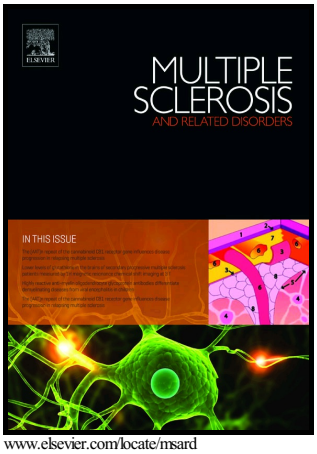

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The effects of intrathecal rituximab on biomarkers in multiple sclerosis

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JT, RD SL, AM and EE have no interests to declare.

DL and HK are employees of Roche

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Contributorship

SL, AM and EE designed the trial, recruited patients and administered medication. SL, AM and EE performed flow cytometry. JT, RD and GG designed and performed biomarker work. DL and HK conceived and arranged rituximab analysis.

JT prepared/wrote the manuscript and was responsible for statistical analysis with assistance from RD and GG. All authors had access to the data and provided editorial input into the manuscript.

All authors approved the final version of the manuscript.

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Abstract

Objectives: Clinical trials of IV-rituximab have proved successful. It is unclear whether intrathecal (IT)-rituximab is more efficacious at lower doses. We examine its effects on B-cell biomarkers.

Methods: 9 MS patients received IT-rituximab at 3 time-points. CSF and serum samples were obtained at up to 5 time-points (days 0, 7, 14, 56 and 112).

Serum and CSF BAFF and CXCL13, and CSF kappa and lambda free light chains (FLC) were measured. Flow cytometry was performed, examining effects on lymphocytes, CD3-19+ and CD3-20+ cells.

Results: CSF BAFF fell following rituximab (p=0.0091 absolute values, p=0.0284 change from baseline) whilst serum BAFF increased across time-points 1-4 (p=0.0005 absolute values, p=0.0017 change from baseline).

There were significant reductions in CD20+ and CD19+ cells in blood from baseline (p<0.0001) but not in CSF. CSF kappa FLC levels significantly increased (p=0.0480).

Conclusions: BAFF levels fall in CSF but increase in serum following IT-rituximab. Rituximab appears to act peripherally with dramatic decreases in peripheral CD20+ and CD19+ cells. It is likely that CSF B-cell counts were too low to enable differences to be seen. The rapid reduction in B-cells suggests rituximab has immediate effects. The profound depletion of B-cells, despite low doses of rituximab, underlines rituximab's efficacy.

Introduction

Increasing evidence suggests that the pathology of Multiple Sclerosis (MS) is not solely T-cell mediated, with B-cells appearing to contribute. The discovery of intrathecal oligoclonal immunoglobulin (Ig) production in around 90% of MS patients (1-3) led to research centering on humoral immune responses. Since then, B-cell aggregations into lymphoid-like structures have been discovered in the brain, meninges and demyelinating lesions of MS patients (4-7), and a correlation between localization of ectopic follicles and severe cortical pathology and clinical deterioration has been described in patients with secondary progressive MS (8). Latent EBV infection was found in a considerable proportion of these B-cell and plasma cell accumulations by one group (6), but this finding has not been replicated by other groups (7). B-cell infiltration short of follicle development has also been widely described, and is thought to be a common feature of disease (10). This evidence, alongside the success of B-cell targeted therapies, such as Phase I/II/III clinical trials of intravenous (IV) rituximab (11-14) (an anti-CD20 monoclonal antibody), demonstrates potential clinical benefits in targeting B-cells in MS.

CD19 and CD20 cells are phosphoproteins expressed on the surface of B-lymphocytes, pre-B cells and mature B-cells, during B-cell development (11, 15, 16). Plasma cells do not express CD20 or CD19 in contrast to plasmablasts which express both cell surface markers. Memory B-cells express CD20; but unlike naive B-cells, memory B-cells also express toll-like receptor 9 through which they can be non-specifically reactivated. The mechanisms involved in the effects of rituximab remain unknown. Current theories include antibody-dependent cell-mediated cytotoxicity involving apoptosis, cell lysis through complement activation, or activation of macrophages, monocytes or natural killer cells (15, 17-20).

