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Classification of individuals based on *ex-vivo* glatiramer acetate-induced interferon-γ and interleukin-4 response

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Abstract

Background: Glatiramer acetate (GA) in multiple sclerosis acts through the induction of GA-specific T-helper 2 cells. Nevertheless, the phenomenon is not universal in patients, explaining individual differences in clinical response.

Objective: The objective of this article was to categorize GA-treated patients.

Method: An enhanced quantitative PCR assay was used for measuring *ex-vivo* GA-induced IFNγ and IL4 mRNA responses in mononuclear cells from 23 healthy donors, 27 untreated patients, 33 short-term (≤6 months) and 77 long-term (>6 months) GA-treated patients. Thresholds for IFNγ and IL4 transcriptional response were calculated by ROC analysis and long-term treated patients were compared in terms of prognostic stratification.

Results: Thresholds for IFNγ and IL4 transcriptional response were calculated at 5.36 and 1.41 relative expression (RE). Finally, 67% of long-term treated patients scored above both response thresholds. These patients had a higher proportion of relapse-free subjects (74% vs 40% when compare to patients who scored below both thresholds) and a significantly better relapse-free survival rate (p=0.006; CI 0.29–0.75). The negative predictive value to predict adverse clinical outcome was 79% (CI 0.63–0.90), meaning that by a positive response, there is a 79% chance that the patient will not experience a negative outcome at 3 years.

Conclusions: Our enhanced quantitative PCR assay produced clinically significant results for GA-treated patients. As such, if patients have a positive response, it means they have less chance of a relapse, while patients with a negative response have a greater probability of a worse outcome.

Keywords

Glatiramer acetate, nonresponsive patients, interferon-gamma, interleukin-4, transcriptional response, real-time PCR

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Introduction

Glatiramer acetate (GA) approved for the treatment of multiple sclerosis (MS),[1–4] acts through the induction of GA-specific T-cells characterized by protective anti-inflammatory T-helper (Th)2 responses. Nevertheless, the induction of these T-cells is not universal in patients,[5] a phenomenon that could explain individual differences in clinical response.[1–4,6–8]

To date, evaluation of responsiveness to GA has been tested mostly by monitoring clinical and neuroradiological changes.[1–4,8,9] An attempt to introduce a biological approach was made through an enzyme-linked immunoadsorbant spot (ELISpot) assay, used to analyse changes of expression of the Th1-type cytokine, interferon-gamma (IFNγ) and Th2-type cytokine, interleukin-4 (IL4), in peripheral blood mononuclear cells (PBMCs) from patients treated with GA.[6,10] This assay was shown to correctly discriminate between GA-treated and untreated subjects,[6] and its prospective use as a biomarker of clinical response was reasonably suggested.[10]

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Table 1. Distribution of patients and blood samples among the different timing categories.

	Patients tested within three categories (samples)	Patients tested within two categories (samples)		Patients tested within one category (samples)			TOT patients (samples)	
Untreated patients with multiple sclerosis	4 (4)	15 (15)		8 (8)				27 (27)
Short-term treated patients	4 (4)		12 (12)		17 (19)[a]			33 (35)
Long-term treated patients	4 (4)	15 (15)	12 (12)			46 (53)[b]		77 (84)
Actual TOT patients	4	15	12	8	17	46	**102**	**137 (146)**

[a]Two patients were tested twice within the short-term treatment category.
[b]Seven patients were tested twice within the long-term treatment category.

Nevertheless, although ELISpot has yielded important data regarding the immunological responsiveness to GA, this technique has proven to be unsuitable for routine clinical practice. An advantage of this technique is its high throughput; but high throughput implies the concurrent testing of many samples obtained from different patients at the same time. Clearly cryopreserved PBMCs may be tested, but in most cases cells responses are substantially reduced compared with those from freshly isolated PBMCs. Another disadvantage of the ELISpot method is that scoring the wells for positive reactions involves the manual enumeration of large numbers of coloured spots, which can vary greatly in size and shape; thus, although computer-based image analysis systems are now being developed, this method is subject to operator bias.

It is our goal in this paper to suggest an alternative approach for analysing IL4 and IFNγ transcriptional responses. To accomplish this goal, we first propose to determine whether IL4 and IFNγ transcriptional responses of PBMCs after *ex-vivo* exposure to GA, correlate with the biological response as previously shown by ELISpot. To this end, we first assessed the agreement between the two technical approaches. Then we compared GA-treated patients in terms of prognostic stratification.

