

**Rituximab-induced T-cell depletion in patients with rheumatoid arthritis: association  
with clinical response**

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**Disclosures**

**JM** was invited to attend an international congress by UCB. **DM** participated on behalf of his institution in clinical trials sponsored by Abbott, Roche, BMS, Pfizer, UCB and MSD; his hospital received a grant for research from Abbott in 2004; he has acted as a consultant and given lectures on behalf of his institution for MSD and Pfizer; he has been invited to attend international congresses by MSD, Roche, BMS and Abbott. **PG** participated on behalf of his institution in clinical trials sponsored by Abbott, Roche, BMS, Lilly, Novartis, Pfizer, UCB and MSD; he has acted as a consultant and given lectures on behalf of his institution for Abbott, BMS, MSD, Pfizer, UCB; he has been invited to attend international congresses by MSD, Roche, BMS and Abbott. **BR**, **HW** and **GT** declared no conflict of interest

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### **Author contributions**

**JM** participated in the clinical assessment, performed data collection and statistical analysis, and wrote the manuscript. **DM** supervised the study design, participated in the clinical assessment and wrote the manuscript. **BR** participated in the analysis of data, **HW** participated in discussing the results and improving the manuscript. **PG** participated in improving the manuscript and clinical assessment. **GT** supervised the study design and the flow cytometry analysis and wrote the manuscript. All authors read and approved the final manuscript.

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**Full-Length Article**

**Abstract**

**Objective:** Rituximab, a monoclonal antibody specifically targeting CD20, induces B-cell depletion and is effective in rheumatoid arthritis (RA). We aimed to study whether routine monitoring of lymphocyte subpopulations, especially T cells, may be useful in patients receiving rituximab for RA.

**Methods:** We examined data for all RA patients receiving rituximab between July 2007 and November 2012 in our centre. Peripheral blood CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD3<sup>+</sup>CD56<sup>+</sup> and CD19<sup>+</sup> lymphocyte counts before and during the first course of rituximab were measured by flow cytometry. Mann-Whitney non-parametric test was used to compare lymphocyte subpopulation counts before and during treatment.

**Results:** We examined data for 52 patients. Rituximab induced unexpected and substantial depletion of T cells, mainly CD4<sup>+</sup> cells, in most patients. The CD4<sup>+</sup> count decreased by mean 37%  $\pm$  33 as compared to baseline at week 12, reaching < 200 cells/ $\mu$ L in 3 patients. Importantly, lack of CD4<sup>+</sup> cell depletion was associated with no clinical response, therefore the mechanism of action of rituximab may depend at least in part on T cells.

**Conclusion:** Rituximab induces substantial T-cell depletion mainly CD4<sup>+</sup> cells, which is associated with the clinical response in RA. Routine monitoring of T cells may be useful in the clinical setting in RA.

**Keywords:** rituximab, T cells, rheumatoid arthritis, clinical response, therapeutic monitoring.

T cells, especially CD4<sup>+</sup> T cells, and B cells are both considered involved in the pathogenesis of rheumatoid arthritis (RA). Decreasing lymphocyte activity with conventional or monoclonal antibody-based treatments can reduce disease activity. The first evidence that CD4<sup>+</sup> T lymphocytes were involved in the pathogenesis of RA was the association of polymorphic human leukocyte antigen (HLA) class II alleles, such as HLA-DRB1, which present antigenic peptides to CD4<sup>+</sup> T cells (1). In accordance, targeting CD4<sup>+</sup> T cells with anti-CD4 monoclonal antibodies has resulted in clinical improvement, although modest, in preliminary clinical trials (2, 3). Moreover, abatacept, a CTLA-4-Fc recombinant fusion protein inhibiting CD4<sup>+</sup> T-cell activation by antigen-presenting cells (APCs), has been approved for RA (4).