Studies have shown that treatment with rituximab in relapsing remitting MS (RRMS) depletes CD20+ B-cells, with subsequent reductions in CD4+ and CD8+ T-cells, as well as in proliferative and pro-inflammatory cytokine responses, such as Th1 and Th17. This suggests a possible effect via reduced

T-cell trafficking into the CNS (2, 21). Whether this is a direct or indirect effect of B-cells remains questionable. It has been suggested that B-cells of the CNS may alter the blood brain barrier (BBB) or secrete chemokines promoting T-cell recruitment (11) possibly explaining the reduced T-cell response to diminished circulating B-cells as a result of rituximab treatment.

BAFF is a B-cell activating factor that regulates B-cell survival, differentiation, development, production of Immunoglobulins (Ig) and size of peripheral B-cell pool. BAFF levels are increased in MS patients in the serum, and within the CSF in some studies however, the latter still remains questionable and varies between studies. In MS brains, BAFF may promote BAFF-R-expressing B cell survival, enabling expansion of CNS B-cells, contributing to plasma cell survival (22). Both interferon-beta (23) and alemtuzumab (24) increase serum BAFF when used in the treatment of MS. CXCL-13 is alternatively known as B lymphocyte chemoattractant factor, and has a key role in regulating B-cell trafficking into the CNS (25). Both CSF and serum levels are increased in patients with MS (26); first-line disease modifying treatment has not been shown to affect CXCL-13 levels. CXCL-13 appears to significantly decline after IV rituximab treatment (375mg/m^2 weekly for 4 doses) in both blood and CSF (27).

Previous clinical trials of intravenous (IV) rituximab in MS have shown dramatic reductions in gadolinium enhancing brain lesions at doses of 1000mg on days 1 and 15 (12, 13) and a repeat course administered at weeks 24 and 26 (12), with one or two trials involving smaller doses of 375mg/m^2 IV weekly for 4 doses (14, 28). Current trials are beginning to emerge in which 1000mg rituximab has been administered both intravenously and intrathecally in secondary progressive MS (SPMS) (29). There is an ongoing open label trial of low dose intrathecal rituximab (**ClinicalTrials.gov identifier NCT01719159) in progressive MS** (30) and a single case report of intrathecal use in a patient with severe progressive MS (31). It remains uncertain whether intrathecal rituximab is more efficacious at lower doses than are administered peripherally.

We therefore set out to evaluate the efficacy of intrathecal rituximab at three low doses (5, 10 and 15mg), and studied the effects on B-cell biomarkers BAFF, free light chains (FLC) and CXCL13, and on CSF and peripheral B-cell populations. These B-cell biomarkers were chosen as representative markers for B-cell survival [BAFF], B-cell trafficking [CXCL13] and B-cell activation [FLC].

Methods

Recruitment

A total of 9 patients with clinically definite MS (4 RRMS and 5 SPMS; EDSS 4-8, table 1) were recruited. Subjects were recruited at the MS Centre (City Clinical Hospital 31) in Saint-Petersburg, Russia, using specific inclusion and exclusion criteria documented in tables 1 and 2 in Appendix 1 (full criteria documented in Appendix 2). Ethical approval for this study was granted by the Research Council of Pavlov State Medical University, St. Petersburg. All subjects provided written informed consent. Details regarding clinical history and previous MS medications are given in table 1 [Table 1].

Rituximab administration and dosage

Intrathecal rituximab was administered to all patients at 3 timepoints at increasing doses: 5mg on D0, 10mg on D7 and 15mg on D14 [Appendix 1 table 3]. Serum and CSF samples were taken at up to 5 timepoints; baseline and days 7, 14, 56 and 112 (immediately prior to the initial and subsequent intrathecal rituximab treatments administered on D0, D7 and D14). Expanded Disability Status Scores (EDSS) were documented throughout the trial at 5 timepoints (Baseline, D7, D14, D56, D112).

Flow cytometry of peripheral blood and cerebrospinal fluid cells

Flow cytometry was performed on peripheral blood and CSF, at the MS Centre (City Clinical Hospital 31) in Saint-Petersburg, Russia, to examine the effects of IT rituximab on total white blood cell count, lymphocytes, CD3-CD19+ and CD3-CD20+ cells, using BD FACSAria II and BD FACSDiva Software (BD Biosciences, USA). CSF samples were taken by lumbar puncture immediately prior to

rituximab administration and standard CSF analysis was performed. Peripheral blood was taken at the same time. Cells were spun down at 1000g centrifugation and 1ml pellets examined using flow cytometry.