Patients and control subjects

Patients who agreed to participate and signed the consent form were eligible for the study if they had a diagnosis of clinically definite relapsing–remitting MS,[11] at least a 4 year history of follow-up and an Expanded Disability Status Score (EDSS) ≤6.0. Moreover, patients were included if they were initiating therapy with GA (Copaxone, Sanofi-Aventis, Paris, France) or they were already receiving GA therapy. Exclusion criteria were: immunosuppressive therapy during the 12 months before the study and concurrent therapy with corticosteroids. Since glucocorticosteroids might alter immune responses, blood samples were drawn at least 30 days after the last relapse therapy.

A total of 146 samples from 102 patients were collected between November 2006 and November 2008. Of the 146 samples, 27 were obtained from patients who had never been treated with GA before entering this study (treatment-naive patients), 35 were obtained from 33 patients with ≤6 months of GA therapy (short-term treated patients) and 84 samples were obtained from 77 patients with >6 months of therapy (long-term treated patients). The 6 month time-point was chosen based on previous research.[6] Notably, 25 patients were tested more than once with different category placement (Table 1).

For routine clinical monitoring, subjects were required to visit the clinic for a baseline evaluation and every 3 months for neurological examination, with completion of EDSS and relapses recorded. A relapse was defined as the appearance of a new symptom or a worsening of a pre-existing symptom, lasting more than 24 hours and producing a modification in the functional system of the EDSS score.[12]

A total of 23 healthy volunteers were included as a control. Demographic and clinical characteristics of patients and healthy controls are shown in Table 2.

Methods

Ex-vivo *stimulation with GA*

PBMCs were isolated by centrifugation of whole blood on a density gradient. Leukocytes were harvested, pooled and washed with PBS. Cells were resuspended in 20 mL of RPMI medium supplemented with 10% FBS and split into 2 equal volumes, resulting in ~10^6 cells/aliquot: an aliquot was stimulated with 50 μg/mL of GA (Copaxone, Sanofi-Aventis, Paris, France), while the second aliquot was untreated. Suspensions were incubated at 37°C for 18 hours.

After incubation, cells were collected by centrifugation and subjected to total RNA extraction. RNA was finally reverse transcribed by random priming with the High Capacity Reverse Transcription Kit (Lifetechnologies, Monza, Italy) at a final concentration of 5 ng/μL.

Table 2. The demographic and clinical characteristics of patients and controls at baseline.

Characteristics	Healthy Controls	Treatment-naïve patients	Short-term treated patients	Long-term treated patients		
				TOT	Clinical responders	Clinical nonresponders
Sample size	23	27	33	77	46	31
Females/males (%)	16/7 (70%/30%)	18/9 (67%/33%)	20/11[a] (65%/35%)	52/18[b] (74%/26%)	31/9 (77%/23%)	21/9 (70%/30%)
Median (range) age at start of therapy, y.	34 (26–58)	35 (17–56)	37 (18–62)	38 (18–61)	38 (18–51)	37 (25–61)
Median (range) disease duration at start of therapy, mo.	/	11 (2–39)	12 (4–42)	12 (4–59)	12 (6–59)	12 (4–50)
Median (range) EDSS score at start of therapy	/	1.0 (0–3.5)	1.5 (0–3.0)	1.0 (0–4.0)	1.0 (0–3.0)	1.0 (0–4.0)
Median (range) number of relapses 1y. before therapy		1 (0–2)	1 (0–2)	1 (0–3)	1 (0–2)	1 (0–3)

EDSS: Extended Disability Status Scale; RR: relapse rate; y.: year; mo.: months.
[a]Two females were tested twice within this category.
[b]Five females and two males were tested twice within this category.

Pre-amplification and real-time PCR analysis

Since during preliminary experiments IL4 expression was not detectable in most samples, cDNA was pre-amplified according to the optimized protocol of the TaqMan PreAmp Master Mix Kit (Lifetechnologies, Monza, Italy), using pooled gene-specific primers (i.e. IL4, IFNγ and reference gene glyceraldehyde-phosphate-dehydrogenase, GAPDH).

Before proceeding with the real-time PCR analysis, pre-amplification uniformity was evaluated by calculating the ΔCt, i.e. the difference between the Ct of the target gene and the reference gene, and the ΔΔCt between cDNA and pre-amplified cDNA; thus subtracting the ΔCt value for cDNA from the ΔCt for multiplex pre-amplification. Both targets produced ΔΔCt values close to zero and within ±1.5, indicating that pre-amplification was uniform. Next, the pre-amplification product was used as a template for the real-time 5'-nuclease assay. TaqMan gene expression assays (Lifetechnologies, Monza, Italy) were utilized as primers and probes. Data were normalized using GAPDH, and relative expression of *ex-vivo* GA-treated samples to respective untreated samples was determined by the $2^{-\Delta\Delta Ct}$ formula.

