Journal of Neuroimmunology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Predictive cytokine biomarkers of clinical response to glatiramer acetate therapy in multiple sclerosis☆

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ARTICLE INFO

Article history:
Received 11 April 2016
Received in revised form 13 June 2016
Accepted 21 June 2016
Available online xxxx

Keywords:
Multiple sclerosis
Glatiramer acetate
Cytokines
Biomarkers

ABSTRACT

A prospective study of 62 patients with relapsing-remitting multiple sclerosis (RRMS) treated with Glatiramer acetate (GA) was conducted to evaluate the value of baseline and treatment-modulated cytokines in predicting the clinical response to the drug after 2 years of therapy. There were 32 responders and 30 non-responders. GA upregulated Th2/regulatory cytokines and inhibited Th1 cytokines in sera or PBMC supernatants 3 and 6 months into treatment. We found two prognostic models with clinical utility. A model based on IL-18 at baseline, the change in TNFa from baseline to 3 months, the change in IL-4 from baseline to 6 months, and the change in the log of the ratio of TNFa/IL-4 from baseline to 6 months had an area under the curve (AUC) of 0.80. A high IL-18 level at baseline and a reduction of TNF-alpha over time are associated with a response to GA. Although the study identified predictive biomarkers of clinical response to GA, the results will need to be validated in other data sets.

1. Introduction

Glatiramer acetate (GA) is an approved drug for the treatment of relapsing-remitting (RR) multiple sclerosis (MS) and is effective in reducing relapse rate and disability accumulation (Martinelli et al., 2003; Mikol et al., 2008; Cadavid et al., 2009). GA is generally well tolerated; however, response to the drug is variable. The therapeutic effect of GA is supported by the results of magnetic resonance imaging (MRI) findings from various clinical trials (Comi et al., 2001; Zivadinov et al., 2015; Khan et al., 2012). However, to date there are no validated predictive biomarkers of response to GA treatment.

The exact mechanism of action of GA in MS is still unclear. Although we believe that mechanisms of action of GA do not seem to be antigen specific, or even multiple myelin antigen specific, we theorize that antigen-based therapy generating GA-specific immune responses seem to be a prerequisite for GA therapy. A possible initial mode of action of GA is associated with binding to MHC molecules and consequent competition with various myelin antigens for their presentation to T cells (Fridkis et al., 1999; Arnon et al., 1996). A further aspect of its mode of action is the induction of T regulatory cells (T-regs) that presumably can migrate to the brain and lead to in situ bystander suppression (Dhib-Jalbut et al., 2003). We and others have previously shown that GA treatment in MS results in the induction of GA-specific T cells with predominant Th2 phenotype both in response to GA and cross-reactive myelin antigens (Chen et al., 2001; Weber et al., 2007; Aharoni et al., 2003). Furthermore, the ability of GA-specific infiltrating cells to express anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta (TGF-β) together with brain-derived neurotrophic factor (BDNF) seem to correlate with the therapeutic activity of GA in EAE (Sarchielli et al., 2007).

Initial studies by Balabanov et al. also demonstrated that GA-monocyte activation through urokinase plasminogen activator receptor (uPAR) could be a possible mechanism of GA in relapsing remitting MS (Balabanov et al., 2001; Stern et al., 2008). Subsequent studies indicated that GA induces immunomodulatory activity exerted by cells of monocytic lineage including antigen presenting cells (APC) through an increase in IL-10 and reduction in IL-12 and IL-1B.

In addition, most investigators currently believe that the immunomodulatory effect of GA is linked to its ability to alter T-cell differentiation, in particular promotion of Th2 polarized CD4 cells. These GA-induced cells are believed to mediate bystander immunosuppression through the induction of IL-10 producing T-regs (Stern et al., 2008).

☆ This manuscript contains original work that has not been published or submitted for publication elsewhere.

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http://dx.doi.org/10.1016/j.jneuroim.2016.06.005
0165-5728/

Current data also provide evidence that Tregs contribute to GA's therapeutic action in EAE and possibly MS. Recent reports indicate that the deficiency in CD4(+)CD25(+)FoxP3(+) regulatory T-cells observed in MS and EAE is restored by GA treatment (Jee et al., 2007; Hong et al., 2005). These findings represent a plausible explanation for GA-mediated T-cell immune modulation and may provide useful insight into the mechanism of action of GA in EAE and MS.

Since the induction of regulatory T-cells and clinical benefit are not universal among GA treated MS patients, it is important to determine if the immunological effects of GA treatment are predictive of the clinical response.

