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Original Article

BAFF level increased in patients with autoimmune hemolytic anemia

Yu-Bing Zhao[1], Jun-Min Li[1], Bei-Wen Wei[2], Zi-Zhen Xu[2]

[1]Department of Hematology, Ruijin Hospital, Shanghai Jiao-Tong University School of Medicine, Shanghai, China; [2]Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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Abstract: Introduction: BAFF (B-cell activating factor of the TNF family), an important regulator of B-cell, has been observed to be over-expressed in a variety of autoimmune diseases. Autoimmune hemolytic anemia (AIHA) is an acquired autoimmune disease occurred when antibodies directed against autologous red blood cells. We assessed serum levels of BAFF in AIHA patients with different serological characteristics. Methods: Serum BAFF levels were measured in 44 AIHA patients with different direct antiglobulin test (DAT) results and 25 healthy controls. The correlation of BAFF expression with DAT results and serological characteristics was assessed. Results: Serum levels of BAFF in AIHA patients were significantly higher than in healthy subjects (AIHA: 1382.7 ± 1412.8 pg/ml, healthy control: 725.0 ± 415.7 pg/ml, P = 0.0057). Serum BAFF levels were significantly higher in patients with IgG(+)C3(+) or IgG(+) than healthy controls (DAT: negative) (P = 0.012, 0.004, respectively). No significant correlations were presented between serum BAFF levels and four serological parameters: hemoglobine, percentage of reticulocyte, total serum bilirubin, and lactate dehydrogenase. Conclusions: AIHA patients present higher serum BAFF levels than healthy controls, especially for those of IgG(+)C3(+) DAT result. This might lead to a new approach of AIHA treatment.

Keywords: B-cell activating factor, autoimmune hemolytic anemia, direct antiglobulin test

Introduction

BAFF (B-cell activating factor of the TNF family), is an important regulator of B-lymphocytes proliferation, differentiation and survival, which is normally expressed by macrophages, monocytes, and dendritic cells [1]. It plays critical roles in B-cell homeostasis, variability and malignant transformation by binding and activating three receptors: BAFF receptor (BAFF-R; also known as BR3), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and B-cell maturation antigen (BCMA) [2, 3]. Aberrant BAFF expression has been observed in a variety of autoimmune diseases, i.e. systemic lupus erythematosus, rheumatoid arthritis, and several types of lymphoma, and presented correlations with clinical activity or survival status of several diseases [4-7]. Autoimmune hemolytic anemia (AIHA) is an acquired autoimmune disease occurred when antibodies directed against autologous red blood cells [8], which can be either primary or secondary to lymphoproliferative disease, infections, immunodeficiency, and tumors. It was suggested that the loss of immunologic tolerance to RBC self-antigens may arise by various mechanisms: ignorance against RBC self-antigens, molecular mimicry, poly-clonal T- and/or B-cell activation, errors in central or peripheral tolerance, and immunoregulatory disorders including cytokine network alteration [9]. As the BAFF is associated with B-cell and autoimmune diseases, we have reason to believe that the BAFF is correlated with AIHA. However, few studies in the literature had been focused on the expression of BAFF in AIHA patients. We therefore concentrated on the expression of BAFF in peripheral blood of AIHA patients and the correlation of levels of BAFF expression with clinical and serological characteristics of AIHA patients.

Table 1. Clinical and serologic characteristics for 44 patients with AIHA

Parameters		Total (N = 44)
gender	Female/male	28/16
Age (years)	Median	48.5
	Range	2-85
Primary AIHA		33
Secondary AIHA		11
Lymphoproliferative disorders		7
Follicular lymphoma		1
Diffuse large B-cell lymphoma		1
Angioimmunoblastic T-cell lymphoma		3
Waldenstrom's macroglobulinemia		1
Others		4
Myelodysplastic syndromes		1
Primary biliary cirrhosis		1
Panhematopenia		1
Pure red cell aplasia		1
Serologic characteristics		
Hemoglobin	< 9 g/dl/≥ 9 g/dl/NA	28/14/2
Percentage of reticulocyte	> 2%/≤ 2%/NA	31/11/2
Total serum bilirubin	> 2 mg/dl/≤ 2 mg/dl/NA	14/17/13
LDH	> 500 IU/ml/≤ 500 IU/ml/NA	0/10/34

*AIHA: autoimmune hemolytic anemia, LDH: lactate dehydrogenase, NA: not available; *Serologic information at diagnosis with several missing data was involved.

