$CD4^+CD25^{high}FOXP3^+$ T regulatory cells as a biomarker of disease activity in systemic lupus erythematosus: a prospective study

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Abstract

Objective

Several studies have reported low numbers of T regulatory cells (Tregs) in active systemic lupus erythematosus (SLE). However, it is not evident if these cells may be utilised as a biomarker in assessing disease activity.

Methods

Tregs ($CD4^+CD25^{high}FOXP3^+$) were prospectively assessed by flow cytometry in 285 separate blood samples from 100 white Caucasian SLE patients and 20 healthy controls. Patients were divided, according to disease activity (as measured by SLEDAI) into groups A (n=39, samples=94, SLEDAI=0), B (n=33, samples=92, SLEDAI=1-5), C (n=10, samples=53, SLEDAI=6-10) and D (n=18, samples=46, SLEDAI>10). Longitudinal measurements were performed in 131 cases (37 relapses, 44 remissions and 50 cases with stable disease activity) during three years. Statistics were performed by Student's t-test or one-way ANOVA; correlations with Pearson co-efficient, while $p<0.05$ was considered significant.

Results

Tregs were found significantly lower in severely active disease (group D), compared to healthy controls, inactive disease, mild and moderate disease activity (0.57±0.16% vs. 1.49±0.19%, 1.19±0.34% and 1.05±0.36%, 0.72±0.21%, $p<0.05$, respectively). There was a strongly inverse correlation between Tregs and SLEDAI ($r=-0.644$, $p<0.001$). Alterations in disease activity were characterised by inverse alterations in Tregs: relapse (from 1.23±0.44% to 0.64±0.19%, $p<0.001$, mean change 0.59±0.41%), remission (from 0.65±0.27% to 1.17±0.30%, $p<0.001$, mean change 0.52±0.35%). In cases with unaltered disease activity, Treg numbers remained stable (from 0.98±0.35% to 1.03±0.34%, $p=0.245$). Tregs were practically halved during relapse (mean reduction 42.6±22.2%), and doubled during remission (mean increment 113±120.9%). Mean change of Tregs in stable disease was significantly lower (7.3±20.6%, $p<0.001$). A clinically significant change in SLEDAI (sum of cases with relapse and remission, n=81) was followed by a significant (>20%) inverse change in Tregs in 71/81 cases (sensitivity 87.7%). In 50 cases of stable disease activity, Tregs were significantly changed (>20%) in 13 cases (specificity 74%). Positive predictive value (PPV) was 84.5% and negative predictive value (NPV) was 78.7%.

Conclusion

$CD4^+CD25^{high}FOXP3^+$ T regulatory cells displayed a strongly inverse correlation to disease activity in the long term. Treg alterations reflected changes in SLEDAI with high sensitivity. These cells may be a reliable biomarker for the assessment of disease activity in SLE by longitudinal measurements.

Key words

systemic lupus erythematosus, activity, SLEDAI, T regulatory cells, biomarkers

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Introduction

Systemic lupus erythematosus (SLE) represents the prototype of systemic autoimmune diseases with protean clinical manifestations and a wide variety of immunological and other laboratory findings (1). The clinical course of the disease is characterised by unpredictable flares and remissions. For the precise assessment of disease activity, several composite indices, such as Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), along with certain biomarkers (cytokines, autoantibodies, complement fragments and specific cellular subpopulations) have been proposed (2). However, in regard to biomarkers, none has been proved to reliably and uniformly reflect disease activity (3).

Disease pathogenesis is considered to be multifactorial, given the fact that dysregulated function of practically every component of the immune system has been described (1, 4). It is believed that the breakdown of immune tolerance is the critical factor that leads to autoreactivity and, subsequently, tissue damage (5). The mechanisms of peripheral tolerance are mainly mediated by the regulatory T cells (Tregs). Tregs comprise a quite heterogeneous population and derive either from thymus (natural Tregs) or by naïve T cells in the periphery (inducible Tregs) under certain circumstances (6). The latter include mainly the so-called Th1 cells (mainly acting through IL-10 secretion), Th3 cells (functioning via TGF-β secretion), $CD8^+$ Tregs and other subpopulations (7). Natural Tregs are characterised by high surface expression of CD25, while Foxp3 represent the master regulator for their functional differentiation. Thus, the phenotype $CD4^+CD25^{high}FOXP3^+$ is believed to better define natural Tregs; on the contrary, CD25 and Foxp3 are up-regulated upon stimulation in inducible Tregs, making their study rather complicated (8).

Many studies have demonstrated quantitative and/or qualitative defects of Treg populations in lupus patients (9-11). However, the utility of Tregs as a biomarker of disease activity in SLE has not been extensively investigated. This question has been addressed in the present study, by prospective and longitudinal evaluation of $CD4^+CD25^{high}FOXP3^+$ Tregs in SLE patients in different time points and phases of disease activity.

Patients and methods

One hundred consecutive white Caucasians lupus patients (89 female, 11 male), who were regularly followed up at the Clinical Immunology Unit, 2nd Department of Internal Medicine, Hippokration General Hospital, Aristotle University of Thessaloniki were included and followed up for 4 years (January 2009 to December 2012). All patients fulfilled the updated American College of Rheumatology classification criteria for SLE (12, 13). Mean age was 45.4±13.9 years at study entry (range 23 to 80) and 36.4±12 years at disease onset (range 17 to 68). Mean disease duration was 108.3±101.5 months. Demographic data, cumulative clinical manifestations and use of immunomodulating drugs are presented in Table I.

The Human Ethics Review Committee of Aristotle University of Thessaloniki approved the study protocol and a signed informed consent was obtained from each subject.

Evaluation of disease activity and Tregs

At every visit (at 6-monthly intervals or sooner, depending on disease activity), history taking, thorough clinical examination and SLEDAI assessment by the same physician, according to the initial description were performed (14). Laboratory investigations included the parameters demanded for SLEDAI (C3, C4d, anti-dsDNA antibodies, full blood count and the level of proteinuria). Complement fragments (C3, C4d) were evaluated by nephelometry (Dade Behring, Newark, DE, USA). Anti-dsDNA antibodies were assessed by indirect immunofluorescence (IFA) on a *Crithidia luciliae* substrate. Other laboratory parameters included erythrocyte sedimentation rate (ESR) and IgG levels.