Statistical analysis

Optimal cut-offs for mRNA responses were calculated by receiver operating characteristic (ROC) analysis.

Data were tested for distribution by the Kolmogorov-Smirnov test. Since values from treated patients were non-normally distributed, non-parametric statistical tests were applied: GA-induced responses were compared by either the Mann–Whitney *U*-test or the Wilcoxon test for pairwise comparisons and the Kruskal-Wallis test for multiple comparisons. A mixed effect modelling was used to evaluate evidence for differences between subjects and items as a random effect.

For clinical analyses we considered time to first relapse and time to disease progression as measured by changes from baseline in EDSS. Relapse-free survivals were estimated by the Kaplan-Meier method and compared by the log-rank test. Fisher's exact test was used to compare the number of relapse-free patients. *P*-values <0.05 were considered significant. Analyses were performed using SPSS version 19.0 (Chicago, USA).

Results

Differential ex-vivo *GA-induced mRNA responses*

Comparing healthy controls, untreated and GA-treated patients, Kruskal-Wallis rank sum test yielded a significant difference among conditions from both IFNγ (p=0.03) and IL4 (p=0.005) analysis. In detail, IFNγ and IL4 transcriptional changes in response to *ex-vivo* GA-stimulation were higher in treated patients compared to both other groups (Figure 1). A mixed model with subjects and items as random effects yielded a similar advantage.

To verify whether those responses were related to the duration of GA therapy, we divided treated patients into short-term and long-term treated individuals. IFNγ and IL4 transcriptional changes in response to *ex-vivo* GA

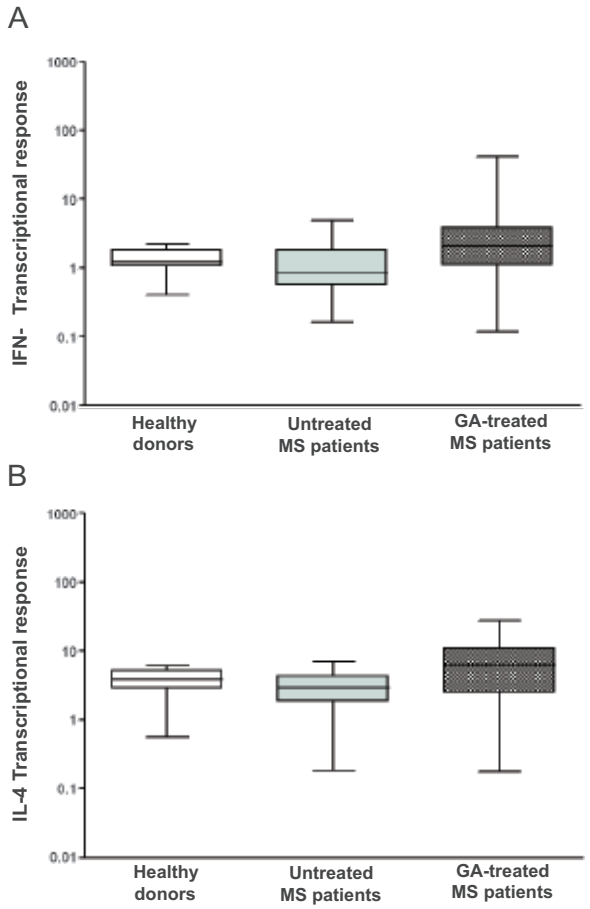

Figure 1. Comparison of IFNγ and IL4 mRNA responses after *ex-vivo* stimulation with GA. Blood samples were obtained from 23 healthy donors, 27 untreated MS patients and 110 patients treated with GA. GA-induced mRNA levels of both (a) IFNγ and (b) IL4 are significantly higher in patients treated with GA than in both healthy controls and untreated patients (both $p \leq 0.03$).

stimulation were lower in short-term treated patients compared to long-term treated patients, being comparable to both untreated patients and healthy controls ($p_{IFN\gamma}$=0.03 and p_{IL4}=0.002). The mixed model showed similar differences.

Defining biological responsiveness

We next asked whether *ex-vivo* GA-induced IFNγ and IL4 mRNA changes could represent a marker for the detection of treatment effects. Predictive discriminating transcription values were calculated by ROC analysis. Estimates of optimal cut-offs were made comparing IFNγ and IL4 mRNA responses in long-term treated patients and healthy controls, taking into account sensitivity and specificity. Thresholds were calculated at 5.36 and 1.41 relative expression (RE) for IFNγ and IL4, respectively.