However, B cells are responsible for production of auto-antibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), the hallmarks of RA. Rituximab, a chimeric anti-CD20 monoclonal antibody inducing the depletion of mature B cells and pre-B cells is effective and currently used in RA (5, 6). The role of B and T cells in the pathogenesis of RA and autoimmune diseases may be tightly linked because of the APC functions or cytokine secretion capacities of B cells. Activated B cells are thought to be fundamental in coordinating T-cell functions. Indeed, transgenic mice prone to a spectrum of autoimmune diseases (glomerulonephritis, vasculitis, skin disease) were protected after B-cell depletion, which is itself associated with a marked decrease of CD4<sup>+</sup> and CD8<sup>+</sup> populations (7). Sfrikakis *et al.* highlighted that after B-cell depletion, decreased T-helper cell activation was associated with clinical remission of lupus nephritis and suggested that B cells have a role in promoting the disease, independent of auto-antibody production (8). Saadoun *et al.* showed that T-cell abnormalities found in mixed cryoglobulinemia such as CD8<sup>+</sup> T-cell activation can be reversed after treatment with rituximab, in relation with complete clinical response (9).

Although rituximab seems to be modestly more effective in RF-positive than in -negative patients, its efficacy is not restricted to patients producing auto-antibodies, so T-cell function may also be hampered by rituximab treatment (10, 11). Several cases of opportunistic infections, usually observed in CD4<sup>+</sup>-deficient patients, have been reported in rituximab-treated RA patients, which argues for an action of rituximab on the T-cell arm of the pathogenesis (12-17). In addition, some of these patients, with low pre-treatment CD4<sup>+</sup> levels, showed a further decrease in CD4<sup>+</sup> counts at the time of the opportunistic infection (12, 14).

Although lymphocyte phenotyping can be recommended before each treatment course of rituximab to identify patients at high risk of infection (18), lymphocyte subpopulation count changes during rituximab treatment have been essentially documented for the B-cell compartment. In a pilot study, Vital *et al.*, using highly sensitive flow cytometry, showed that the degree of B-cell depletion rather than dose of rituximab determined the clinical response (19). However, an increase in IgD<sup>+</sup>CD27<sup>+</sup> memory B-cell subset at the time of recovery was associated with non-response (20).

We aimed to 1) study lymphocyte count changes, with a particular attention to T cells, during rituximab treatment and 2) examine the association of changes in lymphocyte population counts and clinical response in RA patients.

### **Patients and methods**

#### **Patients and study protocol**

We considered patients with RA who received rituximab between July 2007 and November 2012 in the rheumatology department of the University Hospital Centre of Tours, France. The treatment protocol was in accordance with the guidelines of the French Society of Rheumatology (18). Patients received two 1000 mg infusions of rituximab preceded by a 100 mg intra venous pulse of methylprednisolone unless cons-indication at an interval of 2 weeks

and were reviewed in consultation at weeks 12 (W12), 24 (W24) and between weeks 36 and 48 (W36-48) with the recurrence of symptoms. Disease activity was assessed by the disease activity score in 28 joints (DAS28) before treatment and at each follow-up visit. In our center, lymphocyte phenotyping by flow cytometry (FCM) is performed to check the level of B-cell depletion induced by rituximab before the first and second infusion of rituximab and at each follow-up visit as routine. Therefore, ethical approval and written consent was not sought in this analysis. Data on demographic, clinical and biological variables were collected at the time of initiation of treatment. Patients were classified according to European League Against Rheumatism (EULAR) criteria at W24 as good responders, moderate responders or non-responders (21).

### **Lymphocyte phenotyping by flow cytometry**

Phenotype analysis was performed according to a standard no-wash whole-blood procedure with use of a Preplus workstation (Beckman Coulter, Villepinte, France). Blood samples (100  $\mu$ L) were incubated with FITC-conjugated anti-CD3, PE-conjugated anti-CD56 and PE-CY5-conjugated anti-CD19 antibodies (20  $\mu$ L) or with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 or FITC-conjugated anti-CD45RA, PE-conjugated anti-CD45RO and PE-CY5-conjugated anti-CD4 antibodies (all Beckman Coulter) for 15 min at 18-20°C. Red blood cell lysis and cell fixation involved use of a TQ-Prep workstation and ImmunoPrep reagent system (Beckman Coulter). Cells were analyzed by use of an EPICS-XL-MCL flow cytometer (Beckman Coulter). Gates were set on lymphocytes with forward and side scatters and data were collected for a minimum of  $5 \times 10^3$  events for each determination.

### **Statistical analysis**

Mann-Whitney test was used to compare lymphocyte counts before treatment, at week 2 (W2), W12, W24 and W36-48. Absolute lymphocyte counts, percentage depletion and absolute values of depletion were compared before and after treatment. Mann-Whitney test was used to compare percentages and absolute values of depletion at W24 for good responders, moderate responders and non-responders and to analyze the association of patient characteristics for categories and number of CD4<sup>+</sup> T cells before treatment and CD4<sup>+</sup> depletion. Pearson correlation testing was used to analyze the correlation between patient characteristics for continuous variables and number of CD4<sup>+</sup> T cells before treatment and CD4<sup>+</sup> depletion.