T regulatory cells (phenotype $CD4^+CD25^{high}FOXP3^+$) were assessed by triple-colour flow cytometry (EPICS COULTER XL®), in whole blood samples, shortly after venepuncture. Cells were stained with anti-CD4 (13B8.2, Immunotech) FITC (Fluoroscein Iso-

Table I. Demographic data, cumulative clinical manifestations and use of immunomodulating drugs of the patients.

Demographic data	
Patients (n)	100 (89 females / 11 males)
Age at study entry	45.4±13.9 years (23-80)
Age at onset	36.4±12 years (17-68)
Disease duration	108.3±101.5 months
Cumulative clinical manifestations (n)	
Skin rash/cutaneous lupus	32
Arthritis	68
Pleuritis	8
Pericarditis	11
Renal involvement	20
Neuropsychiatric involvement	20
Haematological manifestations	35
Raynaud	21
Constitutional symptoms	63
Immunomodulating drugs (n)	
Steroids	73
Hydroxychloroquine	34
Azathioprine	46
Mycophenolate mofetil	10
Cyclophosphamide (iv)	10
Intravenous immunoglobulins	5

thiocyanate), anti-CD25 (B1.49.9, Immunotech) ECD (Phycoerythrin-Texas-Red-X) and anti-FOXP3 (PCH101, e-Bioscience) PE (Phycoerythrin). Samples were elaborated according to the respective protocol with Intraprep TM solutions (Beckman-Coulter) for red blood cell lysis and intracellular FoxP3 staining. A representative FACS figure is presented in Figure 1.

In total, 285 blood samples were assessed (36 patients once, twice in 14 patients, thrice in 22, four times in 8, five times in 6, six times in 9, seven times in 3, eight times in one and ten times in one patient). Treg values are given as absolute numbers and as a proportion (%) of the $CD4^+$ T cells.

Study design

Patients were subdivided into four groups according to disease activity. Group A consisted of 39 patients with no disease activity (SLEDAI=0), group B included 33 patients with minimal disease activity (SLEDAI=1-5), group C 10 patients with moderately active disease (SLEDAI=6-10) and group D 18 patients with severely active disease (SLEDAI>10) (Fig. 2). Blood samples (n=285) were accordingly stratified in groups A1 (n=94), B1 (n=92), C1 (n=53) and D1 (n=46) (Fig. 2).

$CD4^+CD25^{high}FOXP3^+$ Tregs were re-evaluated in 37 distinct cases of disease relapse (31 patients, 6 patients relapsing twice) and 44 separate cases of disease remission (35 patients, 9 patients remitting twice). A reassessment of Tregs was additionally performed in 50 individual cases with stable disease (20 cases with permanently inactive disease, SLEDAI=0, 12 cases with insisting minimal disease activity, SLEDAI=1–5 and 14 cases with persistently moderate to severe disease, SLEDAI>5). Twenty age- and sex-matched healthy volunteers were used as controls.

Statistical analysis

Analysis was performed using the Student's *t*-test for independent samples or the one-way analysis of variance (ANOVA) test. Post hoc analysis was made using the Bonferroni multiple comparisons test. In every occasion, the equality of variances was assessed by Levene's test. Correlations were made with Pearson correlation coefficient. Before these tests, normal

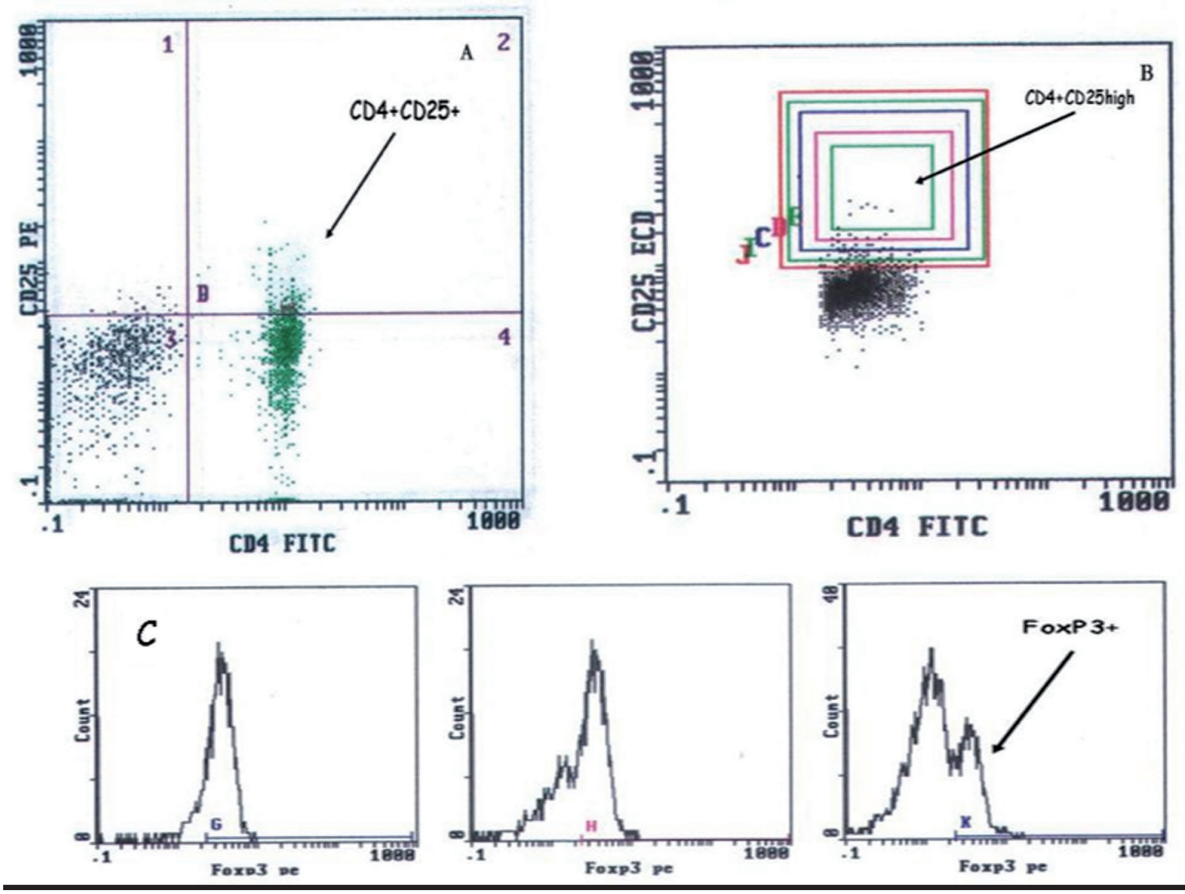

Fig. 1. Representative FACS (fluorescence activated cell sorter) figure.