ELISAs

Commercially available ELISAs were used to measure **BAFF** [R&D Systems Inc.,USA] and **CXCL13** [R&D Systems Inc, USA] levels in serum and CSF at each timepoint. **Kappa and lambda free light chains (FLC)** [BioVendor, Czech Republic] were measured in CSF only. All ELISAs were performed at the Blizard Institute (QMUL, Whitechapel), in line with the manufacturer's instructions with the exception of CSF dilution for BAFF and FLC ELISAs (unspecified by manufacturer). For the BAFF ELISA a 5-fold dilution was used for serum samples, and a ratio of 20µml CSF to 30µl diluent for CSF samples. For the FLC ELISA (kappa and lambda) CSF samples were diluted 1:10. For the CXCL13 ELISA kit neither serum nor CSF samples were diluted.

RItuximab concentration

CSF and serum rituximab concentrations were measured using a validated ELISA by a qualified analyst at Genentech (Genentech; San Francisco, CA, USA). The reporting range of this ELSA is 5-125ng/mL for both serum and CSF. A summary of the method is as follows: test samples, quality controls, and standards (rituximab in buffer) were incubated on plates pre-coated with a polyclonal goat anti-rituximab antibody, followed by washing. Bound samples were detected by incubation with goat anti-mouse IgG F(ab')2 conjugated to horseradish peroxidase. Following a further wash to remove any unbound conjugate, a substrate solution (tetramethyl benzidine/hydrogen peroxide) was added to the wells, resulting in a color development in proportion to the amount of rituximab in the samples. The reaction was stopped and absorbance was measured photometrically.

Statistical analysis

All data was tested for normality, and log transformed if necessary. Data from flow cytometry, ELISAs and clinical examination (including EDSS scoring) were analysed between timepoints and percentage difference from baseline using repeated measures ANOVA. Graph Pad Prism 5 was used for all analysis.

Results

CD20+ and CD19+ B-cell population

A significant reduction was found in the percentage of CD20+ and CD19+ B-cells in blood from baseline ($p<0.0001$ for both, timepoints 1-5) (Figures 1 and 2 respectively; see also supplementary figures 1 and 2). A non-significant decrease was seen in CSF ($p= 0.8702$; $p=0.6475$ respectively) (Appendix 1 table 4).

BAFF levels

CSF BAFF levels were significantly reduced ($p=0.0091$ absolute values, $p=0.0284$ for change from baseline) whilst serum BAFF levels showed an opposite effect and significantly increased ($p=0.0005$ for absolute values, $p=0.0017$ for change from baseline), for timepoints 1-4 [D0 (baseline) - D56]. Immediate significant changes were observed in CSF BAFF levels between D0 (baseline) vs. D7, 14 and 56 ($p<0.05$) (Figure 3 and supplementary figure 3). There was a gradual increase in serum BAFF levels, with significant increases between D0 vs. D56 ($p<0.001$) and D7 vs. D56 ($p<0.01$) (Figure 4 and supplementary figure 4).

Rituximab levels

Rituximab was not detectable in the CSF of any subject at any of the timepoints (data not shown). Rituximab was detectable in the serum in 5 subjects. In all subjects in whom rituximab was detected in the serum it was present at D14 (mean concentration at D14 1580ng/ml; range 909 – 2510ng/ml). In one subject (subject number 5), serum rituximab was also detectable at D7 (557ng/ml) and D56 (983ng/ml).

EDSS and clinical progression

EDSS decreased over the treatment period in 4/9 patients (44%) and remained stable in 5/9 patients (56%) (Appendix 1 table 5). EDSS improvements ranged from 0.5-3.5. EDSS continued to improve even after the last treatment (T3) in Patients 3 and 5 but began to increase in Patient 1 at T5. There was no significant change in EDSS across timepoints. No relapses were documented during the course of this study.

FLC and CXCL13 levels

CSF kappa FLC levels moderately increased during timepoints 1-5 ($p=0.048$ for change from baseline, absolute values not significant $p=0.38$). There were no significant effects on either serum or CSF CXCL13 or CSF total or lambda FLC levels (data not shown). There were no significant effects on either blood or CSF total WBC count (data not shown).