Materials and methods

Patients and control donors

44 patients primarily diagnosed as AIHA in Shanghai Ruijin hospital in 2014 were included in this study. Direct antiglobulin test (DAT) for the detection of IgG and complement bound to RBCs was performed with the tube technique using the standard method with poly-specific anti-human globulin and monospecific anti-IgG and anti-C3 antisera. Only patients with a positive DAT test (IgG, C3 or both positive) were involved in this study. Complete clinical examination, complete blood counts, hemolytic markers (reticulocytes, total and unconjugated bilirubin, lactate dehydrogenase, and hematoglobin), were performed. Control blood samples were taken from 25 healthy human volunteers. Informed consent was obtained from all patients in accordance with the regulations of the Shanghai Jiao Tong University School of Medicine Institutional Review Boards.

Processing of patient plasma

Whole blood was drawn into standard EDTA-containing collection tubes. Plasma was separated from whole blood cells by centrifugation at 2500 g. Plasma was stored in aliquots at -80°C and used after first thaw for BAFF measurements.

Enzyme-linked immunosorbent assay

The concentrations of BAFF were measured with human BAFF/BlyS/TNFSF13B immunoassay kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. ELISA plates were coated overnight with monoclonal mouse IgG anti-human BAFF at 1 μg/mL in phosphate-buffered saline. After nonspecific binding had been blocked with 0.5% bovine serum albumin, the samples were added, followed by the detection antibody, biotinylated goat anti-human BAFF (R & D Systems). Streptavidin horseradish peroxidase and 3, 3', 5, 5'-tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO, USA) were used for detection. The reaction was stopped with 0.5 M H_2SO_4, and the enzyme activity was read at an optical density of 450 nm. A seven-point standard curve starting at 20 pg/mL of recombinant BAFF was generated.

Statistical analysis

All data are shown as means and standard error (mean ± standard error) in the text. We used the Mann-Whitney U test to compare unpaired data. For multiple comparisons, the ANOVA was used. Linear model was applied for regression analysis. Correlation coefficients were calculated by Spearman's test. A two-sided *P*-value < 0.05 was considered statistically significant.

Results

Clinical and serologic characteristics of the patients

The main clinical and serologic characteristics of the patients are summarized in **Table 1**.