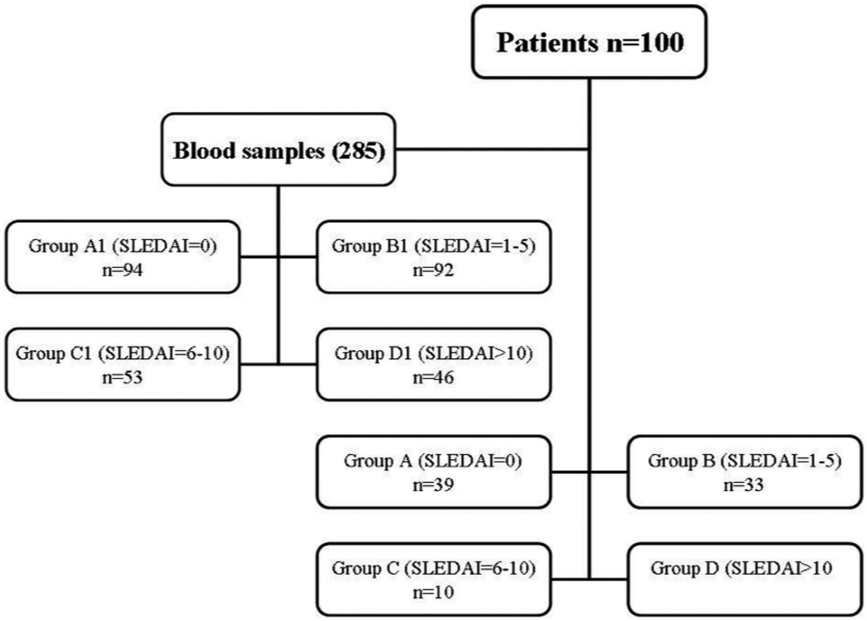

Fig. 2. Patients' and samples' stratification according to the level of disease activity.

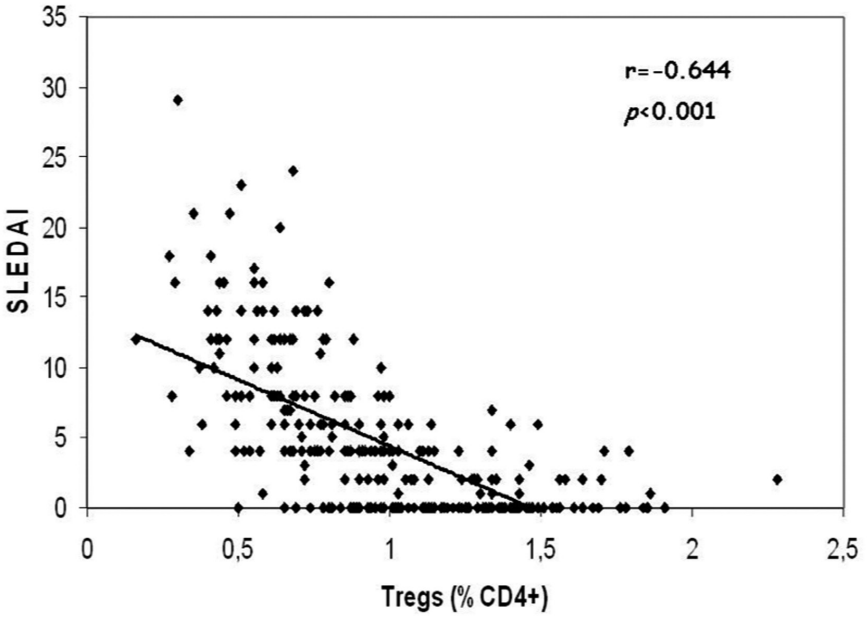

Fig. 3. Tregs were inversely correlated to SLEDAI (r=-0.644).

distribution was assessed by Kolmogorov-Smirnov test. All *p*-values were 2-tailed and $p<0.05$ was considered to be statistically significant. The SPSS software for Windows (version 20.0) was used for statistics.

Results

1. Tregs are decreased in SLE patients relatively to disease activity

There was a strongly inverse correlation between $CD4^+CD25^{high}FOXP3^+$ Tregs and SLEDAI (r=-0.644, $p<0.001$), (Fig. 3). Tregs were found significantly lower in severely active disease (groups D and D1), compared to healthy controls, inactive disease, mild and moderate disease activity (0.57±0.16% *vs*. 1.49±0.19%, 1.19±0.34%, 1.05±0.36% and 0.72±0.21%, $p<0.05$, respectively), (Fig. 4). Absolute Treg numbers were significantly lower in patients with moderate and severe disease in comparison to inactive patients and mild disease activity (5.3±2.5cells/mm^3 *vs*. 11.3±5.2cells/mm^3, $p<0.05$). Interestingly, in 9 newly diagnosed patients, Tregs were found to be 0.78±0.36% (absolute count 7.4±4.2cells/mm^3) before treatment initiation and were significantly lower than healthy controls and inactive patients ($p<0.001$, data not shown).

Further analysis, in regard to the levels of complement fragments C3 and C4d, showed that Tregs were significantly lower in patients with low C3 (n=42, 0.72±0.39% *vs*. 1.02±0.37%, $p<0.001$) and low C4d (n=41, 0.65±0.23% *vs*. 1.03±0.39%, $p<0.001$) (normal values 90–180mg/dl for C3 and 10–40mg/dl for C4d), as shown in Figure 5A and 5B, respectively. Concerning the presence of anti-dsDNA antibodies, Tregs were found marginally lower in patients with positive anti-dsDNA (0.79±0.34% *vs*. 1.02±0.39%, $p=0.009$), compared to those with negative antibodies, as shown in Figure 5C.

Analysis of Treg numbers with regard to other activity parameters, such as the absolute lymphocyte count, ESR, IgG and the level of proteinuria did not reveal any significant correlations (data not shown).