## **Results**

### **Patients**

Among the 64 RA patients who received rituximab during the study period, 52 patients for whom FCM data were available before and at least at one time during the first course of rituximab were included in this study. The characteristics of these 52 patients are shown in

#### **Table 1.**

### **Depletion of T lymphocytes during rituximab treatment**

As expected, a total B-cell depletion occurred at W2 and persisted for 6 months followed by a partial B cell recovery observed between 9 and 12 months (**Figure 1**). Accordingly, the percentages of T cells and natural killer cells were slightly increased from W2 to W24, whereas that of CD4<sup>+</sup> and CD8<sup>+</sup> cells remained stable (data not shown). Importantly, although the CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> absolute cell counts were unchanged at W2 as compared with baseline, they were substantially decreased later on. At W12, the averages of depletion were 35%, 37% and 24% for CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells, respectively. One half of the patients

showed a decreased in percentage of CD4<sup>+</sup> T cells ranging from 21% to 62%, and for the lowest quartile, the depletion ranged from 62% to 77% (**Figure 1**). By contrast, some patients showed a substantial increase (up to 57%) in CD4<sup>+</sup> T cells. The results observed at W24 were similar, and the decrease was less marked although still significant at W36-48 with a trend toward a recovery (Figure 1C). Thus, the T-cell depletion, which mainly affected CD4<sup>+</sup> cells, was delayed as compared to the very early B-cell depletion, but the pattern of T- and B-cell count change was similar in that both lasted up to 6 months, with partial recovery later on.

When expressed as absolute counts, several patients showed > 2000 CD4<sup>+</sup> cells/μL depletion (data not shown). Of note, the CD4<sup>+</sup> T-cell count was ≤ 200 cells/μL in 3 (4.7%) of our patients, one presenting with an extensive oropharyngeal candidiasis. In some patients who received successive courses of rituximab, the depletion–reconstitution of T cells occurred after each courses with the same time-related pattern, so T-cell changes, although delayed as compared to B cells, were unambiguously related to rituximab. An example is presented in **Figure 2**.

Finally, we analyzed the change in number of naïve and memory CD4<sup>+</sup> T cells by examining CD45RA and CD45RO markers in 10 RA patients with CD4<sup>+</sup> T-cell depletion. The proportion of CD4<sup>+</sup>CD45RA<sup>+</sup> and CD4<sup>+</sup>CD45RO<sup>+</sup> cells was unchanged during treatment, so rituximab-induced CD4<sup>+</sup> T-cell depletion affected naïve and memory cells similarly (**data not shown**).

### **Lack of T-lymphocyte depletion is associated with no clinical response to rituximab**

To examine the association of change in T-cell count and response to rituximab, we used data for 41 patients with data for FCM and for clinical response at W24. We compared changes in CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cell counts for patients divided into 3 groups by their EULAR responses (*i.e.*, non-responders [n=11], moderate responders [n=15] and good responders



[n=15]) (**Figure 3**). The decrease in CD4<sup>+</sup> and CD3<sup>+</sup> cell counts was significantly greater for moderate responders (on average, 43% and 37%, respectively) and good responders (on average, 47% and 38%, respectively) than non-responders (on average, 7% and 7%, respectively). The groups did not significantly differ in CD8<sup>+</sup> T-cell changes although there was a trend toward a decrease in CD8<sup>+</sup> cell counts in good responders compared to non-responders. Thus, the lack of CD4<sup>+</sup> T-cell depletion was associated with lack of response to rituximab at W24.

Moreover, we analyzed the association of CD4<sup>+</sup> T-cell depletion at W24 and time between the first and second course of rituximab for 24 patients with available data (**supplementary figure 4**). There was a trend toward a greater CD4<sup>+</sup> T-cell depletion relative to baseline for patients who received a second course of rituximab after 12 months compared to those who received a second course between 6 and 12 months (46% vs. 15%; p=0.06).

