

# Skewed expression profile of receptors for BAFF on peripheral blood B lymphocytes in Graves' Disease

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## Research article

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# Abstract

## Background

Previous studies have demonstrated altered serum B lymphocyte activating factor (BAFF) level and expression of BAFF and BAFF receptor (BAFF-R) in thyroid infiltrating lymphocytes in Graves' disease (GD) patients. The aim of the present study was to investigate expression of receptors for BAFF on the peripheral blood B lymphocytes in addition to serum BAFF concentrations.

## Methods

Fifty-two GD patients and twenty-three healthy controls (HC) were recruited in this study. Serum BAFF concentrations were measured by ELISA. Expression of receptors for BAFF on peripheral blood B lymphocytes, including BAFF-R and transmembrane activator and calcium-modulating and cyclophilin ligand interactor (TACI), was analyzed by flow cytometry. Serum BAFF levels, expression of BAFF-R and TACI on peripheral blood B lymphocytes were compared between GD and HC groups. Correlations between serum BAFF concentrations and thyroid function and thyrotropin receptor autoantibody (TRAb) titers were analyzed in GD patients. In 10 patients with Graves' orbitopathy (GO) receiving steroids therapy, effects of steroids on serum BAFF levels and expression of BAFF-R and TACI were observed.

## Results

Serum BAFF levels were significantly higher in GD patients ( $1.18 \pm 0.33$  ng/ml) than those in HC ( $0.93 \pm 0.24$  ng/ml,  $P = 0.0011$ ). In GD patients, serum BAFF levels correlated positively with free thyroxine concentrations (Spearman  $r = 0.2460$ ,  $P = 0.0394$ ), but not TRAb titers ( $P = 0.4734$ ). BAFF-R expression on peripheral B lymphocytes were elevated in GD (mean MFI:  $4.52 \pm 2.06$  in GD vs  $3.00 \pm 0.87$  in HC,  $P < 0.0001$ ), while TACI expression on peripheral B lymphocytes were decreased in GD (mean MFI:  $7.96 \pm 4.06$  in GD vs  $9.10 \pm 3.37$  in HC,  $P = 0.0331$ ). Steroids significantly suppressed serum BAFF concentrations (from  $1.18 \pm 0.27$  ng/ml to  $0.97 \pm 0.10$  ng/ml,  $P = 0.0364$ ) and BAFF-R expression on peripheral blood B lymphocytes in GO patients (mean MFI from  $6.26 \pm 4.91$  to  $4.05 \pm 1.58$ ,  $P = 0.0083$ ). Change of TACI expression on B lymphocytes was not statistically significant after steroids therapy.

## Conclusions

Skewed expression profile of receptors for BAFF on B lymphocytes may mediate the autoimmunity in GD. Restoring the normal expression profile of receptors for BAFF could be a new strategy for treating and cueing GD.

## Introduction

Graves' disease (GD) is an autoimmune disease mediated by thyrotropin receptor specific autoantibody (TRAb)[1]. TRAb secreted from the autoreactive B lymphocytes binds to thyrotropin receptor and stimulates the over production of thyroid hormone [1]. The persistent existing of the TRAb secreting B lymphocytes lead to the high relapse rate of GD. Targeting these autoreactive B lymphocytes could be a new strategy to treat GD [1].

B lymphocyte activating factor (BAFF, also known as Blys), a member of the tumor necrosis factor (TNF) family, promotes B lymphocytes proliferation, differentiation and survival [2]. BAFF rescue low-affinity auto-reactive transitional B lymphocytes at tolerance checkpoints and promotes their maturation [2, 3]. Overexpression of BAFF in mice induced a dramatic expansion of activated autoreactive B lymphocytes and autoantibody production [4]. In humans, over produced BAFF was also linked to autoimmune diseases. Serum BAFF concentrations are elevated in autoimmune diseases including systemic lupus erythematosus (SLE) [5], autoimmune hepatitis [6] and primary Sjögren's syndrome [7].