2. Alterations in disease activity are characterised by inverse alterations in Treg numbers

A. Disease flare

Longitudinal measurements of Tregs were performed in 37 particular cases of disease relapse in 31 patients (6 patients experienced two relapses during follow-up). Blood samples were taken before treatment adjustment. Relapse was defined as a more or equal than 3 increment in SLEDAI. Tregs significantly decreased (from 1.23±0.44% to 0.64±0.19%, $p<0.001$, mean change 0.59±0.41%, absolute numbers 10.9±6.7cells/mm^3 to 4.9±2.7cells/mm^3, $p<0.05$), whereas SLEDAI was significantly increased (from 3.1±4.4 to 11.3±6.4, $p<0.001$, mean change 8.3±4.7) (Fig. 6A).

Concerning other activity parameters, ESR was significantly increased (from 28.5±18.8mm/h to 43.3±24.5mm/h, $p=0.003$), while C3 and C4d lev-

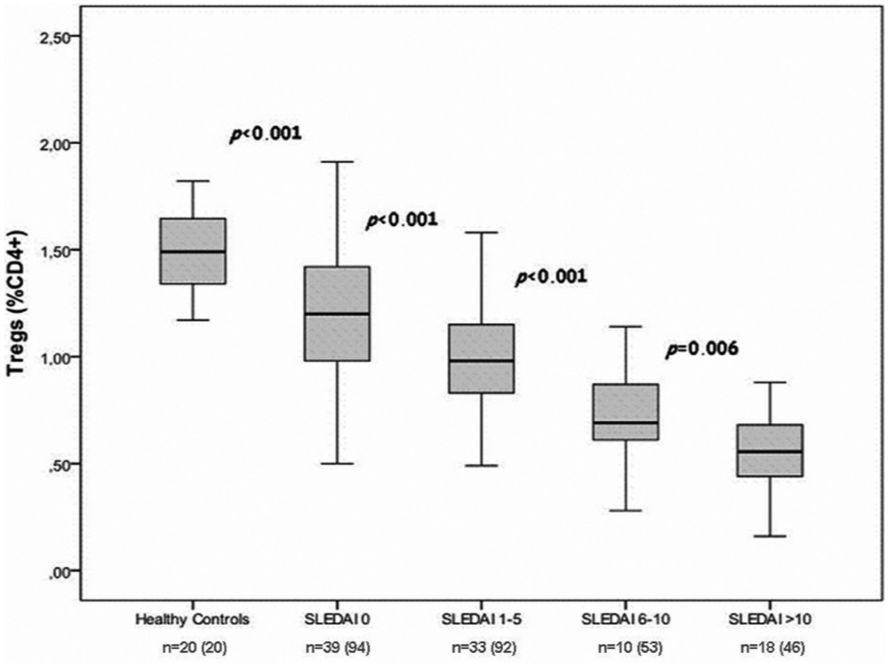

Fig. 4. $CD4^{+}CD25^{high}FOXP3^{+}$ Tregs were significantly lower in patients with severe disease, in comparison to moderate, mild, no disease activity and healthy controls.

els were marginally decreased (from 128.4±33.7mg/dl to 112.9±35.6mg/dl, p=0.041 and from 17.4±7mg/dl to 14.2±7mg/dl, p=0.041, respectively).
Tregs were decreased in 35/37 (94.6%) relapses, suggesting a high sensitivity in confirming disease flare. Interestingly, in the two patients with Treg increment during relapse, Tregs were initially low (0.50% and 0.54% respectively), while their increment was insignificant (0.04% and 0.01% respectively). The mean percentile decrement of Tregs was 42.6±22.2% in total, suggesting that Tregs were practically halved during disease flare.

B. Disease remission

Longitudinal Treg assessment was performed in 44 distinct cases of remission in 35 patients (9 patients achieving two remissions), after appropriate pharmaceutical management. Remission was defined as no disease activity (SLEDAI=0) or as a clinically important SLEDAI decrement (>5 points), in patients with previously severe disease. Tregs were significantly increased at remission (from 0.65±0.27% to 1.17±0.30%, p<0.001, mean change 0.52±0.35%, absolute numbers 5.3±3cells/mm^3 to 10.9±5.4cells/mm^3, p<0.05), while SLEDAI was decreased (from 11.4±6.3 to 1.5±2.1, p<0.001, mean change 9.9±5.5) (Fig. 6B).
Treg increment was independent of the administered pharmaceutical regimen; specific drugs used were cyclophosphamide (pulse therapy in 10 patients), methylprednisolone (pulse therapy in 8 patients, oral therapy in 7 patients), methylprednisolone plus azathioprine (oral therapy in 8 patients), hydroxychloroquine (6 patients) and intravenous immunoglobulins (IVIGs, 5 patients). Intravenous regimens (cyclophosphamide, methylprednisolone and IVIGs) resulted in a significant increase of Tregs after therapy completion (4.2±1.6 *vs*. 10.1±5.7 cells/mm^3, 2.9±1.3 *vs*. 10.6±4.8 cells/mm^3 and 5.6±2.7 *vs*. 15.2±6.3 cells/mm^3 respectively, p<0.05). Oral methylprednisolone, alone or combined to azathioprine, led to significant Treg increment in three months (7.4±2.5 *vs*. 11.8±3.8 cells/mm^3 and 5.1±2.4 *vs*. 9.4±3.6 cells/mm^3 respectively, p<0.05). Likewise, hydroxychloroquine resulted in significant Treg increase after three months (8.2±2.4 *vs*. 12.8±2.7 cells/mm^3, p<0.05).
Concerning other activity parameters, ESR was significantly decreased (from 43.7±28mm/h to 22±16.4mm/h, p<0.001), while the levels of C3 and C4d increased insignificantly (from 125.1±50mg/dl to 142.7±35.8mg/dl, p=0.061 and from 16.1±8.1mg/dl to 17.8±7.4mg/dl, p=0.183, respectively). Tregs were increased in all remissions, suggesting an absolute sensitivity in confirming disease remission (100%). The mean percentile increment of Tregs was 113±120.9%, suggesting that Tregs were practically doubled during remission.