There is currently no practical in vitro assay for monitoring the immunological effects of GA. However, a triad of immune responses were proposed by Hohlfeld et al. that identify GA-treated from untreated patients: (1) a significant reduction of GA-induced PBMC proliferation; (2) a positive IL-4 ELISPOT response mediated predominantly by CD4 cells after stimulation with GA; and (3) an elevated IFN-gamma response partially mediated by CD8 cells after stimulation with high GA concentrations (Farina et al., 2001, 2002). In an earlier study, we demonstrated that lymphoproliferation to GA did not differentiate GA-responders (GA-R) from GA- non-responders (GA-NR). However, reduced IFN-gamma expression and stable IL-4 expression in peripheral blood mononuclear cells (PBMC) and an increased IL-4/IFN-gamma ratio were associated with a favorable clinical response (Valenzuela et al., 2007). More recently we identified an HLA-class II haplotype to be highly predictive of response to GA (DR15 + DQ6+; DR17-DQ2-) where as the DR15-DQ6-; DR17 + DQ2+ was predictive of a poor response (Dhib-Jalbut et al., 2013).

To determine whether GA-induced immunological changes in vivo can predict the clinical response to GA therapy, we conducted a prospective 2-year study in which cytokine levels (Th1, Th2, and Th17) from GA-treated MS patients were correlated with the clinical response to the drug at the end of at least 2 years of therapy. The laboratory personnel were blinded as to whether the patients were clinical responders or non-responders.

2. Methodology

2.1. Subjects

Seventy-one patients diagnosed with definite RRMS according to the 2010 revised McDonald criteria (Polman et al., 2011) were enrolled in the study. Patients were treated with GA, 20 mg subcutaneous daily. Subjects were followed up at four MS centers: 15 from the University of Medicine and Dentistry New Jersey (UMDNJ)-Robert Wood Johnson Medical School MS Center, 31 from the University of Maryland Center for MS in Baltimore Maryland, 8 from the Gimble MS Center in Teaneck, New Jersey and 17 from the Carolinas Medical Center-MS Center, Charlotte, North Carolina. The study was approved by the Institutional Review Board for Human Subjects at each Center. Enrollment criteria included RRMS with at least one clinical relapse in the year preceding enrollment. Multiple sclerosis relapse was defined as new or worsening neurological deficit lasting 24 h or more in the absence of fever or infection (Poser et al., 1983). Patients with relapse were typically seen within 1–2 weeks, and followed closely every three months thereafter. Relapses were determined by an MS specialist at each of the MS center. Relapses and progression were measured by Expanded Disability Status Scale (EDSS) recorded by two independent evaluators. Patients were examined and an expanded disability status scale (EDSS) was determined at base line. Annualized relapse rate (ARR) at baseline was determined historically based on chart review of at least 2 years preceding enrolment. ARR and EDSS were also determined at the end of 2 years. Sixty-two patients completed treatment with GA for at least 2 years. Nine patients either did not complete treatment or were lost to follow-up. This is shown in Supplementary Table 1 (Table 1S). Although there are recommendations on follow-up imaging while on disease modifying therapy, the practice of performing imaging varies significantly among MS centers. Since this was a multicenter trial, we had no control over neuro-imaging time points. Therefore, we did not include MRI scans in this study to determine "no evidence of disease activity" (NEDA).

2.2. Classification of clinical responders and non-responders

After at least 2 years on GA therapy, patients were classified as GA-R (n = 32) or GA-NR (n = 30) based on a clinical criteria more stringent than those recently reported in the literature (Rio et al., 2006). A responder (R) is a patient with no relapses and no evidence of disease progression as measured by EDSS (expanded disability status scale) at the end of two years of treatment with GA. A non-responder (NR) is a patient with one or more relapses or with progression in the EDSS of at least 1 point sustained for 6 months.

2.2.1. Blood samples

Approximately 60 cm^3 of heparinized blood and 10 cm^3 of blood in serum separation tubes were obtained by venipuncture from each MS patient pre-treatment and at 3 and 6 months during treatment. PBMC were purified using Ficoll-Hypaque gradients as described in the supplier's protocol (ICN Biomedicals Inc. Ohio, USA). Samples from collaborating centers were sent via overnight delivery at room temperature and processed immediately upon arrival. The cells and sera from baseline, 3 months, and 6 months were saved frozen and then run simultaneously. The same is true for samples taken at 9 and 12 months.

2.3. Immune markers assays

All cytokine levels were assayed in sera except for IL-17 which was measured in supernatants of GA-stimulated PBMC (Two × 10^5 PBMC/well were seeded in 96 well U-bottom micro titer plate in the absence of antigen (unstimulated condition) (US) or the presence of GA at 40 and 100 μg/ml (Teva Pharmaceutical Industries, Ltd., Israel). Delta IL-17 (difference between US and GA-stimulated supernatants levels) was used in the analysis. Interleukin-18 and Caspase-1 levels were detected by human ELISA kits (Sandwich ELISA, Bender Medsystems , USA). The sensitivity of the ELISA was 9.2 and 3.3 pg/ml for IL-18 and Caspase-1 respectively. TGF-β, TNF-α, IL-4, IFN-γ, IL-17 and IL-10 levels were detected by human ELISA kits from ebioscience (San Diego CA, 92,121 USA). The sensitivity of the ebioscience kits were 60 pg/ml, 4 pg/ml, 2 pg/ml, 4 pg/ml, 4 pg/ml and 2 pg/ml respectively. According to assay instructions, the limit of detection of the cytokines was defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (calculated as the mean plus 2 standard deviations).