Accumulated evidences support BAFF is associated with autoimmune thyroid diseases (AITD). BAFF gene polymorphisms are susceptibility variants for the occurrence of GD [8, 9]. Patients with either GD or autoimmune thyroiditis showed elevated serum BAFF concentrations [10–12]. BAFF also associated with the onset of thyroid autoimmunity in chronic hepatitis C patients receiving interferon alpha therapy [13]. Blocking BAFF function with soluble BAFF receptor fusion protein relief hyperthyroidism in a murine model [14]. Finally, immune suppressive effect of steroids in patients with Graves' orbitopathy (GO) is mediated by blocking BAFF secretion [11].

BAFF exerts its biological effect through interacting with three cell surface molecules: BAFF receptor (BAFF-R, CD268; also known as TNFRSF13C or BR-3) [15], transmembrane activator and calcium-modulating and cyclophilin ligand interactor (TACI, CD267; also known as TNFRSF13B) [16] and B cell maturation antigen (BCMA; also known as TNFRSF17) [17]. They have the different expression profiles based on B cell developmental stages [3, 18]. BAFF-R is the dominant BAFF receptor expressed on almost all B lymphocytes expressing functional B cell receptor (BCR), including naïve B cells, marginal zone B cells and switched memory B cells. TACI is mainly expressed by CD27<sup>+</sup> memory B cells and a small part of plasma cells. Expression of BCMA is restricted on plasmablasts and plasma cells. Campi *et al.* found thyroid infiltrating lymphocytes (TIL) in AITD expressed more BAFF and BAFF-R than those in multinodular goiter [19]. However, it is not clear whether expression of receptors for BAFF on peripheral blood B lymphocytes are altered in the background of GD.

In this study, to disclose how the interactions between BAFF and its different receptors promote the autoimmunity of GD, we measured serum BAFF levels and the expression of two BAFF receptors, BAFF-R and TACI, on the peripheral blood B lymphocytes in GD patients. Furthermore, effects of steroid on expression of BAFF and its receptors in GO patients were also observed.

## Materials And Methods

## Power Calculation and Patients

Power was calculated by R package. We set the Type I Error=0.05 and the Type II Error=0.2. Based on data of our initial assay, at least 14 subjects should be enrolled in each group to achieve the hypothesis being tested and these error rates, so 52 newly diagnosed GD patients and 23 healthy controls (HC) were recruited from Jiangsu Province Hospital of TCM/the Affiliated Hospital of Nanjing University of Chinese Medicine.

## Therapy

All patients received methimazole therapy. Initial methimazole dosage was determined by serum free tetraiodothyronine concentration according to the American thyroid association (ATA) guideline for management for hyperthyroidism [20]. Ten patients having clinical activity score (CAS) above 3/7 at the first examination were diagnosed with active GO according to the European Group of Graves' Orbitopathy (EUGOGO) classification system [21]. These patients underwent methylprednisolone iv injection in addition to administration of methimazole. Methylprednisolone was administered 500mg weekly in the first 6 weeks and 250 mg weekly in the following 6 weeks as Zhu *et al.* described [22].

## Thyroid function and TRAb titers assay

Thyroid function, including serum thyroid stimulating hormone (TSH), free thyroxine (FT4) and free triiodothyronine (FT3) levels, and TRAb titers, were measured by Roche Elecsys electrochemiluminescence immunoassay kit.

## BAFF ELISA

Serum BAFF concentrations were determined by ELISA according to the Quantikine ELISA kit instruction (R&D systems, DBLYS0B). Sensitivity of the kit was 2.68 pg/ml. Intra-assay coefficient of variation of the kit was 5.6%. All the samples were measured duplicate. Blank and serially diluted recombinant BAFF from 4,000 pg/ml to 62.5 pg/ml were assayed at the same time to establish standard curve. Concentration of BAFF in each sample was calculated according to the formula deducted from the standard curve.