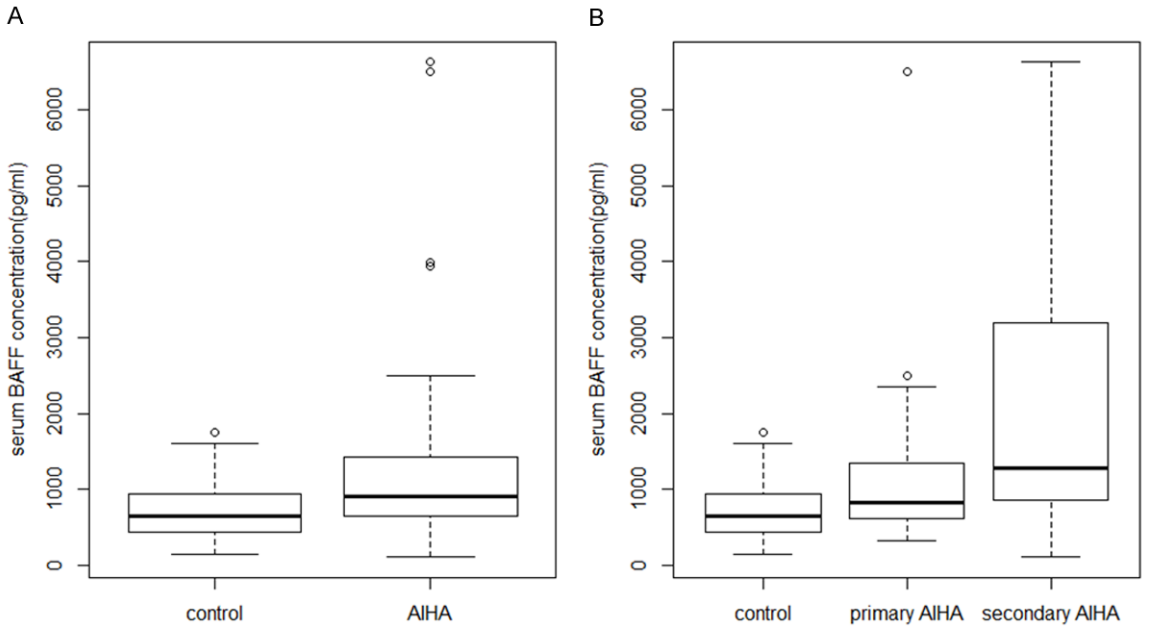

Figure 1. Serum levels of BAFF in AIHA patients and healthy controls. A: Serum levels of BAFF in AIHA patients and healthy controls. Serum levels of BAFF in AIHA patients were significantly higher than in healthy subjects (P = 0.0057). B: Serum levels of BAFF in primary AIHA patients, secondary AIHA patients and healthy controls. There was no significant difference between serum levels of BAFF in secondary AIHA patients and in primary AIHA patients (P = 0.1392). Serum levels of BAFF in secondary AIHA patients were significantly higher than in healthy controls (P = 0.0414). Serum levels of BAFF in primary AIHA patients and in healthy controls were not significantly different (P = 0.0556). AIHA: autoimmune hemolytic anemia. Data are presented as box plots, where the lines inside the boxes represent the medians, the boxes represent the 25th and 75th percentiles and the lines outside the boxes represent the 10th and 90th percentiles.

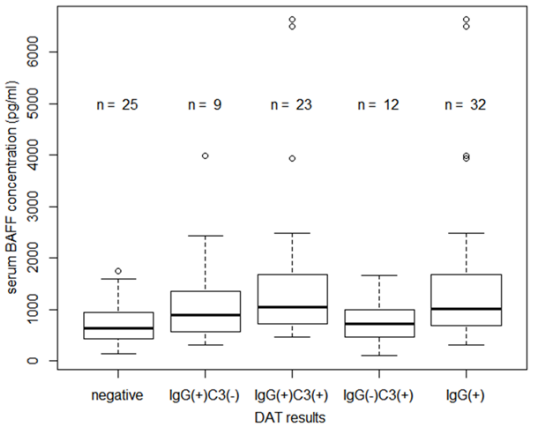

Figure 2. Serum levels of BAFF in AIHA patients with different DAT results and healthy control. Serum BAFF levels were significantly higher in patients with IgG(+) than in patients with IgG(-) (P = 0.008). Serum BAFF levels were significantly higher in patients with IgG(+)C3(+) or IgG(+) than healthy controls (DAT: negative) (P = 0.012, 0.004, respectively). Data are presented as box plots, where the lines inside the boxes represent the medians, the boxes represent the 25th and 75th percentiles and the lines outside the boxes represent the 10th and 90th percentiles.

Totally, 44 patients with AIHA (28 female [63.6%]) were included in this study. The median age at diagnosis was 48.5 years (range, 2-85 years). Secondary AIHA was observed in 25% of the patients (11/44). Their primary diseases were consisted of lymphoproliferative disorders: follicular lymphoma, diffuse large B-cell lymphoma, angioimmunoblastic T-cell lymphoma and Waldenstrom's macroglobulinemia, and other diseases: myelodysplastic syndromes, primary biliary cirrhosis, panhematopenia and pure red cell aplasia. With the limitation of clinic follow-up, some information of several patients was missed. The hemoglobin was < 9 g/dl in 28 of the patients, and ≥ 9 g/dl in 14 of the patients. The percentage of reticulocyte was > 2% in 31 patients and ≤ 2% in 11 patients. The total serum bilirubin was > 2 mg/dl in 14 patients and ≤ 2 mg/dl in 17 patients. The LDH was > 500 IU/ml in 0 patients and ≤ 500 IU/ml in 10 patients.