2.4. Statistical analysis

The R statistical environment was used for statistical analysis (R Development Core Team, 2011). Because 76% of patients were missing at least one measurement, the R package Amelia was used to impute 10 complete datasets (Honaker et al., 2011). The multiple imputation procedure replaced missing values with values randomly drawn from the inferred distribution of that variable. Results of statistical procedures were combined across the imputations. "Rubin's rules" were used to correctly compute standard errors taking into account both the observed variability and the additional uncertainty due to the imputation process (Rubin, 1987).

The prognostic performance of each marker individually was summarized using the AUC (area under the receiver operating characteristic, or ROC, curve). Because we wished to investigate the use of binary predictors, we also dichotomized each variable, using as a cutoff the value minimizing the distance between the ROC plot and the upper left corner of the unit square. For each dichotomized variable, the

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AUC, sensitivity, specificity, positive predictive value, and negative predictive value were determined.

A naïve application of this procedure can be expected to be over optimistic, in the sense of showing better results that would be obtained with subsequent independent data since the same data was used to set the cutoff and to determine the performance. To adjust for this phenomenon, we used the computationally intensive bootstrap statistical method to determine "optimism-corrected" values of the test performance measures (Smith et al., 2014). The bootstrap procedure included the cutoff-determining step (that is, the cutoff was recalculated for each bootstrap draw, thus recapturing the original process) and, via the application of the bootstrap-sample-derived cutoff to the original data, provided an estimate of the performance of the dichotomized predictors on future data.

Because of the large number of measures, one would expect that some measures would perform well by chance. To quantify that, we ran 1000 permutations of the data for which we calculated the maximum AUC across all measures (in their continuous, not dichotomized, form). Percentiles of these maxima-per-permutation were then used to determine significance thresholds. For example, the 95-th percentile corresponds to a multiplicity-corrected level of $\alpha = .05$.

To examine the predictive value of the markers in combination we used logistic regression with Bayesian model averaging (BMA) (Raftery, 1995). BMA estimates the posterior model probability of many regression models that variously include different sets of predictive markers. In doing so, BMA in turn provides estimates of the posterior probability that any given marker has a non-zero effect, by adding the posterior probabilities of all the models that the marker appears in. This approach seamlessly incorporates multiple imputation, since the BMA-derived probabilities can simply be averaged over the imputed data sets.

3. Results

The demographics of the responders and non-responders are shown in Table 1. The mean disease duration was 9.8 ± 7.8 years for the GA-R group and 11.2 ± 8 years for the GA-HR/NR group. The baseline ARR and EDSS were not significantly different between the GA-R and the GA-HR/NR groups. Response rates did not differ significantly among centers.

Fig. 1 shows means and 95% confidence intervals for those means for all immune markers and their ratios at baseline, 3 months, and 6 months. Note that these statistics use imputed data combined following Rubin's rules. In general, treatment with GA resulted in a tendency for a decrease in levels of the proinflammatory Th17 and Th1 cytokines (IL-17, IFN-g, TNF-a and IL-18) and an increase in the anti-inflammatory/regulatory cytokines IL-4, TGF, and IL-10 at 3 and 6 months into treatment in clinical responders relative to non-responders. A similar tendency was also observed in the ratios of selected Th1 to Th2 cytokine levels. Table 2 shows prognostic measures for the best-performing immune markers as determined by AUC (results for all markers are given in the Supplementary Table 2S). The AUC has a simple interpretation, namely as the proportion of the time that a random pair of a responder and non-responder is correctly ordered by the biomarker. As such, it runs from 0 to 1 with 0.5 corresponding to chance ordering. For comparison, the well-known Framingham Risk Score has an AUC of about 0.75 for predicting coronary heart disease.

Permutation testing indicated that the 90-th and 95-th percentiles for maximum AUC are 0.70 and 0.72, respectively Fig. 2. As shown in Supplementary Table 2, the two measures that surpassed the latter threshold for AUC were Δ TNF-Alpha at 3 months, at 0.73, and Δ log (TNF-Alpha/TGF-Beta) at 3 months, at 0.73. For Δ TNF-Alpha at 3 months, the medians for responders and non-responders were −10.21 and −1.37, respectively, indicating that large decreases in TNF-Alpha from baseline to 3 months are associated with response. For Δ log(TNF-Alpha/TGF-Beta) at 3 months, the medians for responders and non-responders were −0.33 and −0.04, respectively, indicating that a drop in the TNF-Alpha/TGF-Beta ratio from baseline to 3 months is associated with response.