The proposed model postulates that the GA-induced differential Th1 and Th2 cytokine response occurs only when *ex-vivo* post-stimulation mRNA values are above both thresholds. To verify this model, individuals were classified as (IFNγ/IL4)-positive, when showing above-threshold IFNγ and IL4 responses, and (IFNγ/IL4)-negative when showing below-threshold responses. Individuals classified as either IFNγ-negative/IL4-positive or IFNγ-positive/IL4-negative were considered as not informative and requiring further evaluation.

Among 169 samples, 17 (10%) were considered as not informative. Of these, 11 underwent a second evaluation, which then provided an informative result.

According to the threshold-based classification, 50/77 long-term treated patients could be correctly identified, whereas only 3/27 untreated patients and 1/23 healthy donors met the criteria for treatment effects (i.e. false positive). Notably, the great majority of short-term treated patients (19/33) were assigned to the not-treated group.

Overall, for the detection of treatment effects, the combination of *ex-vivo* GA-induced IFNγ and IL4 mRNA responses resulted in sensitivity and specificity of 92% and 67%, with positive (PPV) and negative (NPV) predictive values of 64%, and 93%, respectively (Table 3).

To account for possible omitted-variable bias in the model we determined whether the baseline IFNγ and IL4 (i.e. before *ex-vivo* stimulation with GA) was different between (IFNγ/IL4)-positive and (IFNγ/IL4)-negative patients. The study found no difference between the two groups.

Longitudinal stability

A total of 19 patients were evaluated for *ex-vivo* GA-induced IFNγ/IL4 response, before and after long-term therapy. In 12/19 patients, post-treatment response was increased as compared to pre-treatment response (p=0.008), whereas in the remaining 7 individuals, response was unchanged during therapy (Figure 2).

Table 3. Summary of test performance indicators for *ex vivo* GA-induced IFNγ/IL4 mRNA response under different causal assumptions.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Treatment effect	92	67	64	93
Prediction of relapses	54	79	60	74
Prediction of clinical response	63	87	76	78