Among all AIHA patients, 20.5% patients (9/44) had IgG detected only on the red cell surface,

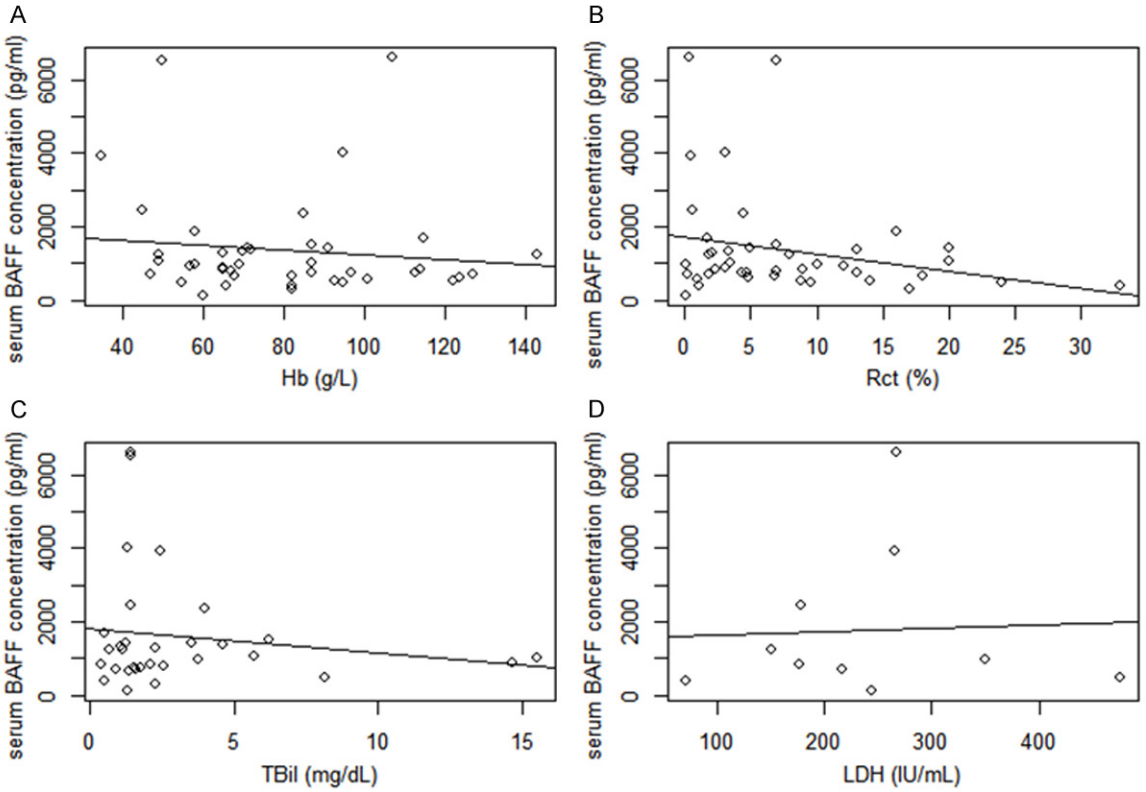

Figure 3. Serum levels of BAFF in AIHA patients according to serologic characteristics (Hemoglobin: Hb, percentage of reticulocyte: Rct, total serum bilirubin: TBil, and lactate dehydrogenase). A: Serum levels of BAFF according to Hb. Serum levels of BAFF decreased with the Hb ($P > 0.05$, R-square = 0.014). B: Serum levels of BAFF according to Rct. Serum levels of BAFF decreased with Rct ($P > 0.05$, R-square = 0.060). C: Serum levels of BAFF according to TBil. Serum levels of BAFF decreased with Tbil ($P > 0.05$, R-square = 0.023). D: Serum levels of BAFF according to LDH. Serum levels of BAFF increased with LDH ($P > 0.05$, R-square = 0.003). The regression line is represented by the solid line.

52.3% patients (23/44) had both IgG and C3 detected on the red cell surface and 27.3% patients (12/44) had C3 detected only on the red cell surface (**Figure 2**).