## Cell Staining and Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from 5ml freshly drawn anti-coagulated blood according to standard procedure on lymphoprep (StemCell Technologies) and blocked with FcR blocking

buffer (Miltenyi Biotec, cat number: 130-059-901) for 10 minutes at 4°C in the dark. Cells were then stained with fluorescein isothiocyanate (FITC) conjugated anti human CD19 (thermofisher, cat number: 11-0199-42) together with either phycoerythrin (PE) conjugated anti human BAFF-R (thermofisher, cat number: 12-9117-42) or PE conjugated anti human TACI (thermofisher, cat number: 12-9217-42) for 30 minutes at 4°C in the dark. After being washed twice with pH 7.4 PBS, cells were immediately acquired by Navios flow cytometer (Beckman, U.S.) and analyzed by Kaluza Flow Cytometry Analysis Software Version 2.0 (Beckman, U.S.). For each test, at least 50,000 PBMCs were acquired. Mean fluorescent intensity (MFI) of expression of BAFF-R and TACI at the surface of CD19<sup>+</sup> cells were determined. Data are represented as Mean ± SD

## Statistics

Statistical analysis was performed by Graphpad Prism Version 6.0. Mann-Whitney U test was used for nonparametric distribution data. Correlations were examined by Spearman's rank correlation tests. Significant was considered when *P* values were not higher than 0.05.

## Results

### 1. Subjects characteristics

Demographic data of the two groups, thyroid function and TRAb titers of the GD group were listed in Table 1. Although more females were recruited in the GD group, there were no differences of age and gender ratio between the two groups.

### 2. Serum BAFF levels were elevated in GD patients and positively correlated with free thyroxine concentrations

Serum BAFF levels were elevated in autoimmune diseases including GD in serial studies. To confirm these findings, we measured serum BAFF concentrations in both GD patients and healthy individuals by ELISA. As shown in Figure 1A, serum BAFF levels in patients with GD were significantly higher ( $1.18 \pm 0.33$  ng/ml) than those in healthy individuals ( $0.93 \pm 0.24$  ng/ml,  $P=0.0011$ , Figure 1A). This was in line with previous studies.

Despite previous reports showed serum BAFF levels were associated with activity of autoimmune disease, there were conflicting conclusions on the relation between BAFF expression and autoantibody titers. We thus plotted correlations between BAFF levels with either thyroid function or TRAb titers. Our data showed in GD patients, serum BAFF levels positively correlated with serum free thyroxine concentrations (Spearman  $r=0.2460$ ,  $P=0.0394$ , Figure 1B), but not the TRAb titer ( $P=0.4734$ , Figure 1C).

### 3. Skewed expression profile of receptors for BAFF on peripheral blood B lymphocytes in Graves' disease

Receptors for BAFF include BAFF-R, TACI and BCMA. Because BCMA is mainly expressed in plasmablasts and plasma cells, which are very few in peripheral blood, we only detected BAFF-R and TACI in this study. Both two molecules were expressed on peripheral blood B cells. Majority of the peripheral B lymphocytes expressed BAFF-R in our assay. Expression of BAFF-R on peripheral blood B lymphocytes in GD patients was higher comparing with that in healthy controls (mean MFI:  $4.52 \pm 2.06$  in GD vs  $3.00 \pm 0.87$  in HC,  $P < 0.0001$ , Figure 2A). TACI was not expressed on all CD19<sup>+</sup> cells. There was no difference of percentages of B cells expressing TACI between healthy controls and GD patients (data not shown). However, as shown in figure 2B, expression of TACI on peripheral blood B lymphocytes decreased in GD patients (mean MFI:  $7.96 \pm 4.06$  in GD vs  $9.10 \pm 3.37$  in HC,  $P = 0.0331$ ). These data suggested skewed expression of receptors for BAFF on peripheral blood B lymphocytes in GD patients.

### 4. Skewed expression profile of receptors for BAFF was corrected after steroids therapy in GO

Steroids therapy was shown to suppress serum BAFF concentrations in GO patients, its effect on BAFF receptors expression needs explored. We therefore measured serum BAFF concentrations, expression of BAFF-R and TACI on peripheral blood B lymphocytes before and after steroids therapy in 10 GO patients. Mean serum BAFF concentrations decreased from  $1.18 \pm 0.27$  ng/ml to  $0.97 \pm 0.10$  ng/ml after a 12-week iv injection of methylprednisolone ( $P = 0.0364$ , Figure 3A). Steroids also decreased BAFF-R expression on peripheral blood B lymphocytes in GO patients from  $6.26 \pm 4.91$  to  $4.05 \pm 1.58$  ( $P = 0.0083$ , Figure 3B). There was a trend of increased TACI expression in B lymphocytes after steroids therapy (TACE MFI:  $6.67 \pm 1.96$  before steroids therapy vs  $7.05 \pm 2.32$  after steroids therapy), however, the difference was not statistical significance ( $P = 0.1974$ , Figure 3C). These results indicated immunol regulation effect of steroids on B lymphocytes is not only on the B cell surviving ligand BAFF, but also on its receptors, mainly BAFF-receptor.