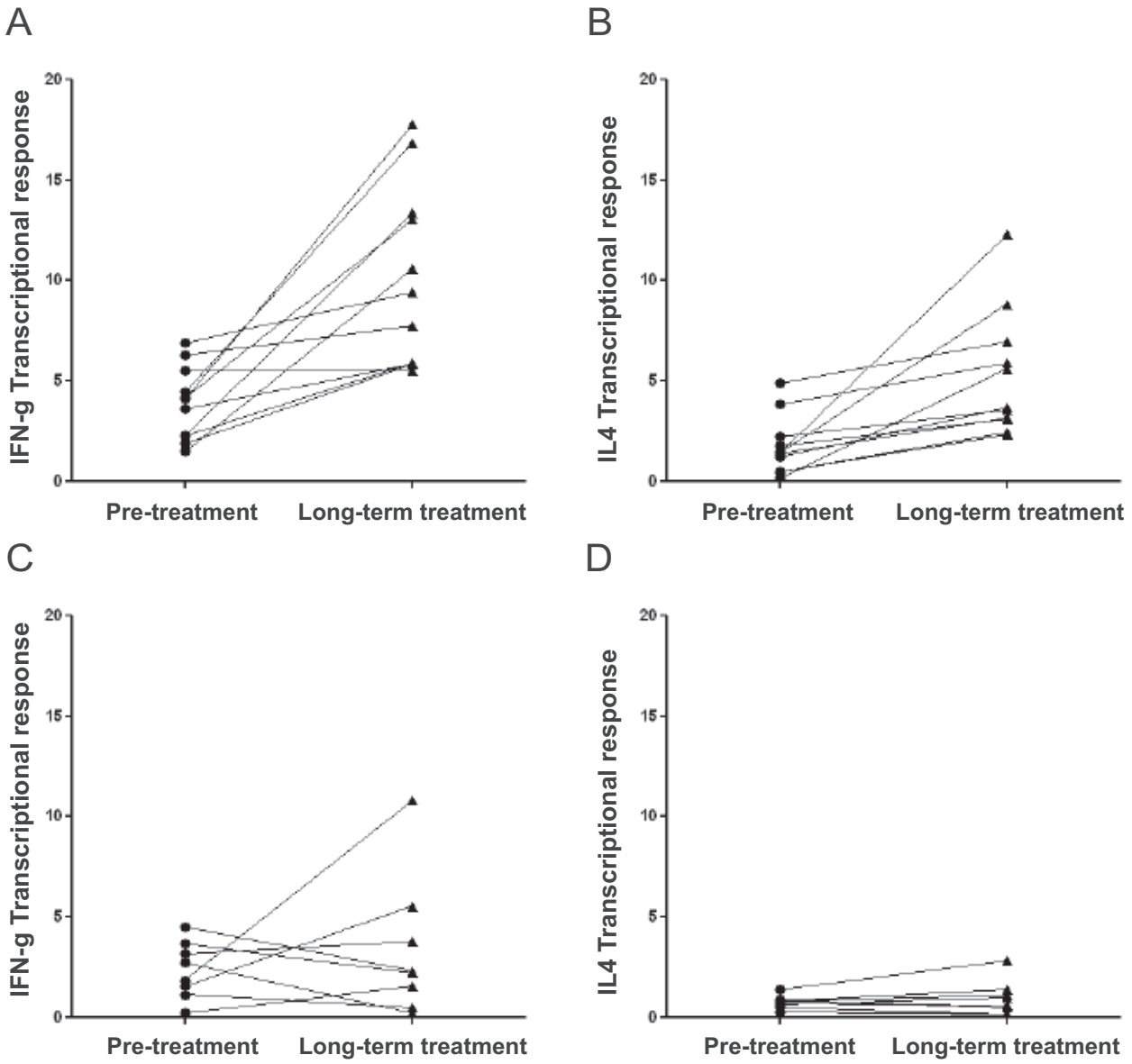

Figure 2. Change of IFNγ and IL4 mRNA response levels in 19 individual patients tested before treatment and after long-term treatment. Patients were sub-categorized based on their clinical responsiveness as (a) and (b) clinical responders (n=11), and (c) and (d) clinical non-responders (n=8).

To assess the longitudinal stability of mRNA response, repeated sampling analysis was performed in four patients. Three patients were classified as (IFNγ/IL4)-positive and one patient as (IFNγ/IL4)-negative. In the (IFNγ/IL4)-negative patient, GA-induced transcriptional responses remained low at all time points, indicating a persistent lack of bioactivity. On the contrary, (IFNγ/IL4)-positive patients showed persistently positive responses after 6 months of treatment.

Predicting a value for relapses

To examine the clinical utility of the *ex-vivo* GA-induced IFNγ/IL4 transcriptional response and its cut-offs, we assessed relapses during therapy, considering informative patients (n=75/77) only. Among the 50/75 (67%) (IFNγ/IL4)-positive patients, 30 (60%) were relapse-free, as well as 8 (32%) of the 25 (IFNγ/IL4)-negative patients. Despite a longer follow-up (49 (12–98) vs 32 (10–76)), the number of relapse-free patients was higher in the positive group compared to the negative group (p=0.03) (Figure 3(a)).

To account for both the 6 month delayed biological effect and treatment duration, a further analysis was conducted in a 6 to 42 month time window. Among the 50 (IFNγ/IL4)-positive patients, 37 (74%) were relapse-free, as well as 10 (40%) of the 25 (IFNγ/IL4)-negative patients. Again, the number of relapse-free patients was higher in the