Bayesian model averaging (BMA) with logistic regression, used to examine the predictive performance of multiple markers, produces posterior probabilities that each biomarker has a non-zero effect, shown in Table 3S (Supplementary data). The optimism-corrected AUC using the entire BMA model, with BMA-estimated model coefficients (not shown) is 0.82; this is roughly the upper bound of what is obtainable with the present data. Fig. 2A shows the ROC curves for that model as well as for logistic regression models using the top two (posterior probability >40%), four (posterior probability >25%), and six (posterior probability >15%) markers (as shown in Table 3S). The optimism-corrected AUCs for these three reduced models are 0.78, 0.82 and 0.83, respectively. A nomogram based on the four markers with posterior probability >25% is shown in Fig. 3A; it has AUC of 0.82.

Fig. 2B shows a similar plot of ROC curves as in Fig. 2A, but restricting to measures from baseline or 3 months. (Restricting to baseline only measures did not give good performance.) The nomogram using markers at baseline or 3 months with posterior probability >25% is shown in Fig. 3B. The AUC for this model is 0.77.

Tables 3a, 3b shows the counts and proportions of non-responders and responders at different risk prediction levels under the two models. For example, for individuals for whom the models predict a 60% to 100% response rate, the observed response rate was 78% and 81% for the 4-parameter and 2-parameter models, respectively.

Table 1
Demographics of individuals completing treatment. Except for sex, values are mean (standard deviation). "EDSS" = expanded disability status scale at baseline. "ARR" = Annualized relapse rate at baseline.

	Overall	Responders	Non-responders
Sex: Female	50 (80.6%)	25 (78.1%)	25 (83.3%)
Male	12 (19.4%)	7 (21.9%)	5 (16.7%)
Age	42.0 (10.6)	41.2 (11.0)	42.9 (10.2)
Disease duration	10.5 (7.9)	9.8 (7.8)	11.2 (8.0)
EDSS	2.01 (0.93)	2.06 (0.97)	1.95 (0.89)
ARR	0.87 (0.34)	0.84 (0.32)	0.90 (0.36)

4. Discussion

Multiple sclerosis patients show varied response to disease modifying treatments. In the last few years, several therapeutic options have become available for patients with MS. Because treatment outcomes are unpredictable, establishing personalized treatment remains one of the biggest challenges in managing MS patients. With the growing number of therapeutic options for relapsing remitting MS, and need for early treatment initiation, a predictive biomarker for treatment response is highly desirable.

In contrast to other immunomodulatory MS therapies, GA has a distinct mechanism of action. It primarily supresses T helper type 1 cells (Th1) and enhances T helper type 2 cells (Th2), referred to as TH2 shift (Ziemssen and Schrempf, 2007; Bakshi et al., 2013; Oreja-Guevara et al., 2012; Pul et al., 2011; Schrempf and Ziemssen, 2007; Weder et al., 2005; Arnon and Aharoni, 2004; Vieira et al., 2003). In addition, GA treatment induces an in vivo change of the frequency, cytokine secretion pattern and the effector function of GA-specific T cells by modifying the properties of antigen presenting cells (APC). Therefore, it is conceivable that measuring different cytokines earlier in the course of treatment can be clinically utilized in therapeutic decision-making. Since Th2 shift is one of GA's main mechanism of action, theoretically, measuring the ratios of Th2/Th1 could correlate positively with responders and less so or not at all with non-responders. In addition, several cytokines maybe useful as surrogate markers in predicting treatment response individually.