## Discussion

More than half of newly generated B lymphocytes have some degree of autoreactivity [23]. These immature autoreactive B cells are extreme sensitive to apoptosis because they express high levels of death receptor Fas, a mechanism to eliminate autoreactive B cells [24]. However, the over expressed BAFF and its receptors may subvert the destiny of apoptosis of these autoreactive B lymphocytes by inducing the expression of anti-apoptotic factors and by blocking the function of pro-apoptotic molecule [25]. The rescued autoreactive B lymphocytes could mediate autoimmune disease through either the secreted autoantibodies or antigen presenting [25]. Altered serum BAFF levels and intra-thyroid BAFF and its receptors expression levels have been observed in autoimmune thyroid disease [10, 12, 19]. Different to

these researches, we measured BAFF receptors BAFF-R and TACI expression on peripheral blood B lymphocytes in addition to serum BAFF concentrations in this study.

We found elevated serum levels of BAFF in GD patients, suggesting a possible role of BAFF in thyroid autoimmunity. This is in line with other studies describing increased serum BAFF levels in thyroid autoimmune diseases including GD and Hashimoto's thyroiditis (HT) [10, 12]. Our data showed serum BAFF concentrations correlated positively with free thyroxine concentrations, confirming serum BAFF levels positively correlating with activity of autoimmune diseases. There was no correlation between serum BAFF concentrations and TRAb titers based on our data. The correlation between serum BAFF levels and titers of autoantibodies are controversial as different results have been reported [7, 12, 26]. In our assay, TRAb was measured by a competing electrochemiluminescence immunoassay method [27]. TRAb measured by this method was only that having the same epitope with monoclonal antibody M22, but not the whole TSH receptor autoantibodies in the blood. BAFF levels may correlate with the whole autoantibodies titers, but not the titer of autoantibody with the specific epitope, this could be the reason we did not observe correlation between BAFF levels with TRAb titers in our study.

The most significant finding of our work was the skewed expression of receptors for BAFF on peripheral blood B lymphocytes in GD patients: the BAFF-R expression was increased while the BAFF receptor TACI expression was decreased. Our results were not in line with reports of reduced BAFF-R expression on peripheral blood B cells in other autoimmune disease as SLE [15]. Conflicting results of TACI expression on peripheral B lymphocytes in SLE patients have been reported [5, 15]. However, B lymphocytes infiltrating in thyroid expressed more BAFF-R, these data combined with our results [19], indicating different expression profiles of receptors for BAFF among autoimmune diseases.

The skewed expression of BAFF-R and TACI in GD may reflect the different functions of these two molecules [2, 3]. BAFF-R and TACI have different binding affinity to BAFF [3]. BAFF-R and TACI oppositely regulates B cell homeostasis [2, 16, 28, 29]. BAFF-R promotes B cells survival while TACI sensitizes B cells to apoptosis. Knocking out TACI led to elevated B cells number and SLE-like symptoms in the presence of over expressed BAFF in mice [28]. TACI activation also mediates immunosuppression through inducing interleukin (IL)-10 production in B lymphocytes [30, 31]. IL-10 is an immunosuppression cytokine. IL-10 secreting B cells are named Bregs (regulatory B cells) or B10 cells. Overexpressed TACI promoted more IL-10 expression in B lymphocytes in SLE patients [30]. Loss of Bregs has been described in GD patients [32]. We may speculate in GD patients, in the context of high levels of BAFF and skewed expression of BAFF-R and TACI on B lymphocytes, BAFF-R pathway activity was enhanced while TACI pathway activity was relatively diminished, which leads to the accumulation of autoreactive B cells and loss of Bregs. The skewed expression of BAFF-R and TACI is the key mechanism leading to autoimmunity in GD. Totally blocking both BAFF-R and TACI signal pathway will only augment inflammation due to the inhibited IL-10 secretion, as observed in multiple sclerosis (MS) patients [33]. To selective inhibit the BAFF-R pathway and to restore the TACI pathway function could be a novel strategy to cure GD. This strategy may eliminate the autoreactive B lymphocytes, thus with the potential to prevent relapse of GD.

