Serum IL-17F does not predict poor response to IM IFN β -1a in relapsing-remitting MS

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ABSTRACT

Objective: There is a significant unmet need for serum biomarkers in relapsing-remitting multiple sclerosis (RRMS) that are predictive of therapeutic response to disease-modifying therapies. Following a recent Stanford study which reported that pretreatment levels of serum interleukin (IL)-17F could predict poor response to interferon- β (IFN β) therapy, we sought to validate the finding using samples from a large clinical trial.

Methods: The validation cohort included 54 good responders (GR) and 64 poor responders (PR) selected from 762 subjects with RRMS from the IM IFN β -1a dose comparison study (Avonex study C94-805). Subjects were classified as GR and PR based on the number of relapses, Expanded Disability Status Scale score, and new and enlarging T2 lesions on MRI. Serum samples were assayed for IL-17F using a multiplexed Luminex assay and for IL-17F/F using an ELISA. Replicate aliquots from the Stanford study were also assayed to assure reproducibility of methods.

Results: Median pretreatment and post-treatment serum IL-17F levels were not statistically significantly different between GR and PR, and serum IL-7/IL-17F ratios were also not predictive of response status. Replicate aliquots from the Stanford study showed good correlation to their original cohort ($r = 0.77$).

Conclusions: We were unable to validate the finding that serum IL-17F is a predictor of PR in a large independent cohort of subjects with RRMS. Differences in patient populations and methodology might explain the failure to validate the results from the Stanford study. *Neurology*® 2012;79:531-537

GLOSSARY

EAE = experimental autoimmune encephalomyelitis; **EDSS** = Expanded Disability Status Scale; **GR** = good responders; **HV** = healthy volunteer; **IFN β** = interferon- β ; **IL** = interleukin; **MS** = multiple sclerosis; **NAb** = neutralizing antibody; **PR** = poor responders; **RRMS** = relapsing-remitting multiple sclerosis; **SLE** = systemic lupus erythematosus.

Relapsing-remitting multiple sclerosis (RRMS) is an inflammatory disease of the CNS of unknown etiology. The underlying pathophysiology as well as the pace and severity of progression vary among patients.¹⁻³ There is substantial evidence, however, that multiple sclerosis (MS) is an immune system disorder and immune-modifying therapies such as interferon- β (IFN β) have shown beneficial effect in patients with RRMS. Despite these advances, our understanding of patient factors that influence the clinical course of disease and biological mechanisms that influence response to therapy is limited.⁴ Identification of serum biomarkers linked to underlying disease heterogeneity that predict therapeutic response would be a significant step toward realizing personalized medicine for patients.

Type I interferons are pleiotropic signaling molecules that regulate both innate and adaptive immune responses.⁵ A recent study of IL-17F from Stanford University (Stanford study) reported a differential treatment effect of IFN β in an experimental autoimmune encephalomyelitis (EAE) model in mice, where disease scores were reduced in the presence of exogenous T-helper type 1 (Th1) cells and exacerbated by T-helper type 17 (Th17) cells.⁶ Based on this

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Supplemental data at www.neurology.org

Supplemental Data

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Table 1 Subject characteristics for the validation cohort (n = 118)

Characteristic	Response category	
	Good responders (n = 54)	Poor responders (n = 64)
Median age (25th, 75th percentile), y	36.0 (28.0, 44.0)	36.0 (31.0, 41.5)
% Female	67	69
% White	100	98
MS classification, %		
Relapsing-remitting	87.0	85.9
Relapsing-progressive ^a	13.0	14.1
Median (25th, 75th percentile) disease duration, y	3.2 (1.6, 7.2)	4.0 (2.0, 7.4)
Median (25th, 75th percentile) age at diagnosis, y	31.5 (25.0, 40.0)	30.7 (27.2, 37.0)
Median (25th, 75th percentile) EDSS score	3.5 (2.5, 4.0)	4.0 (3.0, 4.5)
No. (%) patients with EDSS score		
2.0–3.5	34 (63.0)	30 (46.9)
4.0–5.5	20 (37.0)	34 (53.1)
Median (25th, 75th percentile) prestudy relapse rate ^b	1.0 (0.7, 1.3)	1.3 (1.0, 1.7)
IFN β -1a dose, mean		
30 μ g	25 (46.3)	32 (50.0)
60 μ g	29 (53.7)	32 (50.0)

Abbreviations: EDSS = Expanded Disability Status Scale; IFN β = interferon- β .

^a Patients with early progressive disease who experienced relapses; patients with confirmed progressive disease and no relapses were excluded from the study.

^b Relapse rate per year during the 3 years before study enrollment.

observation, the Stanford study further examined pretreatment serum levels of IL-17F, a cytokine that is highly expressed by Th17 cells, in a retrospective analysis of 26 patients with RRMS on 3 different IFN β therapies: IM IFN β -1a (Avonex, Biogen Idec, Weston, MA), subcutaneous IFN β -1a (Rebif, Merck Serono S.A., Geneva, CH), and subcutaneous IFN β -1b (Betaseron, Bayer AG, Leverkusen, GE). Elevated pretreatment levels of IL-17F were found in a subset of patients classified as poor responders (PR).