Serum levels of BAFF in AIHA patients and healthy controls

Serum levels of BAFF in AIHA patients (n = 44) and healthy controls (n = 25) were presented in **Figure 1A**. Serum levels of BAFF in AIHA patients were significantly higher than in healthy subjects (AIHA: 1382.7 ± 1412.8 pg/ml, healthy control: 725.0 ± 415.7 pg/ml, P = 0.0057). Serum levels of BAFF in primary AIHA patients (n = 33), secondary AIHA patients (n = 11) and healthy controls (n = 25) were presented in **Figure 1B**. There was no significant difference between serum levels of BAFF in secondary AIHA patients and in primary AIHA patients (primary AIHA: 1134.7 ± 1096.9 pg/ml, secondary AIHA: 2026.9 ± 1980.2 pg/ml, P = 0.1392). Serum levels of BAFF in secondary AIHA patients were significantly higher than in healthy controls (P = 0.0414). Serum levels of BAFF in primary AIHA patients and in healthy controls were not significantly different (P = 0.0556).

In **Figure 2**, serum BAFF levels with different DAT results were compared. Serum BAFF levels were significantly higher in patients with IgG(+) than in patients with IgG(-) (P = 0.008). Significant difference was also observed between IgG(+)C3(+) and IgG(-) C3(+) patients (P = 0.017). Serum BAFF levels were significantly higher in patients with IgG(+)C3(+) or IgG(+) than healthy controls (DAT: negative) (p = 0.012, 0.004, respectively), while Serum BAFF levels in patients with IgG(+) only or C3(+) had no significant difference with in healthy controls.

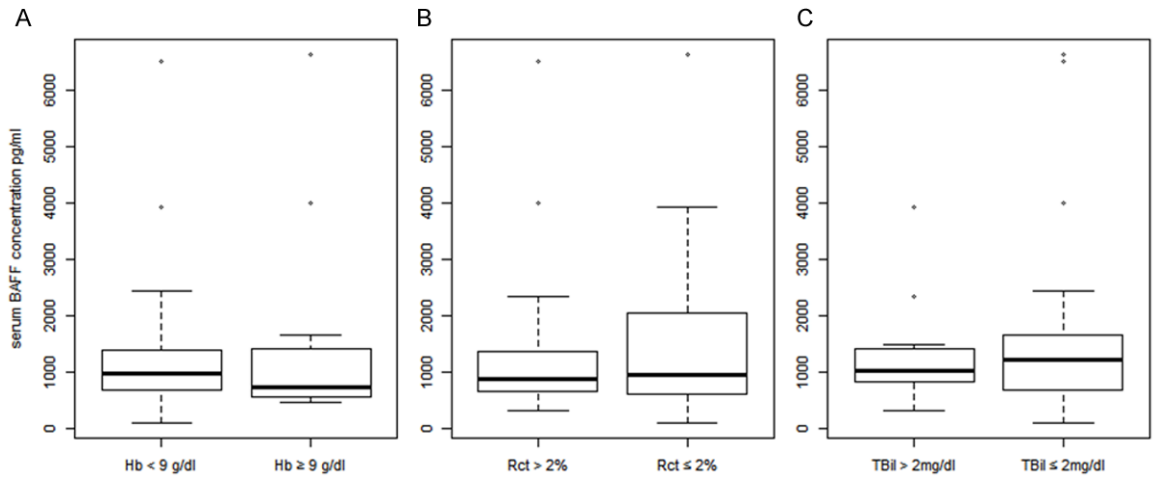

Figure 4. Serum levels of BAFF in AIHA patients with different levels serologic characteristics (Hemoglobin: Hb, percentage of reticulocyte: Rct, and total serum bilirubin: TBil). A: Serum levels of BAFF in patients with different levels of Hb. There was no significant difference between serum levels of BAFF in patients with higher Hb levels: Hb ≥ 9 g/dl and with lower Hb levels: Hb < 9 g/dl. B: Serum levels of BAFF in patients with different levels of Rct percentage. There was no significant difference between serum levels of BAFF in patients with higher Rct levels: Rct > 2% and with lower Rct levels: Rct ≤ 2%. C: Serum levels of BAFF in patients with different levels of serum TBil. There was no significant difference between serum levels of BAFF in patients with higher Tbil levels: Tbil > 2 mg/dl and with lower Tbil levels: Tbil ≤ 2 mg/dl. Data are presented as box plots, where the lines inside the boxes represent the medians, the boxes represent the 25th and 75th percentiles and the lines outside the boxes represent the 10th and 90th percentiles.