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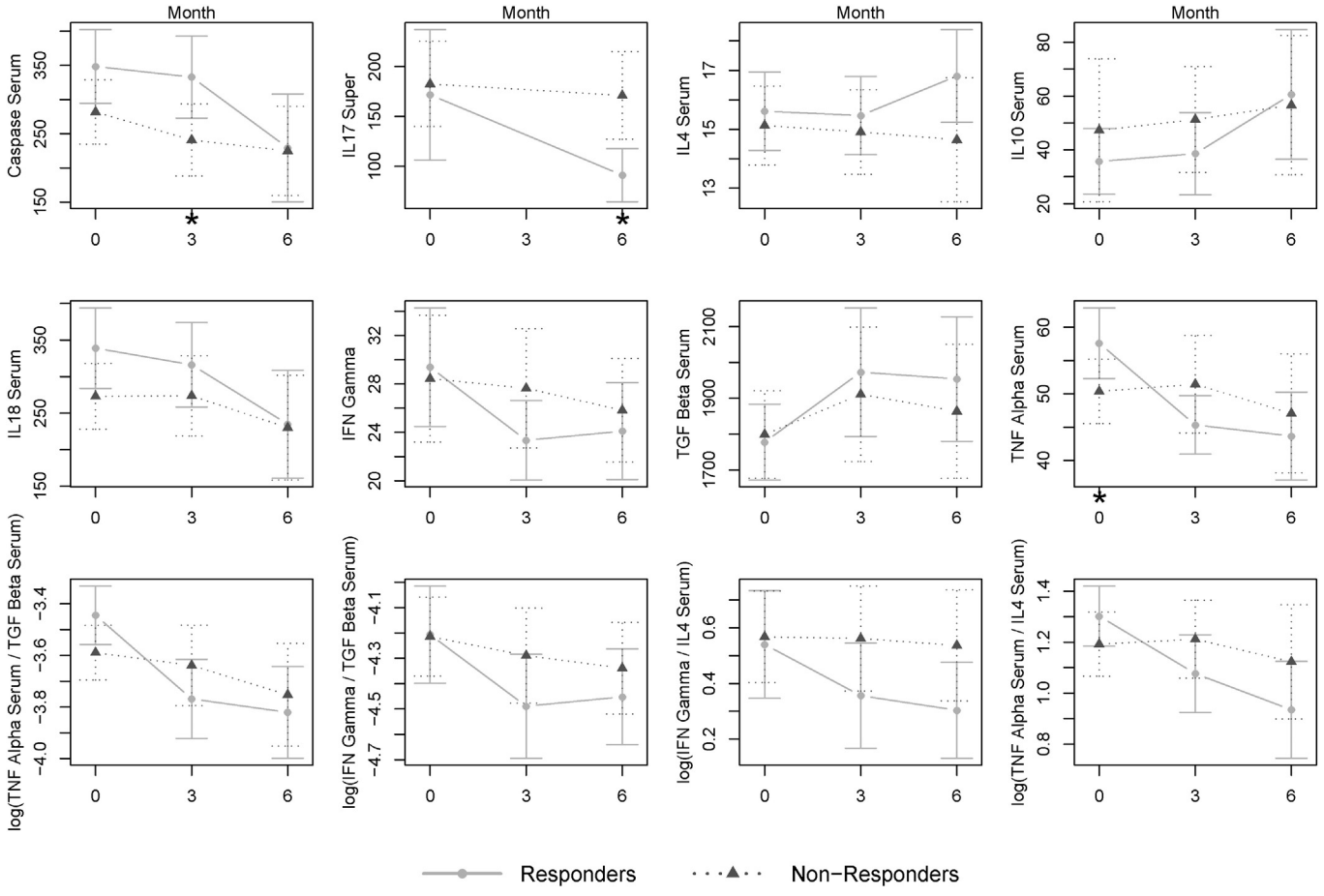

Fig. 1. Means and 95% confidence intervals for those means. Nominally significant differences between the non-responder and responder groups at a given time point are indicated by an asterisk.

The purpose of this prospective study was to determine if cytokine levels at baseline or a change in their levels during treatment predicts the clinical response to GA in patients with RRMS. Of note is that the demographics of the 32 responders and 30 non-responders did not differ significantly between the two groups. Age, sex, disease duration and disease activity prior to treatment did not seem to influence the clinical response.

The immune parameters we elected to study were those that have been reported to be modulated by GA treatment in earlier studies. A reduction in a number of Th1 cytokines and an increase in Th2/regulatory

Table 2
Summary and predictive performance statistics of the top markers (AUC above threshold of 90-th percentile across permutations).

Measure	Δ log TNF Alpha Serum IL4 Serum ratio @ 3 mo	Δ log TNF Alpha Serum IL4 Serum ratio @ 6 mo	Δ log TNF Alpha Serum TGF Beta Serum ratio @ 3 mo	Δ TNF Alpha Serum @ 3 mo
AUC (continuous)	0.71 (0.58, 0.83)	0.71 (0.58, 0.83)	0.73 (0.61, 0.85)	0.73 (0.61, 0.85)
Median (responders)	0.17	0.37	0.33	−10.21
Median (Non-responders)	−0.04	0.10	0.04	−1.37
Response associated with measure dichotomized as	>0.04	>0.24	>0.11	<2.00
Dichotomized predictors:				
Sensitivity	0.69 (0.33, 0.81)	0.64 (0.33, 0.88)	0.71 (0.23, 0.78)	0.65 (0.28, 0.69)
Specificity	0.64 (0.34, 0.76)	0.68 (0.28, 0.74)	0.65 (0.32, 0.86)	0.64 (0.42, 0.87)
Positive predictive value	0.67 (0.40, 0.76)	0.69 (0.39, 0.75)	0.69 (0.37, 0.78)	0.65 (0.41, 0.83)
Negative predictive value	0.67 (0.37, 0.77)	0.64 (0.35, 0.81)	0.69 (0.34, 0.71)	0.64 (0.37, 0.72)
AUC (Dichotomized)	0.67 (0.46, 0.71)	0.66 (0.43, 0.71)	0.68 (0.43, 0.67)	0.64 (0.45, 0.70)

'Δ' in measurement name refers to change from baseline (negative = decrease); in particular, "Δ log" refers to the change in the log of the ratio. Area under the ROC curve (AUC), sensitivity, specificity, positive predictive value, and negative predictive value are all optimism-corrected using the bootstrap. Bootstrap-determined 95% confidence intervals are shown in parentheses. The optimum threshold was determined by minimizing the distance between the ROC plot and the upper left corner of the unit square. The inequality in the optimum threshold row indicates the condition predictive of responder status. Numbers in parenthesis are 95% confidence intervals. Log refers to natural log.