3. Treg numbers are not significantly changed during stable disease activity, regardless of the initial SLEDAI

Longitudinal Treg assessment was performed in 50 separate cases with stable disease activity, based on SLEDAI. In this case, Tregs were not significantly changed (from 0.98±0.35% to 1.03±0.34%, p=0.245 (Fig. 6C), absolute Treg count 9.3±5.6cells/mm^3 *vs*. 10±5.8cells/mm^3), as well as the other parameters studied (ESR, C3, C4d, IgG, absolute lymphocyte count, proteinuria). The mean percentile change of Tregs was 7.3±20.6%.
These cases were subdivided, according to the initial SLEDAI, in continuously inactive disease (SLEDAI=0, n=20), persistently mild disease (SLEDAI=1-5, n=15) and persistently moderate-to-severe disease (SLEDAI>5, n=15). Treg variations were insignificant in inactive (from 1.10±0.34% to 1.17±0.33%, p=0.263, mean percentile change 5.3±14.2%), in mild (from 1.06±0.33% to 1.10±0.26%, p=0.349, mean percentile change 4.8±13.4%), as well as in insistently active disease (from 0.73±0.27% to 0.75±0.27%, p=0.394, mean percentile change 0.2±29.8%). These changes were significantly lower compared to the relevant alterations in Treg numbers in the cases of disease flare (p<0.001) or remission (p<0.001) (Fig. 7).

4. Tregs in specific SLE phenotypes: lupus nephritis (LN), neuropsychiatric SLE (NPSLE) and antiphospholipid syndrome (APS)

Patients with LN (n=20) were divided, according to disease activity, in active (defined by the presence of proteinuria>500mg/24h and/or haematuria and/or casts and/or active urinary sediment) and inactive LN. Diagnosis

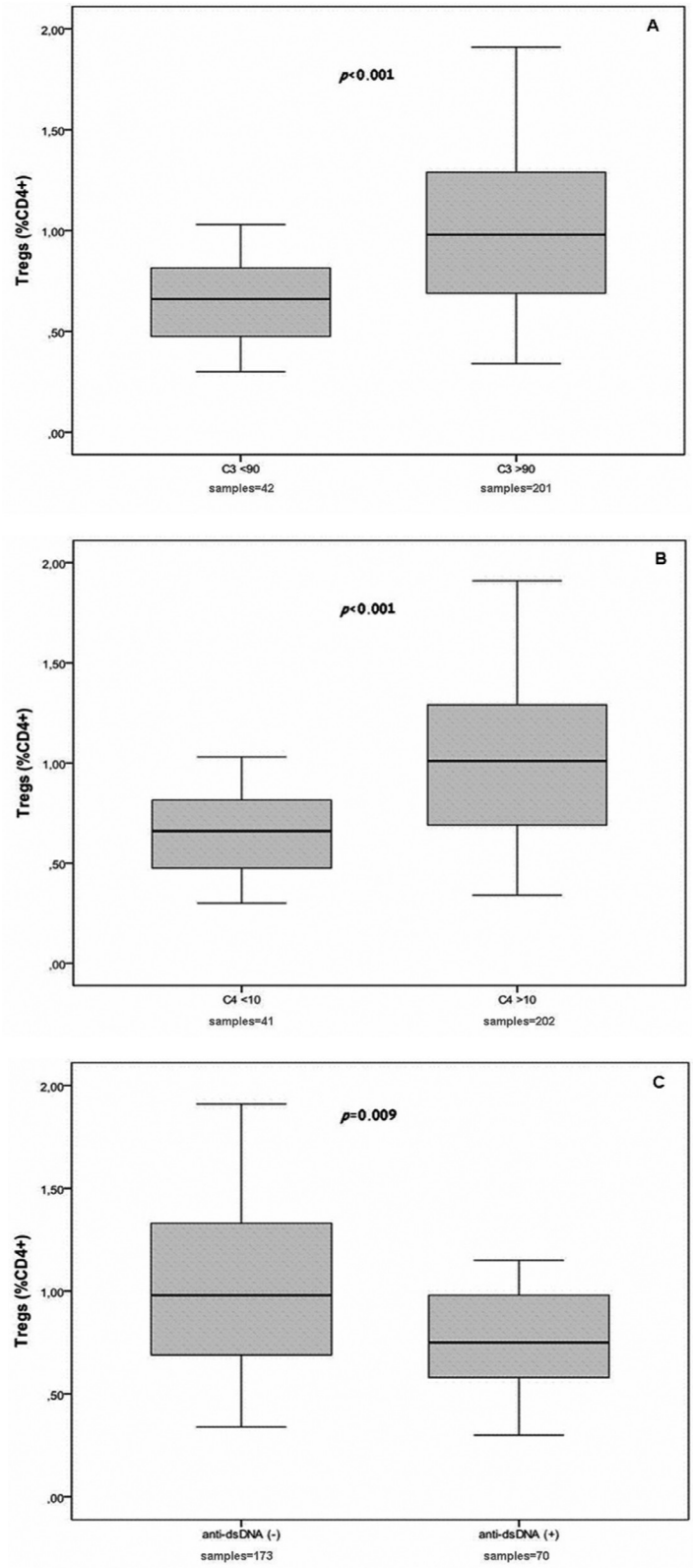

Fig. 5. Tregs were significantly lower in patients with low levels of complement fragments C3 **(A)** and C4d **(B)**. Tregs were lower in patients with positive anti-dsDNA autoantibodies **(C)**.

was histologically confirmed in 12/20 patients (10 with LN class IV-V and 2 with LN class V, according to the ISN/RPS classification) (15), while one patient had developed end-stage renal disease and was on dialysis from diagnosis. Twelve patients had active disease (44 blood samples evaluated) and 8 had inactive disease (17 assessed samples). Tregs were found significantly lower in patients with active LN compared to inactive disease (0.71±0.29% *vs.* 1.14±0.19%, $p<0.001$, absolute count 4±2.3cells/mm^3 *vs.* 8.9±4.4cells/mm^3), (Fig. 8A).

NPSLE was diagnosed in 20 patients, from whom 17 suffered from central nervous system (CNS) involvement and 3 from peripheral nervous system involvement (2 with Guillain-Barre syndrome and one with mononeuritis multiplex). CNS involvement was manifested as psychosis in 4/17, multi-infarct dementia in 6/17, multiple (>1) strokes in 8/17, seizures in 7/17, acute confusional state in 2/17 and reversible posterior leukoencephalopathy syndrome (RPLS) in 1/17 patients. In all cases, alternate diagnoses were thoroughly excluded. Headache, in any form, was not considered as an NPSLE manifestation. Tregs were evaluated in 88 different samples from these patients (26 with active and 62 with inactive disease). Active NPSLE was characterised by significantly lower Tregs compared to inactive disease (0.57±0.17% *vs.* 1.05±0.39, $p<0.001$, absolute count 4.6±3.4cells/mm^3 *vs.* 9.3±6.6cells/mm^3) (Fig. 8B).