Discussion

IT rituximab appears to act peripherally, with dramatic decreases in peripheral CD20+ and CD19+ cells but no significant changes in the CSF. The peripheral mechanism of action is highlighted by the fact that rituximab concentrations in the CSF were sufficiently low as to be undetectable in all patients, whilst a number of patients had detectable rituximab in the peripheral serum compartment. The profound depletion of peripheral CD20+ and CD19+ cells, despite the low dose of

rituximab given (5-15mg IT administration, compared to the usual dosage of 375-1000mg IV administration), underlines the efficacy of this product. Previous studies have demonstrated that peripherally administered rituximab crosses the blood-brain barrier and is detectable in the CSF, albeit at far lower concentrations than seen in the serum (32). Similar results have been seen in an open-label trial of intrathecal rituximab given at similar doses (30). Previous studies in the oncological and haematological literature have demonstrated rapid clearance of rituximab from the CSF compartment (33, 34). The very low doses used when compared to peripheral IV dosing regimes in addition to the fact that samples were taken immediately prior to next dosage may explain the low and negative serum concentrations seen.

It is possible that CSF CD20+ and CD19+ cell counts were too low to enable any significant differences to be seen. IV rituximab has been shown to deplete B-cells in the CSF less effectively than peripheral B-cells, possibly due to more B-cells in the CSF being advanced memory/plasma B-cells with less or no CD20 expression (28). It is clear from our study that IT administered rituximab is also acting peripherally with marked effects on peripheral B-cell populations and levels detectable only in serum. This raises questions regarding both the site of action of rituximab and the doses required for treatment of CNS disease.

BAFF levels fall in CSF but increase in serum following IT rituximab administration. The reasons underlying these changes remain unclear. However, the increased serum BAFF levels may be an indication of B-cell re-population. An alternative explanation may be decreased consumption of BAFF during B-cell depletion. Our data suggests IT rituximab may move immediately to the periphery; however CSF levels of BAFF fall, the reasons for which are unknown. To our knowledge little work exists on the effect of other MS treatments on CSF BAFF levels. No significant effects were found on either serum or CSF CXCL13 levels and minimal effects were seen on CSF FLC at the dose of rituximab given.

Whether the benefits seen by rituximab (anti-CD20) therapy are based directly on B-cell depletion or the indirect effect on T-cells, by the reduction of B-cell to T-cell interaction and communication (ie. B-cell mediated antigen presentation and activation of T-cells), remains unknown. A small population of T-cells express CD20 (CD3+CD20+ cells); it may be that some clinical effects may result from the depletion of these T-cells from the circulation. However, this would be unlikely to explain the extent of the therapeutic effects of rituximab, unless these cells had important immunoregulatory functions. These therapeutic effects may well be a combination of both the direct and indirect effects of this drug on immune cells (B- and T-cell responses).

There was no increase in EDSS from baseline in any patient and no further relapses were documented for any patient during this study (timepoints 1-5). This may indicate rituximab has affected disease activity, as all 9 patients had previously shown resistance to prior therapies with >1 relapses over the preceeding 12 month period. However, this study ran for only 112 days (16 weeks) and a longer study period would be needed to accurately test this hypothesis. It would be interesting to observe whether peripheral B-cell populations and biomarkers increase >16 weeks after the IT rituximab treatment or whether levels continue to decrease further.

Table and figure legends

Table 1: Patient Characteristics

Figure 1: Box and whisker plot demonstrating the effects of intrathecal rituximab treatment on peripheral CD3-CD20+ % cell count over time, with a significant reduction in CD20+ B-cells in blood from baseline. The box indicates the median and standard deviation of CD3-CD20+ % cell count for each timepoint (1-5), and the whiskers represent the range.

Figure 2: Box and whisker plot demonstrating the effects of Intrathecal rituximab treatment on peripheral CD3-CD19+ % cell count over time, with a significant reduction in CD19+ B-cells in blood from baseline. The box indicates the median and standard deviation of CD3-CD19+ % cell count for each timepoint (1-5), and the whiskers represent the range.

Figure 3: Box and whisker plot demonstrating the effects of intrathecal rituximab treatment on CSF BAFF levels over time, with a significant reduction of CSF BAFF levels. The box indicates the median and standard deviation of CSF BAFF levels for each timepoint (1-5), and the whiskers represent the range.