Serum BAFF levels with different serological characteristics

We assessed the relationship between serum BAFF levels and serological parameters obtained from AIHA patients.

There were no significant correlations between serum BAFF levels and four serological parameters: hemoglobine, percentage of reticulocyte, total serum bilirubin, and lactate dehydrogenase (**Figure 3**). This was accordant to results showed in **Figure 4**: there were no significant differences between serum levels of BAFF in patients with higher Hb levels: Hb ≥ 9 g/dl and with lower Hb levels: Hb < 9 g/dl, in patients with higher Rct levels: Rct > 2% and with lower Rct levels: Rct ≤ 2%, and in patients with higher Tbil levels: Tbil > 2 mg/dl and with lower Tbil levels: Tbil ≤ 2 mg/dl, respectively.

Discussions

AIHA is an uncommon disease with the overall incidence being 1 in 80,000 to 100,000 of a given population/year in the Caucasians [10]. More than 70% of new cases are seen annually in patients above 40 years of age, and the frequency of the disorder is usually more in females than in males (male to female ratio: 40:60) [10]. It was observed in our study that 56.8% of the patients were above 40 years old with a female predominance (63.6%), which was accordant to other studies. It was reported in literature that lymphoproliferative diseases such as chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma and Waldenstrom's macroglobulinemia are the leading causes of secondary AIHA cases [10]. In our study, we also found 4 secondary AIHA patients associated with myelodysplastic syndromes, primary biliary cirrhosis, panhematopenia and pure red cell aplasia, respectively, which has not been widely reported in previous studies [11-13].

Although the relationship between BAFF expression and AIHA remains unclear, we first demonstrated that the serum BAFF level was significantly increased in AIHA patients than in healthy controls. As overexpression of BAFF induces B cell hyper activation and autoimmunity in mice [14], BAFF has been considered a promoting factor in the pathogenesis of several autoimmune and allergic diseases. Furthermore, BAFF-targeting therapies are being applied for several human diseases [15].

Recently, Belimumab, a human immunoglobulin G monoclonal antibody specific for soluble human B lymphocyte stimulator protein (BLyS, also known as BAFF), has been applied as a new treatment for systemic lupus erythematosus (SLE) [16, 17]. It is able to inhibit the survival of B cells, including autoreactive B cells, and it reduces the differentiation of B cells into Ig-producing plasma cells. Therefore, Belimumab might be a new solution for AIHA patients, especially for refractory AIHA patients. In AIHA patients, whether red blood cell was coated with IgG, complement (C3) or both was determined by DAT test. It was found that patients with both IgG(+) and C3(+) had significantly higher serum BAFF levels than healthy controls whereas patients with only IgG(+) or C3(+) showed no significant difference of the serum BAFF levels with healthy controls. The mechanism was not clear. However, this finding might be able to give reference for whether monoclonal anti-BAFF antibody could be effective in specific AIHA patients.

We also tested the correlation between serum BAFF levels and different serologic characteristics. The severity of AIHA was determined by hemolysis degree. In some research [18, 19], the hemolysis was classified into moderate and severe on the basis of whether two/there, or all the four laboratory parameters, respectively, were abnormal: 1. Hb (< 9 g/dl), 2. Percentage of reticulocyte (> 2%), 3. Total serum bilirubin (> 2 mg/dl), and 4. LDH (> 500 IU/ml). However, we found no significant correlation between serum BAFF levels and the former 4 serological parameters. This might imply that serum BAFF levels were not correlated with AIHA severity. Nevertheless, that might also be a consequence of limited cases involved and non-ignorable missing data. Further work could be focused on testing the correlation between serum BAFF levels and AIHA severity, finding the correlation between serum BAFF levels and survival status of AIHA patients, and reveling the mechanism of increased serum BAFF levels in AIHA patients, especially in those with IgG(+) C3(+) DAT results.

Taken together, we have found increased serum levels of BAFF in AIHA patients, especially in those with IgG(+)C3(+) DAT results. In addition, increased levels of BAFF in AIHA patients may not associated with disease severity. Our findings lead to the potential implication of applying BAFF-targeting therapies to AIHA patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zi-Zhen Xu, Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. Tel: 86-21-64370045-610506; E-mail: xuzizhen@126.com

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