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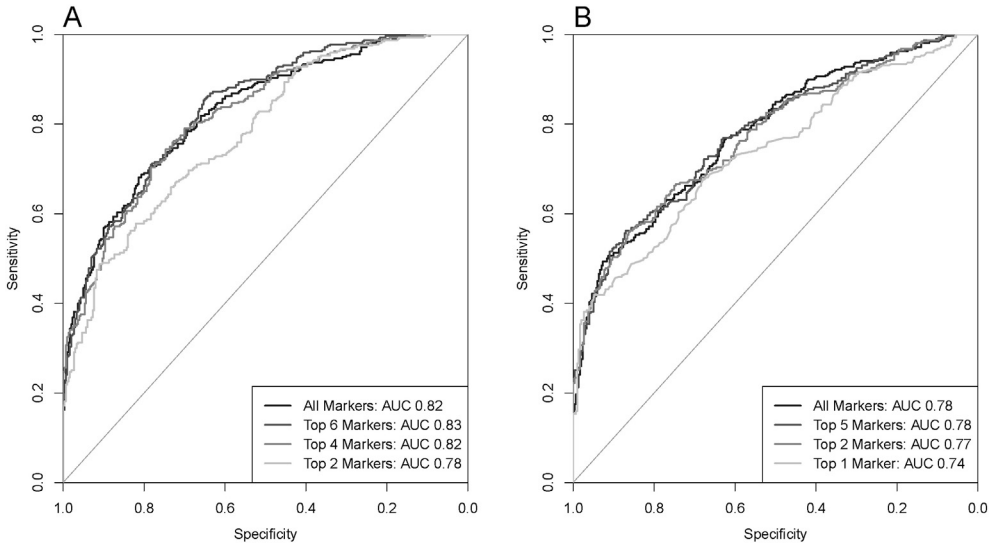

Fig. 2. Receiver operating characteristic curves. AUCs shown are optimism-corrected. Panel A. The top six predictors are, in decreasing order, Δ TNF Alpha at 3 months, Δ IL-4 at 6 months, Δ log(TNF-Alpha/IL-10) at 6 months, IL-18 at baseline, log(TNF-Alpha/TGF-Beta) at 3 months, log(TNF-Alpha/TGF-Beta) at 6 months. Panel B. The top five predictors restricted to models using baseline and 3 month measures are, in decreasing order, Δ TNF Alpha at 3 months, IL-18 at baseline, log(IL-18/IL-4) at baseline, log(TNF-Alpha/TGF-Beta) at 3 months, log(IL-18/TGF-Beta) at baseline.