Figure 4: Box and whisker plot demonstrating the effects of intrathecal rituximab treatment on BAFF levels in serum over time, with a significant reduction of BAFF levels in serum. The box indicates the median and standard deviation of BAFF levels in serum for each timepoint (1-5), and the whiskers represent the range.

Table 1: Patient Characteristics

Patient	Age/Gender	MS type	Disease duration (years)	Baseline EDSS	Therapy prior to RTX		Criteria of resistance to ongoing therapy		
					First line	Second line	Increase EDSS >0.5 point	≥ 1 relapses within 12 months	New T2 lesions (MRI)
1	47/M	RR	4	6.50	INFb-1a 44		x	x	x
2	37/M	SP	12	7.00	INFb-1b 8,6ME	MTX	x	x	x
3	42/F	SP	6	8.00	GA		x	x	x
4	36/F	RR	5	4.00	GA			x	x
5	37/F	SP	9	6.00	GA		x	x	x
6	54/F	SP	19	6.00	INFb-1b 8,6ME			x	x
7	36/F	SP	11	5.00	GA	MTX	x	x	x
8	42/F	RR	3	4.00	INFb-1b 8,6ME			x	x
9	47/M	RR	4	4.00	INFb-1b 8,6ME			x	x

X = Yes, RR - relapsing remitting, SP - secondary progressive INF = Interferon, GA = Glatiramer Acetate (Copaxone), MTX = Mitoxantrone IV.