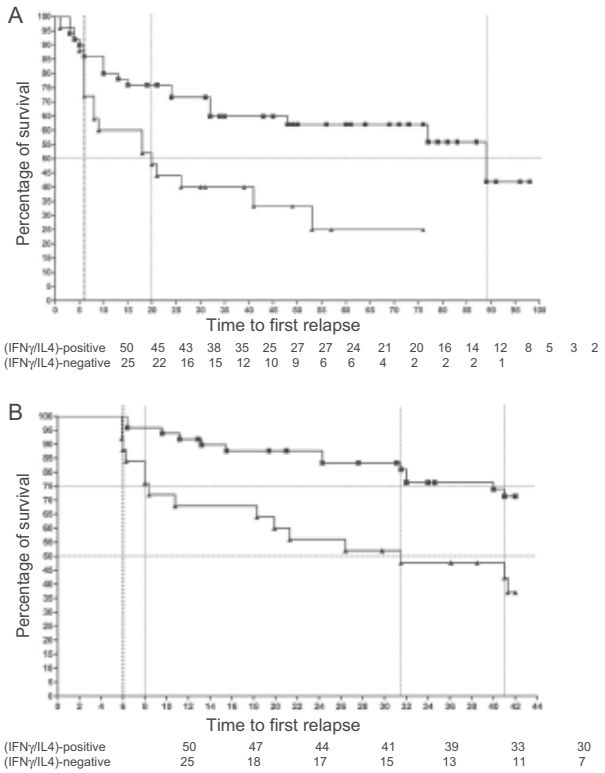

Figure 3. Relapse-free survival of 75 long-term GA-treated patients according to their IFNγ and IL4 mRNA responses. (a) Survival curves overlapped throughout the first 6 months for patients in the (IFNγ/IL4)-positive group (filled circles) and (IFNγ/IL4)-negative group (filled triangles). After 6 months, the rate of survival was higher in the (IFNγ/IL4)-positive than in the (IFNγ/IL4)-negative group. (b) Further clinical analysis was conducted considering a restricted 6 to 42 month time window. (IFNγ/IL4)-positive patients showed a better relapse-free survival (p=0.002; HR=3.04) in respect to (IFNγ/IL4)-negative patients. The negative group showed a median time to first relapse of 31.5 months (25th percentile survival time = 8 months), whereas this value was undefined in the positive group (25th percentile survival time = 41.5 months).

positive group compared to the negative group (p=0.006). Results showed a difference in relapse-free survival (p=0.002) (Figure 3(b)).

Sensitivity and specificity for predicting relapses were 54% and 79%, respectively. Accordingly, the NPV was 74%, meaning that a (IFNγ/IL4)-positive response is highly likely to mean that the person will not experience a relapse over the next 3 years (Table 3).

Predicting a value for a negative clinical outcome

Long-term GA-treated patients were further sub-classified adopting more stringent criteria: patients who had no clinical relapses and stable EDSS in the first 3 years of treatment were defined as clinical responders, whereas patients who had ≥1 relapse (without considering relapses during the first 6 months), or an EDSS deterioration ≥0.5 points of treatment were defined as clinical nonresponders. 45/75 (60%) long-term treated patients were categorized as clinical responders and 30 as nonresponders. Overall, the NPV was estimated at 78%, meaning that by a (IFNγ/IL4)-positive response, there is a 78% chance that the patient will not experience any negative clinical outcome (Table 3).

A comparison between transcriptional responses in clinical responders and nonresponders showed that *ex-vivo* GA-induced IFNγ mRNA responses were higher in responders compared to nonresponders (p=0.004). Likewise, clinical responders showed higher *ex-vivo* GA-induced IL4 mRNA responses compared to nonresponders (p=0.013).

To evaluate the pharmacodynamics of GA in both groups, *ex-vivo* GA-induced IFNγ and IL4 transcriptional responses were analysed as a function of time: IFNγ mRNA response remained stable under long-term therapy in both responders and nonresponders ($r_{spearman} \leq 0.11$; $p \geq 0.32$). In contrast, IL4 mRNA responses correlated positively ($r_{spearman}$=0.57; p=0.004) in clinical responders, but remained stable in clinical nonresponders ($r_{spearman}$=-0.017; p=0.092) (Figure 4).

Discussion

Over recent years, efforts have been made to identify GA nonresponsive patients through the evaluation of GA-induced differential IFNγ and IL4 responses, i.e. the principal cytokines in the Th1/Th2 paradigm.[13] Accordingly, earlier reports have shown that changes in both IFNγ and IL4 expression are associated with GA treatment, and that a correlation with clinical drug response could exist.[6,7,10] Farina et al.[6] report a triad of immune responses to GA that predict treated from untreated patients. This triad consisted of: (1) a decline in GA-induced proliferation; (2) positive IL4 CD4$^+$ cells; and (3) positive IFNγ CD8$^+$ cells in response to a high dose of GA. Subsequently, Valenzuela et al.,[10] determined that the proliferation of GA-stimulated PBMCs did not differentiate GA-responders from the hypo/nonresponders. However, reduced IFNγ expression and stable IL4 expression in stimulated PBMC, and an increased IL4/IFNγ ratio was associated with favourable clinical response. In these contexts, ELISpot was mostly used, but this technique has proven to be unsuitable for routine clinical practice. Thus, we proposed an alternative approach to analyse transcriptional responses by real-time PCR, i.e. a more suitable approach for routine clinical practice.