## Discussion

To our knowledge this report is the first showing that rituximab induces a substantial T-cell depletion, mainly affecting CD4<sup>+</sup> cells, in most RA patients, for a final  $\leq 200$  CD4<sup>+</sup> cells/ $\mu$ L in some. Moreover, lack of CD4<sup>+</sup> T-cell depletion was associated with no clinical response.

We found that rituximab has a long-lasting and reversible depletion effect on CD4<sup>+</sup> counts (both naïve and memory cells) and to a lesser extent CD8<sup>+</sup> cells. To our knowledge, only 2 studies have reported results on the peripheral T-cell compartment in rituximab-treated RA patients(22, 23). The aim of the first one was to investigate the impact of rituximab treatment on regulatory T cells. The absolute number of CD3<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> cells remained unchanged during B cell depletion and regeneration phase compared to baseline (22). However this study involved only 17 patients and 6 of them received lower dose than in our study. Moreover, the values of CD3<sup>+</sup> cell observed in these patients before treatment were

unusually low (mean 867/ $\mu$ L), a finding that has not been reported previously. As stated by the authors this may be due to extensive treatment preceding rituximab in their patients. In addition, T cell counts were obtained using a dual platform method after Ficoll-Paque separation which may alter T cell counts compared to the single platform method used in our study. In the second study the main objective was to investigate synovial tissue in 24 patients with RA treated with rituximab to identify predictors of clinical response. The total numbers of T cells and T cell subsets in peripheral blood were not significantly decreased at week 16, by contrast to the number of T cells in synovial tissue (23). It is of note that patients included in this study were selected on the basis of a very active disease (DAS28 = 6.5 versus 5.4 in our patients) with a joint amenable for arthroscopy. In addition all patients had erosions, were on methotrexate and were RF and/or ACPA positive. Thus, these differences in the patient's characteristics and the limited number of patients in their study may explain the discrepancy with our results.

Beside the major quantitative effect of rituximab on T cells that we observed in RA patients, few studies have reported qualitative modifications in patients suffering from autoimmune diseases. For instance expression of T cell activation markers such as CD40L, CD69 or HLA-DR is decreased along with B-cell depletion in lupus patients treated with rituximab (8, 24, 25). Different mechanisms have been postulated to explain this phenomenon. First, rituximab targets some CD20-expressing T cells. However Leandro *et al.* highlighted that only 3.2% of peripheral CD3<sup>+</sup> T cells expressed low levels of CD20, so a direct effect of rituximab on T cells is unlikely (25). An indirect effect of rituximab on T cells seems more credible. Rituximab is thought to act by reducing auto-antibody production by decreasing auto-reactive B-cell counts. However, rituximab efficacy is not restricted to RF- or ACPA-positive patients, which implies other mechanisms of action. Rituximab may inhibit an activation pathway of CD4<sup>+</sup> cells initiated by the APC function of B cells or by their ability to stimulate CD4<sup>+</sup> cell

proliferation after priming by dendritic cells (26). In addition, B cells are known to produce large amounts of cytokines and chemokines. Rituximab-induced B cell depletion may therefore affect the equilibrium of the cytokines and chemokines involved in cell migration and retention (22). Although this remains to be shown, it could explain why both, naive and memory CD4<sup>+</sup> cells (as well as CD8<sup>+</sup> cells) were affected by rituximab treatment in our patients. Whatever the mechanism(s), this scenario may explain the T-cell decrease occurring after B-cell depletion in some patients and the return to baseline values with diminishing effect of treatment.

An interesting finding is the relationship between the CD4<sup>+</sup> T-cell depletion and clinical outcomes, whereas B-cell depletion affected all patients whatever the response. Indeed, the absence of CD4<sup>+</sup> T-cell depletion was associated with non-response to treatment at W24. In accordance, the extent of CD4<sup>+</sup> depletion at W24 seems to relate to the time between the first and second course (*i.e.*, disease flare). This finding could help clinicians decide to discontinue rituximab for patients with lack of CD4<sup>+</sup> T-cell depletion and poor response after a first course and decide when to re-administer another course in others.

To date, lymphocyte phenotyping in RA patients receiving rituximab aims to identify patients at high risk of infection. We found a 80% decrease in CD4<sup>+</sup> cells leading to < 200 cells/ $\mu$ L in some patients, a threshold below which opportunistic infections may occur (27). Several cases of opportunistic infections, usually observed in patients deficient in CD4<sup>+</sup> T cells, have been reported in RA patients receiving rituximab. Given our results, clinicians should pay particular attention to CD4<sup>+</sup> cell counts before and after a course of anti-CD20 monoclonal antibodies because rituximab may favour the occurrence of opportunistic infection particularly in patients with low CD4<sup>+</sup> counts (12, 14). Considering the primary benefit of antibiotic prophylaxis for these patients seems essential.