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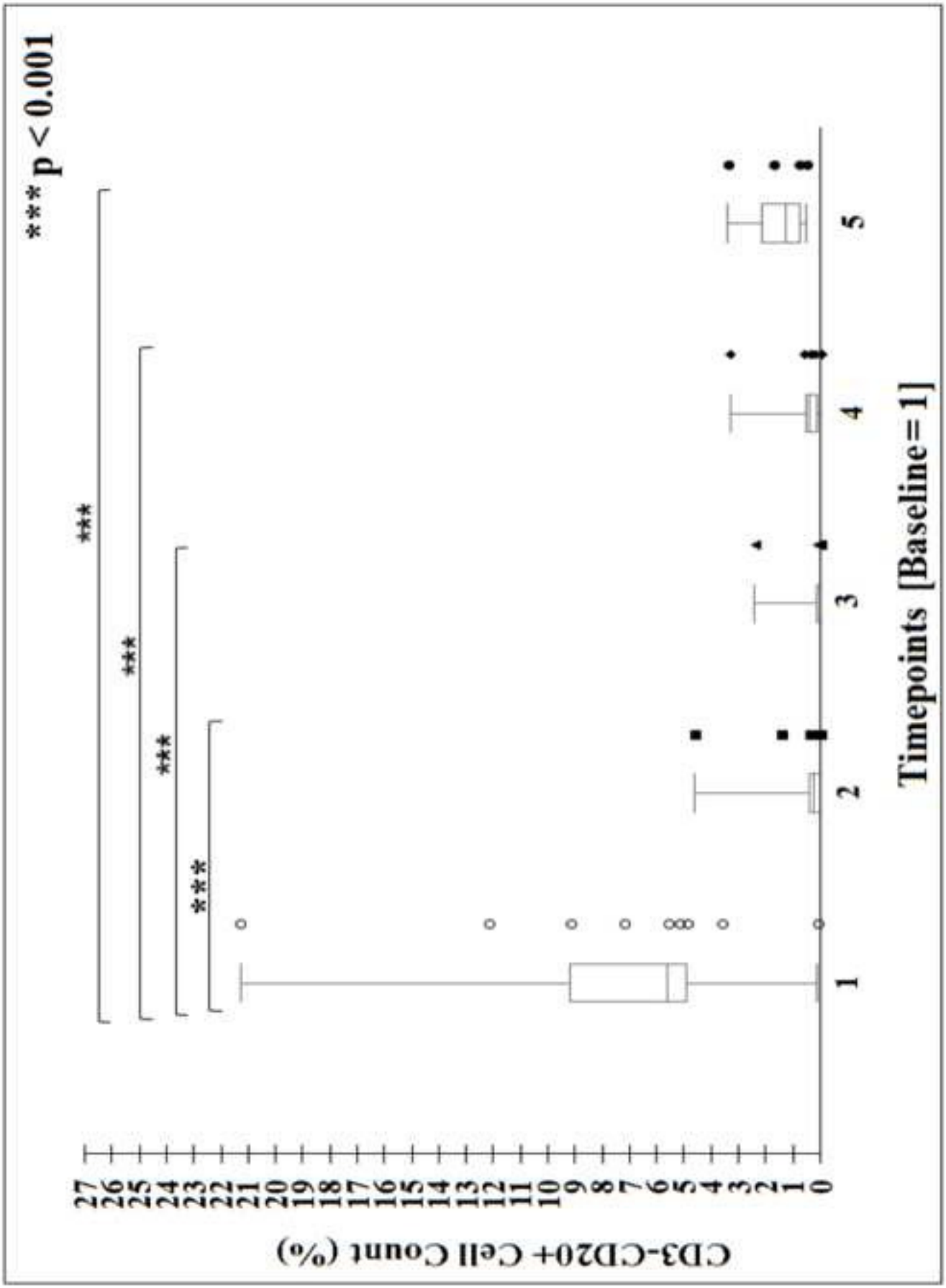
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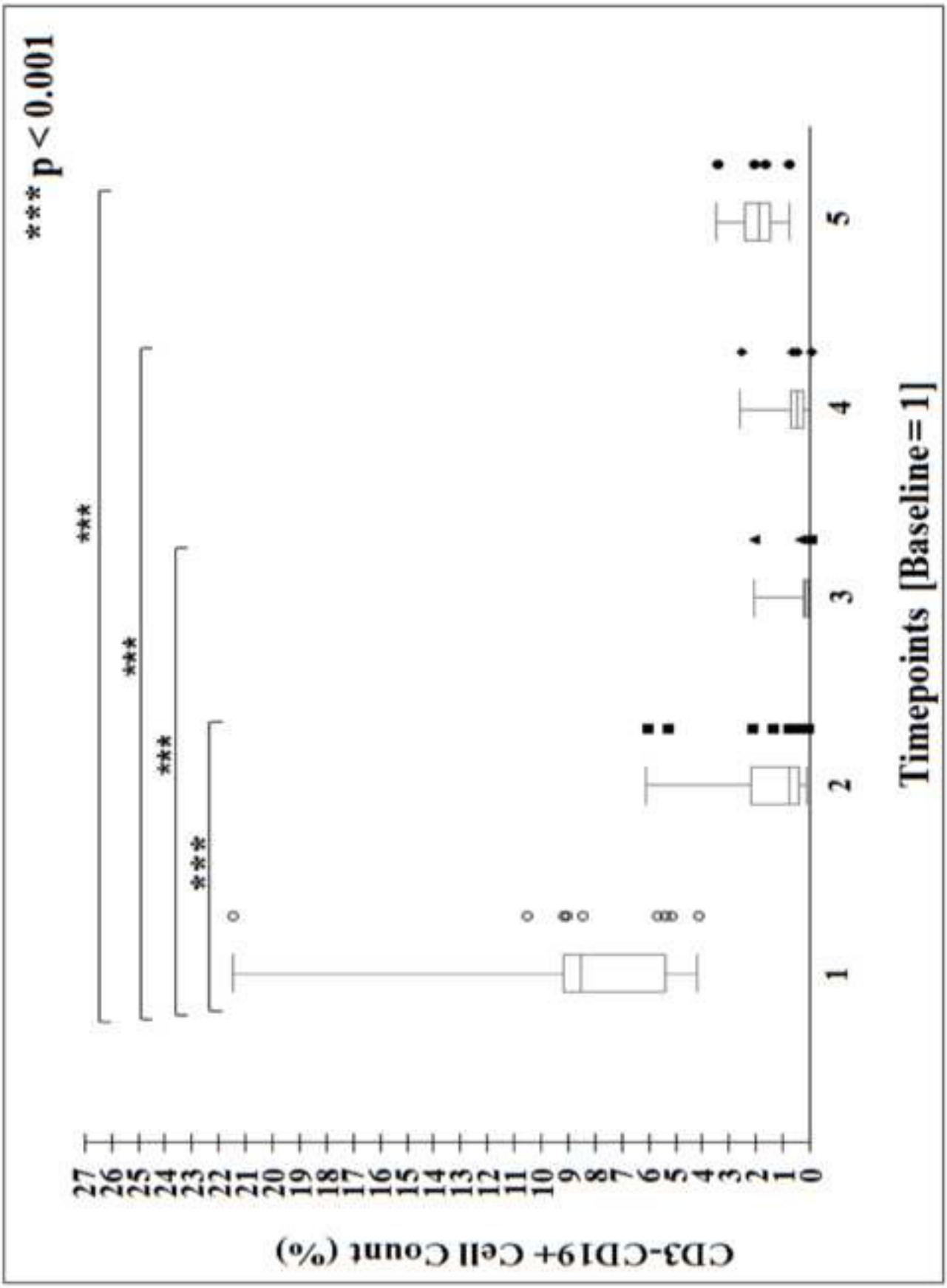