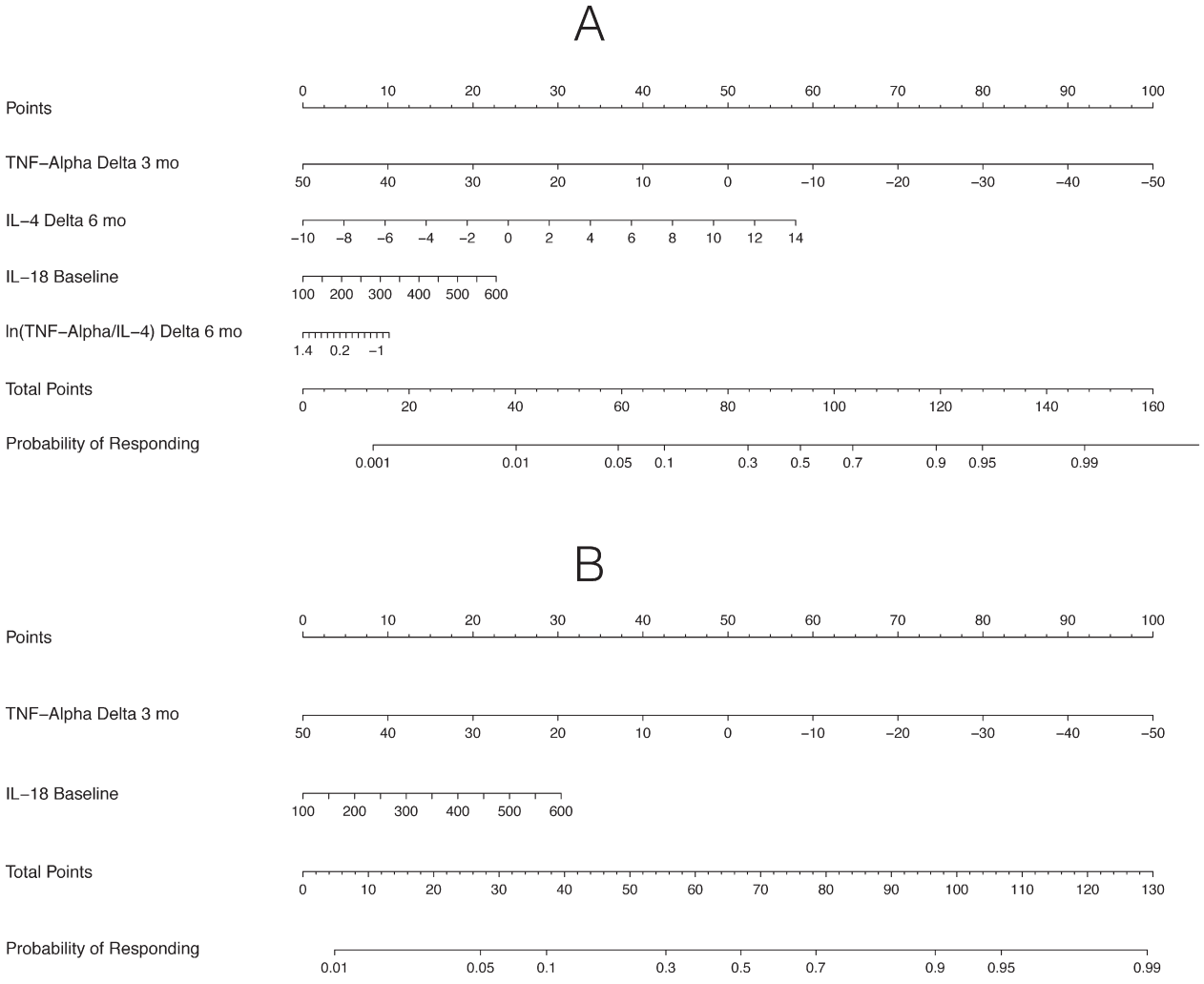

Fig. 3. Nomogram to predict response. For each row, the scale at the top is used to determine the points for that variable. The point total is then used at the bottom to determine the probability of response. Panel A. Using the 4-predictor model, the optimism-corrected AUC is 0.82. Panel B. Using the 2-predictor model restricted to baseline and 3 month measures, the optimism-corrected AUC is 0.77.

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Table 3a
Predicted probabilities versus status for the four-parameter model with nomogram shown in Fig. 3A. Counts shown under "Non-Responders" and "Responders" columns are not whole numbers due to multiple imputation process.

Probability of responding	Non-responders		Responders	
(0.0, 0.4]	17.60	(78%)	5.10	(22%)
(0.4, 0.6]	6.90	(50%)	6.90	(50%)
(0.6, 1.0]	5.50	(22%)	20.00	(78%)

cytokines have been associated with GA treatment, but it was not clear whether these changes correlate with the clinical response and more importantly whether they predict the clinical response to the drug. We found that a reduction in pro-inflammatory parameters including IL-18, Caspase-1, TNFa, and IFNg, and a rise in IL-4, TGF-B, and IL-10 was more prominent in responders to treatment. The group differences were nominally statistically significant for serum TNFa at baseline, in Caspase-1 at 3 months, and in IL-17 PBMC supernatants at 6 months. The ratios of Th2 to Th1 cytokines also showed more of an increase in responders compared to non-responders. Statistical analysis of the predictive value of these immune parameters including sensitivity, specificity, PPV, NPV, and the corresponding AUC shown in supplementary Table 2S were used to develop statistical predictive models of clinical response that have potential clinical utility.

Simple clinical models can be made by looking at any one of the measures above the 90-th permutation percentile for AUC: the change in TNFa prior to initiating GA treatment and then at 3 months into treatment, the change in the log of the ratio of TNFa and TGFB prior to initiating GA treatment and then at 3 months into treatment, or the change in the log of the ratio of TNFa and IL-4 prior to initiating GA treatment and then at either 3 months or 6 months into treatment. Changes <2.00, >0.11, >0.04, and >0.24, respectively, are predictive of response, with sensitivity, specificity, positive predictive value, negative predictive value, and AUC all ranging from 0.64 to 0.71 (Table 2). Better prediction, however, can be made by using several biomarkers and not dichotomizing them.