In conclusion, we found that rituximab may significantly decrease peripheral-blood T cells, particularly CD4<sup>+</sup> cells, in most patients with RA. Interestingly, lack of CD4<sup>+</sup> cell depletion was associated with no response to treatment at W24. Our results support the usefulness of T-cell monitoring in RA patients receiving rituximab. CD4<sup>+</sup> counts may help clinicians in decision making: patients lacking a decrease in CD4<sup>+</sup> cell count are less likely to achieve clinical response than are those with decreased CD4<sup>+</sup> cell count. Patients with low CD4<sup>+</sup> cell count at baseline should be monitored to prevent opportunistic infections.

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**Tables****Table 1: Baseline characteristics of patients with rheumatoid arthritis (RA) and receiving rituximab.**

	All RA patients (n = 52)
Age, years	59 [35-84]
Gender, women	43 (83)
Disease duration, years	16 [1-36]
Previous treatment	
Anti-tumor necrosis factor- $\alpha$	42 (81)
Methotrexate	30 (58)
Prednisone	40 (77)
Disease activity in 28 joints score	5.4 [2.1-7.7]
Swollen joint count	4 [0-19]
Tender joint count	11 [0-27]
Erythrocyte sedimentation rate, mm/hr	25 [4-111]
C-reactive protein level, mg/L	17.4 [1.0-166.0]
Rheumatoid factor	36 (69)
Anti-citrullinated protein antibodies	45 (86.5)
Radiologic evidence of erosion	42 (81)
CD19+ /mm <sup>3</sup>	211 [25-706]
CD3+mm <sup>3</sup>	1777 [323-3378]
CD4+/mm <sup>3</sup>	1247 [233-2882]
CD8+/mm <sup>3</sup>	478 [120 -1114]
CD4+/CD8+	2.5 [0.9-7.4]
CD3-56+ /mm <sup>3</sup>	129 [32-654]
Data are number (%) or median [minimum - maximum].	

### Figures Legends

**Figure 1: CD19<sup>+</sup> (A), CD3<sup>+</sup> (B), CD4<sup>+</sup>(C), CD8<sup>+</sup>(D), CD3<sup>+</sup>CD56<sup>+</sup>(E) lymphocyte count changes (expressed as percentage change relative to baseline value) over time in RA patients receiving a first course of rituximab.** We used data for patients with flow cytometry data before treatment and at least at 1 time during the first course of rituximab. Lymphocyte phenotyping was performed at week 2 (W2), W12, W24, and from W36 to 48 (W36-48) for 51, 38, 41 and 30 patients, respectively. Minimum, maximum, median, mean (+), first and third quartile are represented by box plot. \* =  $p < 0.03$ , \*\* =  $p < 0.0001$ , \*\*\* =  $p < 0.00001$

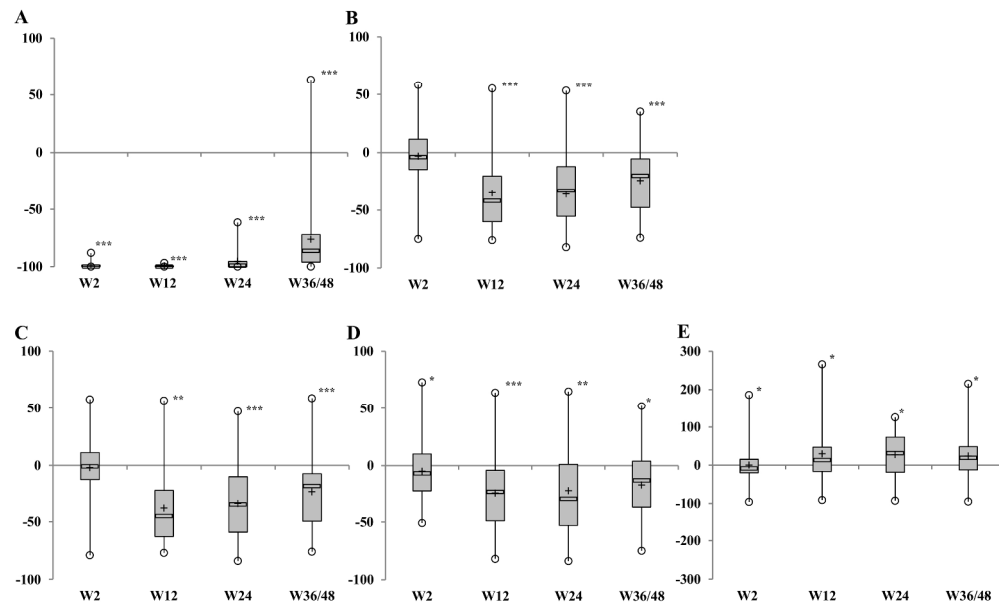
**Figure 2: Lymphocyte count changes over time in 1 RA patient receiving 4 courses of rituximab.** Dotted line corresponds to each course of rituximab. Time is expressed in weeks and cell count is in cells/ $\mu$ L.