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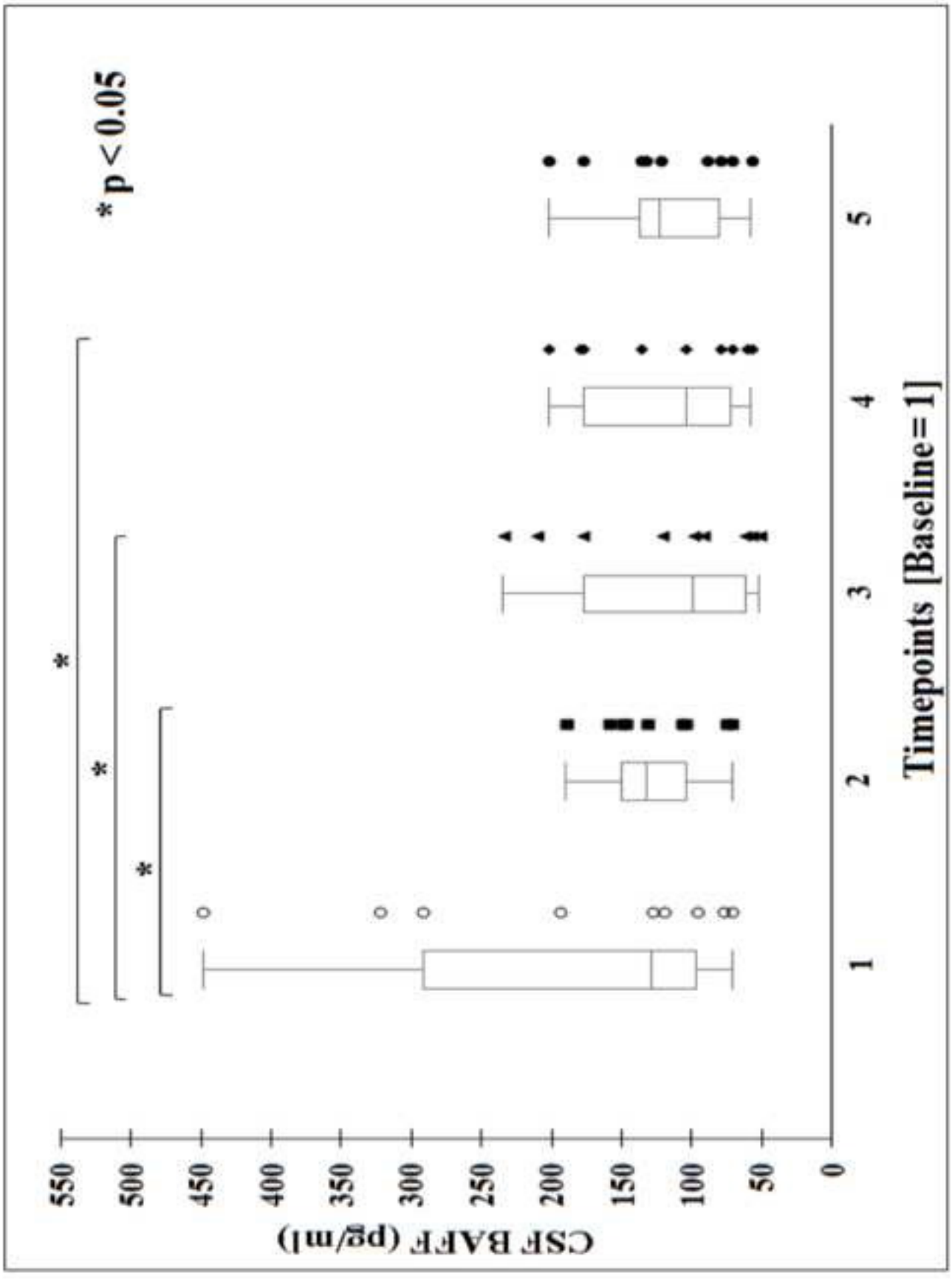
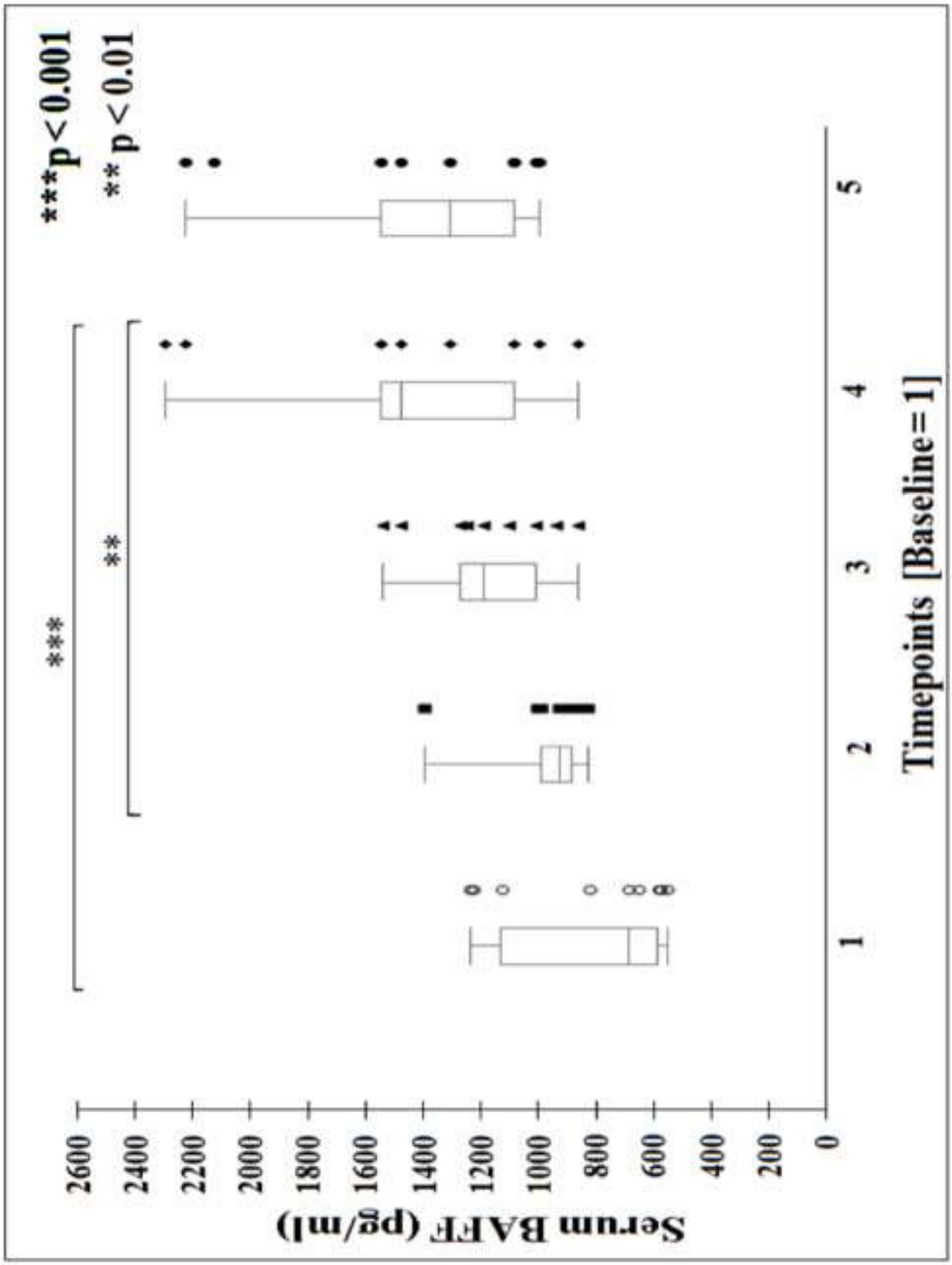
24
25
26
27
1
2
3
4
5
Timepoints [Baseline=1]

Figure



Figure


***p<0.001
**p<0.01
***
**
Serum BAFF (pg/ml)
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
Timepoints [Baseline=1]

Figure