Antiphospholipid syndrome, secondary to SLE, was diagnosed in 25 patients (23 females, 2 males, mean age at study entry 42.1±11.8 years) using the anti-cardiolipin antibodies, the anti-b2GPI antibodies and the lupus anticoagulant assay, according to the respective revised classification criteria (16). It should be mentioned that all these patients had quiescent APS at the time of assessment (defined by the absence of thrombotic manifestations in the previous 3 months) under anticoagulant treatment. There were no significant differences in comparison to non-APS patients in terms of SLE activity (mean SLEDAI 4.8±4.1 *vs.* 4.6±3.9, p=ns, data not shown). Tregs showed a tendency for lower values in APS patients, although difference did not reach statistical significance (0.84±0.45% *vs.* 1.00±0.37%, p=ns), (Fig. 8C).

5. The value of Tregs in predicting SLE flares

Thorough analysis of the disease course was performed in six female patients with inactive disease (SLEDAI=0), who had relatively low Tregs

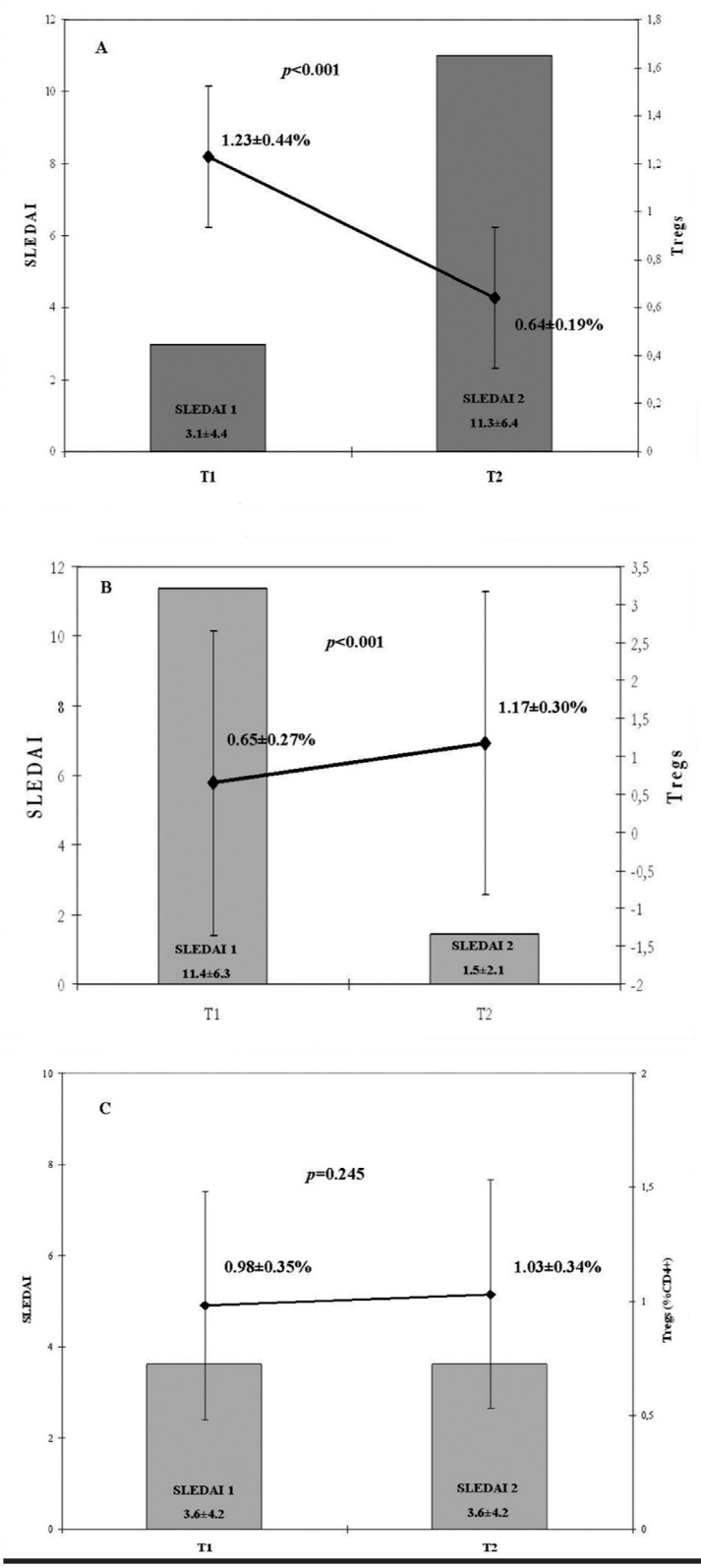

Fig. 6. Tregs (black line) were significantly decreased in cases of disease relapse, as defined by SLEDAI (grey bars) **(A)**, while they were significantly increased in disease remission **(B)**. In cases of unaltered disease activity, their numbers remained stable **(C)**.

(0.74±0.16%), comparable to the levels of patients with moderately active disease (0.72±0.21%). These patients were regularly followed up in six months intervals, for two consecutive years, for capturing any relapse.

After 12 months from the initial evaluation, Tregs were 0.75±0.14%, suggesting no significant changes in their numbers. Disease flare was observed in one patient, in which Tregs were slightly increased from 0.50% to 0.54%. Based on these data, it can be assumed that the positive predictive value of low Tregs in assessing disease relapse is quite low (16.7%).

6. Tregs could be a promising biomarker for SLE activity

Given that SLEDAI represents a meaningful endpoint for lupus patients, a clinically significant change in this index (sum of cases with relapse and remission, n=81) was followed by a significant (>20%) inverse change in Tregs in 71/81 cases, suggesting that the overall sensitivity of Tregs for assessing alterations in SLE activity was 87.7%. In the 50 cases of stable SLEDAI, Tregs were significantly changed (>20%) in 13 cases, thus resulting in a specificity of 74%. Positive predictive value (PPV) was 84.5% and negative predictive value (NPV) was 78.7%.