A well-performing multiple marker model requires measuring three cytokines at up to 3 time points: IL-18 level at baseline, change in TNFa from baseline to 3 months, change in IL-4 from baseline to 6 months, and the change in the ratio of TNFa/IL-4 from baseline to 6 months. The cumulative value of high IL-18 level at baseline, a decrease in TNFa levels from baseline, an increase in IL-4, and a decrease in the TNFa/IL-4 ratio are associated with increasing probability of responding to GA as shown in the nomogram (Fig. 3A). The nomogram depicts the total points associated with the immune parameters levels and the corresponding probability of response. For example, a patient with IL-18 level at baseline of 400, change in TNFa at 3 months of −15 (that is, a decrease), change in IL-4 at 6 months of 2, and a change in the ratio of TNFa/IL-4 from baseline to 6 months of 2.0 (that is, the log ratio increases by 0.69) will have points of 14, 65, 29, and 3, respectively, on the nomogram with total points of 111 and an estimated probability of responding of about 0.80. Table 3a indicates that this model is well calibrated, in the sense of giving estimates close to the observed proportions.

A simpler model to use clinically is to measure serum IL-18 and TNFa at baseline then to repeat TNFa assay at 3 months into treatment. The IL-18 baseline level and the change in TNFa level from baseline are then applied to the nomogram shown in Fig. 3B and a probability of response is calculated. Using the same example as above, the points will be 18 and 65, for a total of 83 and an estimated probability of responding of about 0.75. Table 3b indicates that this model is also well calibrated.

Table 3b
Predicted probabilities versus status for the two-parameter model restricted to baseline and month 3, as shown in the nomogram in Fig. 3B.

Probability of responding	Non-responders		Responders	
(0.0, 0.4]	15.20	(73%)	5.60	(27%)
(0.4, 0.6]	10.70	(55%)	8.60	(45%)
(0.6, 1.0]	4.10	(19%)	17.80	(81%)

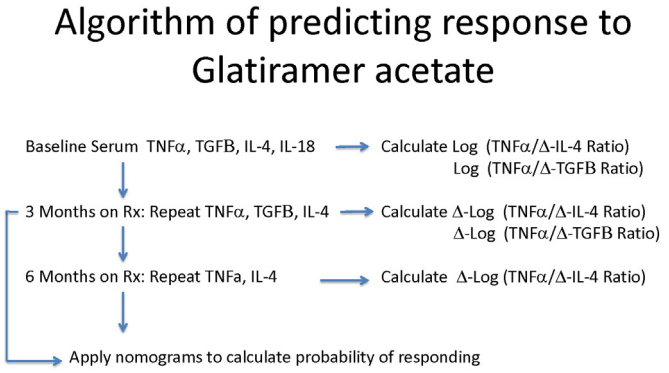

Fig. 4. Algorithm for predicting response based on baseline, 3 and 6 months cytokine measures.

We propose the following overall algorithm to predict the clinical response to GA (Fig. 4). The first step is to measure serum TNFa, IL-4, and IL-18 at baseline. At three months into treatment TNFa levels are repeated. The change in TNFa level from baseline as well baseline measurement of IL-18 can be combined, using the nomogram in Fig. 3B, to estimate the probability of response at 2 years. If the patient continues treatment, at 6 months further assessment of the potential of continued response to GA can be estimated by measuring TNFa and IL-4 only and applying the change in levels to nomogram in Fig. 3A. The AUCs of 0.75 (for the 3 month estimate) and 0.80 (for the 6 month estimate) suggest that this approach will have valuable clinical utility.

Unlike sampling spinal fluid, which is relatively an invasive procedure associated with side effects, using blood based biomarkers is simple, quick, and can be done in the outpatient setting. We believe that these simple blood-based biomarkers can help clinicians and patients make clinical decisions when glatiramer acetate treatment is being considered.

In summary, the normograms we created look at the baseline serum levels of IL-18 and TNF-alpha. A high IL-18 level at baseline and a reduction of TNF-alpha over time are associated with a response to GA. The results look promising, however our findings need a study examining the difference between fresh and frozen samples. If there is no difference in the ELISA results between frozen and fresh samples, it will be more practical to freeze samples, and run tests simultaneously. Laboratories running the assays could then use the proposed nomograms to report to the physician the likely hood that the patient is responding and will continue to respond to GA over the subsequent 2 years.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2016.06.005.

Acknowledgment

This study was supported in part by Wadsworth Foundation and TEVA Pharmaceuticals.

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Please cite this article as: Valenzuela, R.M., et al., Predictive cytokine biomarkers of clinical response to glatiramer acetate therapy in multiple sclerosis, J. Neuroimmunol. (2016), http://dx.doi.org/10.1016/j.jneuroim.2016.06.005

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Please cite this article as: Valenzuela, R.M., et al., Predictive cytokine biomarkers of clinical response to glatiramer acetate therapy in multiple sclerosis, J. Neuroimmunol. (2016), http://dx.doi.org/10.1016/j.jneuroim.2016.06.005