Our data indicated steroids targets BAFF and BAFF-R, but not TACI. There were no previous studies reporting effects of steroids on BAFF-R expression in autoimmune diseases in the literature to our knowledge. In large B cell lymphoma, only BAFF-R, rather than BAFF, was an independent prognostic factor for steroids treatment [34], highlighting BAFF-R is an important target for steroids in eliminating B lymphocytes. Due to the small sample size of patients receiving steroids, the effect of steroids on TACI expression need be studied in the future with larger sample size.

## Conclusions

In conclusions, skewed expression of receptors for BAFF were associated with autoimmunity of GD. Restoring the expression profile of receptors for BAFF could be the new strategy to treat and cue GD.

## Abbreviations

APRIL

a proliferation-inducing ligand

AITD

autoimmune thyroid diseases

ATA

American Thyroid Association

BAFF

B lymphocyte activating factor

BAFF-R

BAFF receptor

BCMA

B cell maturation antigen

BCR

B cell receptor

Bregs

regulatory B cells

CAS

clinical activity score

ECL

electrochemiluminescence

ELISA

enzyme linked immunosorbent assay

EUGOGO

European Group of Graves' Orbitopathy

FITC

fluorescein isothiocyanate

GD  
Graves' disease  
GO  
Graves' orbitopathy  
HC  
healthy controls  
HT  
Hashimoto's thyroiditis  
MFI  
mean fluorescent intensity  
PBMC  
peripheral blood mononuclear cells  
PE  
phycoerythrin  
RA  
rheumatoid arthritis  
SLE  
systemic lupus erythematosus  
TAC1  
transmembrane activator and calcium-modulating and cyclophilin ligand interactor  
TIL  
thyroid infiltrating lymphocytes  
TNF  
tumor necrosis factor  
TRAb  
thyrotropin receptor autoantibody

## **Declarations**

### **Ethnic approval and consent to participate**

This study was in accordance with the Helsinki declaration of 1975, as revised in 2008 and approved by Institutional Review Board (IRB) of the Affiliated Hospital of Nanjing University of Chinese Medicine. Written informed consent was obtained from all participants.

### **Consent for publication**

Not applicable.

## Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

## Declarations

### *Competing interests*

The authors declare that they have no conflict of interest.

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## Authors' contributions

X.W., Q.M. and P.J. contributed to data analysis, manuscript drafting and critical discussion. X.W., J.H. collected the samples. X.W. and P.J. performed flow cytometer assay. A.Z. performed the ELISA assay.

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## Table

Table 1. Demographic characteristics of the study subjects and thyroid function of the Graves' disease group.

	n	Gender	Age	TRAb	TSH	FT3	FT4
		M/F	(M±SE)	(IU/L)	(mIU/L)	(pg/ml)	(ng/dl)
Graves' disease	52	18/34	36.46±11.53	11.98±20.53	0.16±0.41	6.17±4.97	1.88±1.35
Healthy controls	23	12/11	39.96±12.14	N.A.	N.A.	N.A.	N.A.

## Figures

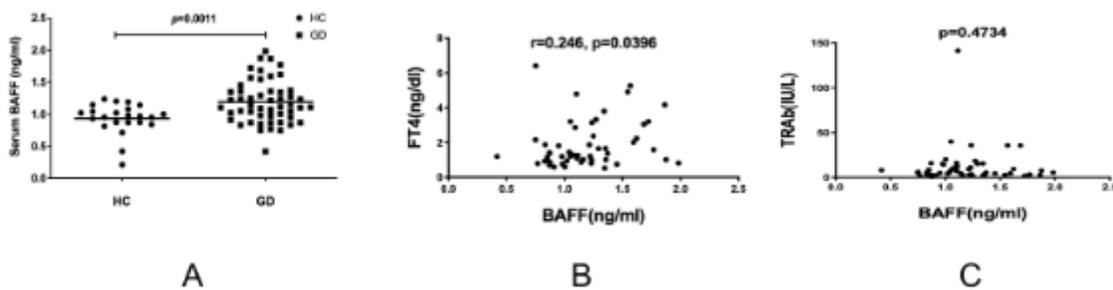
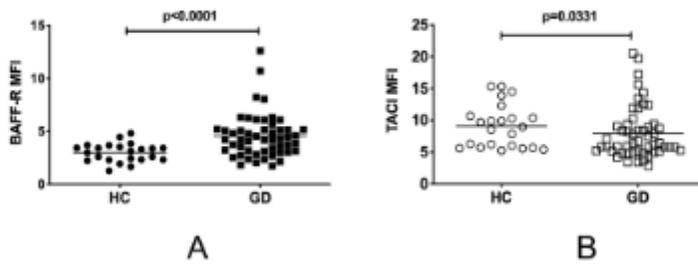