With the aim of testing this approach, we first evaluated *ex-vivo* GA-induced IFNγ/IL4 transcriptional response in PBMCs isolated from GA-treated patients and untreated controls. Conforming to previous studies,[6,7,10] we showed a

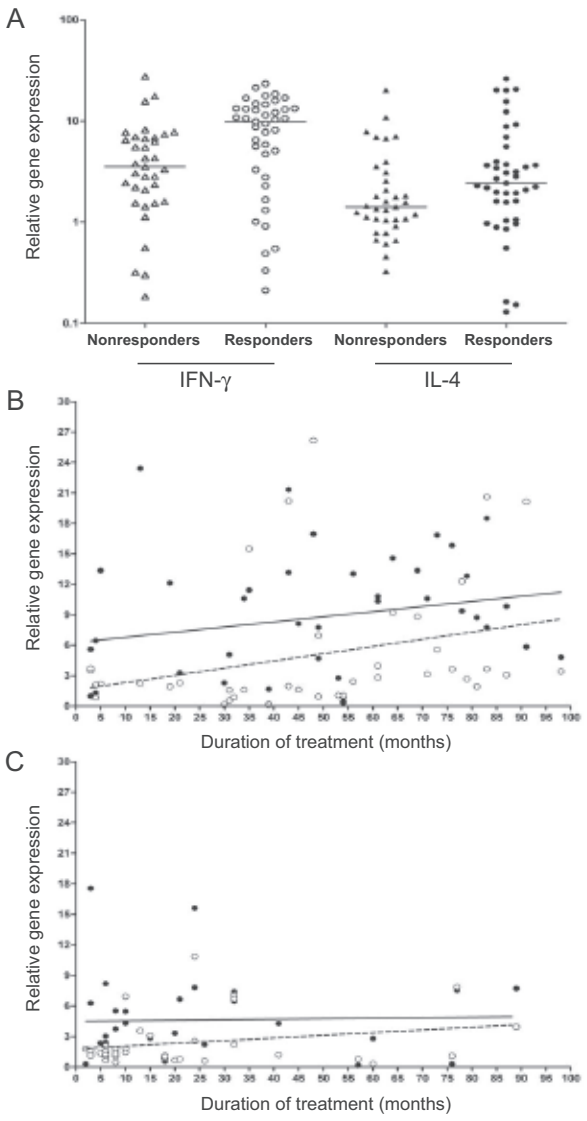

Figure 4. Comparison of GA-induced IFNγ and IL4 mRNA responses in clinically responsive and nonresponsive patients. A total of 75 long-term treated patients were subdivided into clinical responders and nonresponders based on clinical outcomes. (a) Both IFNγ and IL4 mRNA responses were higher in the group of clinically responsive patients compared to nonresponsive subjects ($p \leq 0.013$). Median values are indicated by the horizontal bars and *p*-values are shown. (b) and (c) Pharmacodynamic effects of GA were evaluated in both groups: GA-induced IFNγ (closed circles) and IL4 (open circles) mRNA responses were analyzed as a function of treatment duration (i.e. months). IFNγ mRNA response remained stable under long-term GA therapy, in both (b) clinical responders and (c) clinical nonresponders ($r_{spearman} \leq 0.106$; $p \geq 0.316$). On the other hand, GA-induced IL4 mRNA responses showed a positive correlation ($r_{spearman} = 0.567$; $p = 0.004$) in (b) clinical responders, whereas it remained stable in (c) nonresponders ($r_{spearman} = -0.017$; $p = 0.092$). Solid lines represent a line of best fit for IFNγ and dashed lines represent a line of best fit for IL4.

treatment-related, GA-induced differential Th1 and Th2 cytokine response in PBMCs from treated patients. Particularly, the majority of the latter patients produced high levels of IFNγ and IL4 mRNA in response to *ex-vivo* stimulation with GA. Moreover, IFNγ/IL4 responses were typically and permanently observed in long-term treated patients, whereas they were almost absent in both untreated patients and healthy controls.

As a whole, for the detection of treatment effects, *ex-vivo* GA-induced IFNγ/IL4 transcriptional response resulted in a high sensitivity (92%), which means almost no untreated controls were incorrectly tagged as GA-treated. On the other hand, the relatively low PPV (64%) indicates that in a percentage of long-term treated patients, *ex-vivo* stimulation with GA did not provoke IFNγ/IL4 transcriptional response, meaning an absence of biological responsiveness.