Discussion

The validation of a possible biomarker is a rather complicated process. It has been proposed that any biomarker should be validated for sensitivity, specificity, details of bioanalytical assessment and the probability of false positive and false negative results (17). Despite extensive relative research in SLE, no single index or biomarker has gained universal acceptance over the years for monitoring lupus patients or conducting clinical trials (18). Of note, only a few laboratory parameters are thought to be important in monitoring disease activity; C3 and C4d levels, anti-dsDNA and anti-C1q titers are included in the recent EULAR recommendations for SLE management (19). Obviously, there is an urgent need for improvement of the existing disease activity indices and for the evaluation of possible predictors of disease flares. On this rational, the possible utilisation of $CD4^{+}CD25^{high}FOXP3^{+}$ T regulatory cells in assessing disease activity was evaluated in the present study. Tregs were found to be a reliable biomarker with considerable sensitivity and specificity in the long term; however, their predictive value in timely assessing disease flares is low.

Initial studies, in lupus patients, demonstrated controversial results in regard

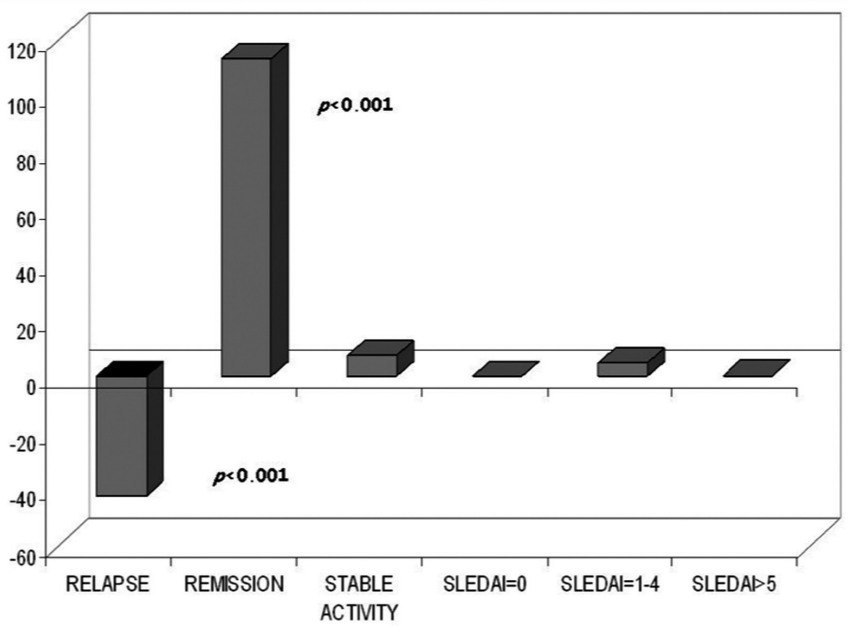

Fig. 7. Alterations in Tregs numbers were significantly greater in cases of altered disease activity (relapse or remission) compared to cases of stable disease.

to Treg numbers and function (9-11, 20-26). Treg numbers ranged from decreased (9-11, 20) to normal (21-23) or even increased (25, 26) in comparison to healthy controls. In the present study, Tregs were significantly lower in lupus patients, compared to healthy controls, while this decrement was analogous to the level of disease activity.

Other investigators also demonstrated the strongly negative correlation of Tregs with disease activity, although there were some contradictory results (20, 21, 27). Previous studies were not designed to address the question of the possible utilisation of Treg numbers in assessing disease activity in the long term. As a result, patients' evaluation was not longitudinal and results were inconclusive (9, 20, 27-33). In the present study, which included significantly more patients (n=100 with 285 samples), r correlation coefficient between Tregs and SLEDAI was strongly negative (r=-0.644, Fig. 3), suggesting the significant impact of Tregs on disease activity.

Important methodological differences do exist between the different studies, concerning, in particular, Treg phenotype. For example in one study with paediatric SLE patients, cells evaluated as Tregs expressed both high and low levels of the CD25 molecule (29). It has been demonstrated that $CD4^+CD25^{high}$ T cells express the FOXP3 molecule (and so they have regulatory capacity) in 85.6% of the cases (9). Thus, in previous studies, the number of measured Tregs may be significantly overestimated. In studies, where the phenotype of evaluated Tregs was $CD4^+CD25^{high}FOXP3^+$, results were comparable (30, 33).

Another important methodological difference lies in the design of previous studies in regard to the estimated level of disease activity. Previous investigators arbitrarily used the SLEDAI=3, as a cut-off to differentiate active disease (9, 34). According to studies on prognosis of short term mortality in SLE, the most relevant stratification is based on a SLEDAI cut-off of 15 for mild disease, 6-10 for moderate disease and >10 for severe disease (35), which was used in the present study.

$CD4^+CD25^{high}FOXP3^+$ Tregs were further assessed in cases of alteration in disease activity during follow-up. In this context, 37 cases of disease relapse and 44 cases of remission were separately analysed. During relapse, a significant reduction in Treg numbers was observed, as these cells were almost halved. Moreover, Tregs were decreased in 35/37 patients with relapse, displaying a high sensitivity (94.6%) in assessing a lupus flare. To our knowledge, there are no published data regarding Treg fluctuations during SLE flares.

On the contrary, in 44 cases, in which remission was achieved, a significant increment in Treg numbers was detected, as these cells were almost doubled. Tregs were increased in all patients, suggesting an absolute sensitivity in assessing remission. These findings come in agreement with initial reports. Miyara *et al*. demonstrated a significant increment of Tregs in 10 lupus patients during remission, while Zhang *et al*. reported similar results after remission induction with autologous stem cell transplantation (9, 36). Other investigators reported a significant increase of Tregs in active LN patients after treatment with rituximab (37, 38). It should be stated that in this study, the increment of Tregs was independent of the administered therapy, as this occurred with different treatment modalities. In this context, it can be assumed that Treg expansion is not drug-specific but rather represents an epiphenomenon to disease remission.

Further analysis was performed in patients with stable disease and at different levels of SLEDAI, thus in permanently inactive, mild and moderate-to-severe disease. In this context, we found no significant differences in the number of Tregs, irrelevantly to the initial disease activity. The mean Treg change in these patients was 7.3±20.6%, arguing in favour of the sufficient reproducibility of the evaluation method. In addition, these variations were significantly lower ($p<0.001$) than the respective alterations in cases of disease relapse or remission. This issue has also not been addressed by previous studies.