Figure 1

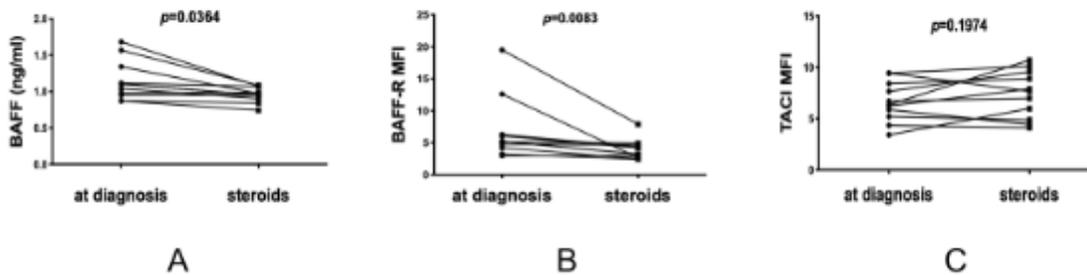
Increased serum BAFF levels and its correlations with thyroid function and TRAb titers in GD patients. (A): serum BAFF levels were elevated in GD patients ( $1.183 \pm 0.334$  ng/ml) comparing with healthy controls ( $0.934 \pm 0.238$  ng/ml),  $P=0.0011$ ; (B): Serum BAFF levels positively correlated with serum free

thyroxine, Spearman  $r=0.2460$ ,  $P=0.0394$ ; (C): No correlation between serum BAFF levels and TRAb titers,  $P=0.4734$ . HC: healthy controls; GD: Graves' disease; Bars: mean BAFF levels.



**Figure 2**

Skewed expression profile of receptors of BAFF on peripheral blood B lymphocytes in GD. (A): Elevated BAFF-R expression on peripheral blood B lymphocytes in GD patients. Mean MFI of BAFF-R on peripheral B lymphocytes in GD patients was  $4.52 \pm 2.06$ , higher than that in HC ( $3.00 \pm 0.87$ ),  $P<0.0001$ ; (B): Decreased TACI expression on peripheral B lymphocytes in GD patients. Mean MFI of TACI on peripheral B lymphocytes in GD patients was  $7.96 \pm 4.06$ , lower than that in HC ( $9.10 \pm 3.37$ ),  $P=0.0331$ . MFI: mean fluorescence intensity. Bars: mean MFI.



**Figure 3**

Restored expression of BAFF and its receptors on peripheral B lymphocytes after steroids therapy in GO patients. (A): Suppressed serum BAFF levels after steroids therapy in GO patients. Mean serum BAFF concentration was  $1.18 \pm 0.27$  ng/ml at the diagnosis of GO (left) and  $0.97 \pm 0.10$  ng/ml at the end of steroids therapy (right),  $P=0.0364$ ; (B): Decreased expression of BAFF-R on peripheral blood B lymphocytes after steroids therapy in GO patients. Mean MFI of BAFF-R on B lymphocytes decreased from  $6.26 \pm 4.91$  before therapy (left) to  $4.05 \pm 1.58$  at the end of steroids therapy (right),  $P=0.0083$ ; (C): Trend of increased TACI expression on the peripheral blood B lymphocytes after steroids therapy in GO patients. Mean MFI of TACI on peripheral blood B lymphocytes was elevated from  $6.67 \pm 1.96$  before steroids therapy to  $7.05 \pm 2.32$  after steroids therapy, without statistically significant ( $P=0.1974$ ).