METHODS Using methods similar to those described in the Stanford study, levels of IL-17F, IFN β , and 47 other analytes were measured using a customized, multiplexed, bead-based assay run on a Luminex instrument (Procarta Cytokine Kit, Panomics, Santa Clara, CA).⁷ The capture antibody used in the assay is specific for both IL-17F/F and IL-17A/F, but not IL-17A.

To assess the sample quality of the validation samples, which had undergone long-term storage, the serum interferon response was assessed using an independent assay that included known interferon response genes. Validation cohort samples were tested in a second Luminex assay at Rules-Based Medicine (RBM, Austin, TX) which included the Human Inflammation MAP and 9 additional cytokines and chemokines. This panel included the serum proteins CXCL10 (IP-10), CXCL11 (IP-9), TNFR2,

B2M, and BAFF, all of which have been reported as IFN signature genes.^{8–12} This panel also included a measurement of IL-17A with a lowest detectable level of 2.7 pg/mL. Concentrations of IL-17F in the validation cohort were also measured using an ELISA specific for the IL-17F/F homodimer with a lower limit of detection of 15.6 pg/mL (ELISA Ready-SET-Go!, eBioscience, Dallas, TX).^{13–15}

Three cohorts of subjects were used in this study: a replication cohort, a validation cohort, and a controls cohort. The replication cohort (table e-1 on the *Neurology*[®] Web site at www.neurology.org) was composed of pretreatment samples from 25 of the 26 patients with RRMS characterized in the Stanford study (1 patient sample was no longer available). Patients who had been receiving IFN β treatment were classified as good responders (GR) or PR to therapy according to a blinded review of 2 neurologists, based on the number of relapses (median of 0 in GR; 2 in PR) and steroid interventions (median of 0 in GR; 2 in PR) during 2 years on treatment. Serum samples were obtained the day prior to starting IFN β therapy. Patients were not excluded based on presence or absence of neutralizing antibodies (NABs) to IFN β . Serum samples were stored at -80°C for between 5 and 16 years with 2 known freeze-thaw cycles.

The validation cohort (table 1) was composed of subjects from an IFN β -1a dose comparison study in subjects with relapsing MS (Avonex study C94-805).^{16,17} Samples were available both pretreatment and following the 3-month dose (12th injection) for 762 of the total 802 subjects. Specimens were stored at -20°C within 1 to 2 hours of collection at the investigative sites and then shipped on dry ice to a central biobank and stored at -80°C for up to 14 years and underwent 2 freeze-thaw cycles prior to analysis.

Two definitions were used to classify patients as GR and PR in the validation cohort. In the first, GR (n = 54) were defined as having no confirmed relapses and no evidence of sustained disability progression (by Expanded Disability Status Scale [EDSS] score) during the first 3 years of treatment. PR (n = 64) were defined as subjects with at least 3 relapses and development of a 6-month sustained progression in disability, defined as a 1.0 or more point increase in EDSS score from baseline in subjects with a baseline score of ≤ 5.5 . Subjects were excluded from the study if they had ≥ 10 new or enlarging MRI T2 lesions in the GR group, or were permanently positive for NABs starting from year 1 at any titer or NAB titers ≥ 20 in either group. New and enlarging T2 lesion data were collected as part of an MRI sub-study (n = 272) in the C94-805 study and was available for 40 (GR = 16, PR = 24) of the 118 subjects in the validation cohort. Using these data, a second classification (called the MRI subset) of GR (n = 11) and PR (n = 19) was defined by requiring the GR and PR to have ≤ 3 and ≥ 4 new or enlarging T2 lesions, respectively, when comparing 3 years MRI to baseline.

To achieve a robust validation, steps were taken to 1) ensure the samples in the validation cohort were of high quality, 2) use assay conditions similar to those in the Stanford study, 3) use stringent definitions of GR and PR to therapy, and 4) use both pretreatment and early post-treatment samples to confirm results. Replicate aliquots of samples from the Stanford study were also assayed alongside the validation cohort at an independent laboratory.

The controls cohort was composed of samples from healthy volunteers (HVs) (n = 30) and subjects with systemic lupus erythematosus (SLE) (n = 20) (BioReclamation Inc., Westbury, NY). These samples were collected and frozen within 3 months prior to testing and were included to provide additional population level control measurements of IL-17F using the Panomics

Figure 1 Serum interleukin (IL)-17F and interferon- β (IFN β) concentrations in the replication cohort

Correlation between IL-17F and IFN β concentrations analyzed by Axtell et al. (Stanford Core Laboratory) and at Panomics: (A) serum IL-17F (ln) (pg/mL), (B) serum IFN β ln (pg/mL).

