and positive predictive values. The negative predictive value was obtained by dividing the number of non-ATR cases that showed negative results by the number of all negative cases, which included non-ATR, mild ATR, and moderate-to-severe ATR cases. The positive predictive value was obtained by dividing the number of ATR cases that showed positive results by the number of all positive cases. As a result, the negative and positive predictive values of pi-BAT were 47% (9/19) and 100% (12/12), respectively.

CD63 and CD203c are representative surface markers for the identification of activated basophils. Although surface expression of CD63 corresponds to the early stage of basophil activation, it is also an activation marker for platelets. Platelet activation leads to the production of platelet microparticles with upregulation of P-selectin and CD63 expression on their surface. These activated platelets and microparticles may bind to basophils via PSGL-1, a ligand of P-selectin, leading to a false-positive result. In contrast, CD203c is expressed only by basophils and their precursors.[15] Additionally, in our previous studies, we observed that CD63 was a less sensitive marker compared with CD203c. Therefore, we believe that CD203c is a better marker for basophil activation compared with CD63.

Since IgA-deficient patients with anaphylactic transfusion reactions were first described in 1968,[19] more than 40 similar cases with immunoglobulin A (IgA)-related anaphylactic transfusion reactions have been subsequently reported. IgA-free blood products are transfused to IgA-deficient patients to prevent IgA-related adverse effects. Moreover, IgA-free blood donors are registered worldwide. However, the clinical entity of IgA-related anaphylactic transfusion reaction has not yet been established by evidence-based medicine.[20] Therefore, the pi-BAT system should also be invaluable in obtaining the etiological evidence for IgA-related and other plasma protein–related anaphylaxis in the future.

We successfully diagnosed ATR using the pi-BAT with a sample size from infantile and neonatal patients in cases of A11 and A19, as shown in Table 1. This provides a great advantage that even quite a limited amount of patient specimens are available for the examination.

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#### CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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