**Figure 3: Association of CD3<sup>+</sup> (A), CD4<sup>+</sup> (B) and CD8<sup>+</sup> (C) T-cell count changes and response to rituximab treatment at W24 in RA patients.** We used data for 41 patients with data for flow cytometry and response to treatment at W24. Patients were divided into 3 groups by treatment response according to European League Against Rheumatism categories (21): non-responders, moderate responders and good responders. Minimum, maximum, median, mean (+), first and third quartile of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> percentage depletion relative to baseline are represented by box plot. NS = non significant.

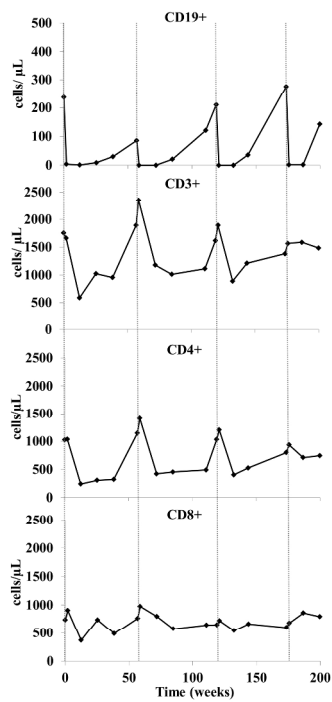
**Supplementary Figure 4: Association of CD4<sup>+</sup> cell depletion at W24 expressed as percentage relative to baseline value and time between the first and second course of rituximab in patients with RA.** In all, 32 patients received a second course of rituximab. The



analysis involved data for 24 patients with flow cytometry data at W24. Minimum, maximum, median, mean ( $^+$ ), first and third quartile are represented by box plot.

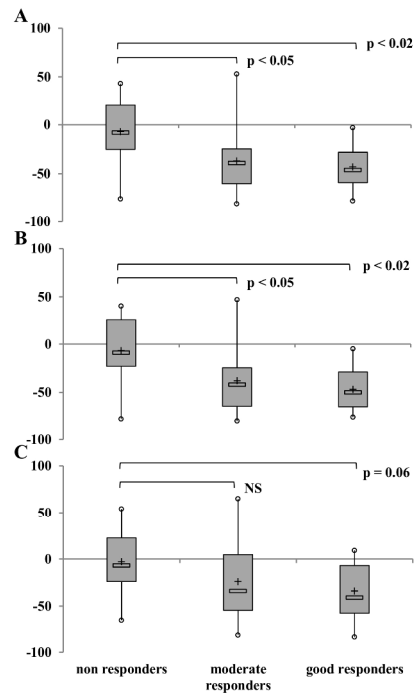


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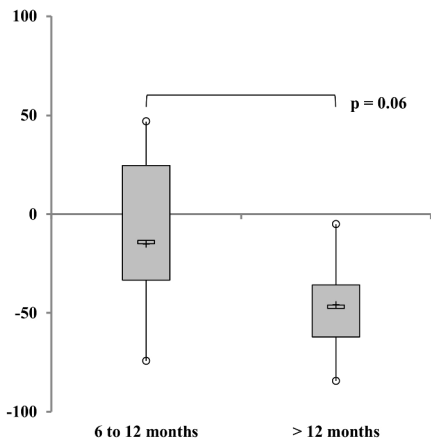


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