As previously demonstrated with interferon-beta therapy,[14] a lack of bioactivity is often associated with the occurrence of relapses and it is therefore likely that patients who do not respond biologically to GA are also predisposed to clinical nonresponsiveness. Thus, to confirm the clinical utility of testing *ex-vivo* induced IFNγ/IL4 transcriptional response, we assessed clinical outcomes during therapy. First, we focused on relapses, showing reduced clinical efficacy in (IFNγ/IL4)-negative patients. Survival curves overlapped throughout the first 6 months for patients in the (IFNγ/IL4)-positive group and (IFNγ/IL4)-negative group. After 6 months, the rate of survival was higher in the (IFNγ/IL4)-positive group than in the (IFNγ/IL4)-negative group. This confirms a significant but 6 month delayed effect of GA, as already observed in many clinical trials, where survival curves for GA-treated patients and placebo controls separated after approximately 180 days.[3,4,9]

To account for both delayed biological effects and treatment duration, we conducted further analyses considering a 6 to 42 month follow-up. Over this time window, similar reductions in clinical efficacy were observed in (IFNγ/IL4)-negative patients, in whom a higher risk for relapse was calculated again. Interestingly, the NPV for predicting relapses was calculated at 74%, meaning that by a (IFNγ/IL4)-positive response, there is a 74% chance that the patient will not experience a relapse at 42 months. Similar trends were reported in further analyses that considered both relapses and disease progression.

In connection with the present study, it is of interest that 79% of (IFNγ/IL4)-positive patients were relapse-free, a percentage that is significantly higher than the ~34% reported in clinical trials.[1,2] Overall, this percentage represents a significant proportion of the GA-treated population, around two-thirds in fact. This suggests a greater benefit of therapy in (IFNγ/IL4)-positive patients with respect to the benefit previously assumed for the whole GA-treated population. We are aware that such a different percentage may

be influenced by distinct patients' selection criteria. Indeed, until recently, aggressive therapies (e.g. Natalizumab or Mitoxantrone) were not available and thus, patients with malignant courses were included indiscriminately in trials. Clearly, this diversity management might have an impact on study outcomes.

The major evidence from a biological perspective lies in the different response patterns observed among patients; *ex-vivo* GA-induced IFNγ/IL4 transcriptional response was higher overall in clinical responsive patients, meaning that in these patients PBMCs are more sensitive to stimulation. In addition, while the IFNγ mRNA response (with Th1 inflammatory properties) remained stable under long-term therapy in both clinical responders and nonresponders, the IL4 mRNA response (with Th2 anti-inflammatory properties) correlated positively with treatment duration in responsive patients only. This is most likely to be explained by the fact that in clinical responders, daily injection of GA induces highly cross-reactive T-cells that secrete Th2 cytokines, while most of the IFNγ-producing cells do not proliferate though they still make IFNγ.[6,15,16] In contrast, this immunological effect seems to be absent in clinical nonresponders.

Although this GA-specific Th2 cytokine response is consistent with several previous studies,[16–18] we cannot rule out the possibility of a response bias. This bias would result from responders having higher IL4 mRNA responses and being required to survive long enough to be assessed for a long-term response, whereas there is no such requirement for nonresponders, thereby overestimating the long-term IL4 response among responders.

In summary, we provide a practical system for monitoring the biological response to GA in patients with MS. For monitoring purposes, the *ex-vivo* GA-induced IFNγ/IL4 mRNA response should be evaluated at least after 6 months of therapy in patients. Clearly, this delay may, to some extent, undermine the usefulness of IFNγ/IL4 mRNA response as an early biomarker. However, it is clear that evaluating biomarkers as biological indicators of the body's response to exposure, it is necessary to have evidence of biological effects. For GA treatment, those biological effects only occur after 6 months of therapy.[6]

As a whole, this assay was shown to produce differing results that may be clinically significant for GA-treated patients. As such, if patients have a positive GA-induced IL4 and IFNγ mRNA response, it means they have a better chance for treatment and a lower chance of a bad outcome. Thus, these patients can be encouraged to continue GA treatment with follow-up on a regular basis. On the contrary, patients with a negative GA-induced IL4 and IFNγ mRNA response have a greater probability of a worse outcome. Hence, these patients need more frequent clinical and neuroradiological monitoring, to better manage their disease, considering also changes in therapy.

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Conflict of interest

Dr Gilli, Dr Malucchi and Dr Bertolotto report that they have been reimbursed by Merck-Serono, Sanofi-Aventis, Biogen Dompe, Biogen Idec, Novartis and Bayer Schering for attending several conferences; Dr Gilli, Dr Malucchi and Dr Bertolotto report that they have received fees for lectures by Merck-Serono, Biogen Dompe, Biogen Idec, Teva and Sanofi-Aventis; Dr Bertolotto reports that he has received funds for research and for staff members from Merck-Serono, Biogen Dompe, Biogen Idec, Bayer Schering, Novartis and Sanofi-Aventis. The remaining authors have nothing to disclose.

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