Concerning the different clinical phenotypes of SLE, we evaluated Tregs in LN, NPSLE and APS patients. Our findings support a significant decrease in Treg numbers in active LN and active NPSLE, while quiescent APS does not seem to influence their population. Several studies were conducted in LN patients, while most of them demonstrated low numbers of these cells in active disease (36-40) and one reported no significant differences (22). We have previously shown that Tregs are significantly lower in active LN and

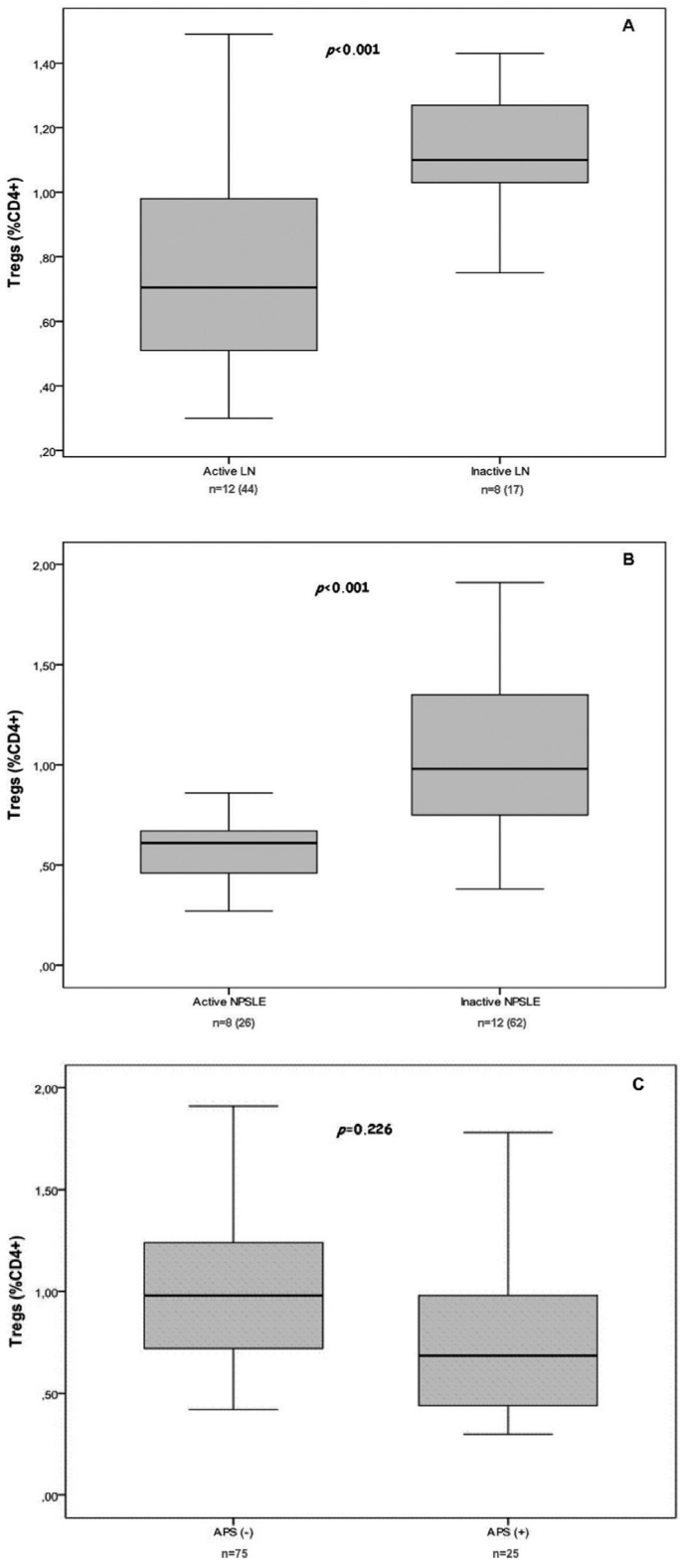

Fig. 8. Tregs were significantly lower in cases of active lupus nephritis (LN) **(A)** and active neuropsychiatric SLE (NPSLE) **(B).** In patients with antiphospholipid syndrome (APS) the differences were insignificant **(C)**.

are inversely correlated with the titers of anti-dsDNA antibodies (41).

Regarding the role of Tregs in NPSLE and APS, the related literature is sparse. In this study, we observed significantly lower Tregs in active NPSLE patients compared to inactive disease. In addition, Tregs were found in lower values in APS patients (secondary to SLE), although insignificantly. The importance of these findings needs further assessment.

The predictive value of Tregs in timely assessing a lupus flare remains unanswered. Although this study did not intend to address this question, a separate analysis of six patients with low Tregs and inactive disease was performed. After 12 months of follow-up, only one patient did relapse, while Tregs remained practically unchanged. In this regard, there is a hint that the positive predictive value of Tregs may be low. Despite the limitation of the small number of studied patients, it can be assumed that Treg value, as a predictive factor in SLE, may be precarious.

The underlying pathogenetic mechanisms of low Tregs in active SLE have not been fully elucidated. In their seminal study, Miyara *et al*. showed that Tregs are not re-distributed to target organs during disease flare (9). Recently, it was demonstrated that this global depletion might be due to an expansion of Th17 cells and increased levels of the relative cytokines, IL-17 and IL-23 (40), along with decreased TGF-β (39). Th17 cells are believed to function in a fine equilibrium with Tregs in autoimmune diseases, under the tight control of cytokine microenvironment (42).

In conclusion, $CD4^{+}CD25^{high}FOXP3^{+}$ T regulatory cells measured longitudinally, may be a reliable biomarker for assessment of disease activity in SLE, as they presented greater sensitivity to disease status change in comparison to other activity parameters, such as ESR, C3 and C4d. Concerning their utility as predictors of lupus flares, further well-designed prospective studies are warranted.

T regulatory cells are strongly depleted in active lupus nephritis and active neuropsychiatric SLE. $CD4^{+}CD25^{high}FOXP3^{+}$ T regulatory cell measurements may give precious information when treating patients with these clinical phenotypes.

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