Procarta assay. All samples, including the replication cohort, the validation cohort, and the controls cohort, were tested in the same assay batch, using identical assay reagents, by a single operator at the Panomics facilities.

Data were analyzed using R 2.11 (The R Foundation for Statistical computing) and SAS v9.1 software package (SAS Institute, Cary, NC). Reproducibility of IL-17F and IFN β in the Stanford cohort ($n = 25$) samples was assessed using the Spearman correlation coefficient. All data were log transformed and the difference for each analyte between GR and PR was assessed by univariate analysis using either a moderated t test or Wilcoxon rank sum test, as appropriate. The Bonferroni correction was used to adjust for multiple comparisons and an adjusted p value ≤ 0.05 was considered to be statistically significant. Unsuper-

vised classification and hierarchical clustering were performed based on the Euclidean dissimilarity and average linkage.¹⁸

Standard protocol approvals, registrations, and patient consents. The research protocol (999MS001) describing the retrospective study and data privacy procedures for consented samples was approved by the Western Institutional Review Board (WIRB #1112290).

RESULTS Measurements of IL-17F and IFN β levels in samples from the replication cohort by an independent laboratory (Panomics) showed good interlaboratory correlation with the results obtained by the Stanford Core Laboratory (correlation coefficients 0.77 and 0.64, respectively) (figure 1). We also observed elevated levels of IL17F in a subset of PR using the same IL-17F cutoff (60 pg/mL) as the Stanford study. We were able to confirm 4 of 6 patients with IL-17F^{high} concentrations as PR, 6 of 8 patients with IL-17F^{low} concentrations as PR, and 9 of 11 patients with IL-17F^{low} concentrations as GR. A significant correlation between IL-17F and IFN β levels was also observed (data not shown). These classification results are, in general, in agreement with the original findings of the Stanford study.

Sample quality from the validation cohort was assessed using 2 independent Luminex assays. The RBM Inflammation Map panel showed that levels of 18 cytokine/chemokines in serum were statistically different between HVs and pretreatment samples from relapsing MS subjects in the validation cohort, after adjusting for multiple comparisons. Unsupervised clustering analysis showed that the RBM panel had a 97% accuracy to correctly classify HVs and relapsing subjects with MS (figure e-1). Overall, approximately 80% of 55 serum inflammation proteins were upregulated in the relapsing MS pretreatment samples compared to the levels observed in HVs (data not shown).

Since the validation cohort included pretreatment and 3-month post-treatment samples, the RBM panel allowed for the assessment of the serum interferon signature gene response. After adjusting for multiple comparisons, 5 IFN signature genes were shown to be statistically significantly upregulated during therapy, including CXCL10 ($p < 0.0001$), CXCL11 ($p = 0.00013$), TNFR2 ($p = 0.001$), B2M ($p = 0.003$), and BAFF ($p = 0.034$) (data not shown). Subgroup analysis of the cytokine dose response to IFN β dose showed a statistically significant increase in the magnitude of the interferon response between the 30 μ g and 60 μ g doses for CXCL10 ($p = 0.0077$), sVCAM ($p = 0.0079$), BAFF ($p = 0.025$), and B2M ($p = 0.026$) (figure 2).

Serum IL-17F levels varied across the sample cohorts (figure 3); higher mean values were seen in the replication cohort (36.3 pg/mL) and the validation

Figure 2 Induction of serum CXCL10 (IP-10) and BAFF in the validation cohort

Pre- and post-treatment serum concentrations of CXCL10 (IP-10) and BAFF in the validation cohort: (A) CXCL10 and (B) BAFF.

cohort (35.2 pg/mL pretreatment and 38.3 pg/mL 3 months post-treatment) than in HVs (19.5 pg/mL) and SLE patients (21.5 pg/mL).

The ability of serum IL-17F concentrations to discriminate between GR and PR in the validation cohort was examined using 2 response definitions. There was no statistically significant difference in IL-17F concentrations under either definition. Under definition 1, median pretreatment levels of IL-17F in GR and PR were 35.7 pg/mL and 35.2 pg/mL, respectively. In the more stringently defined MRI subset (definition 2), the median pretreatment levels of IL-17F in GR and PR were 34.3 pg/mL and 32.5 pg/mL, respectively (figure 4, A and B). Similar findings were observed for the 3-month results (data not shown).

As the IL-17F reagents in the Procarta multiplex panel detects both IL-17F/F and IL-17A/F, we measured the serum concentrations of IL-17F/F using an ELISA specific for the IL-17F/F homodimer in the 118 patients in the validation cohort and 30 HVs (figure e-2). In the replication cohort, only 1 of 25 patients were above the limit of quantitation and this sample was from a PR. In the validation cohort and in HVs, fewer than 5% of samples were above the limit of quantitation.

DISCUSSION RRMS is an inflammatory disease of unknown pathogenesis and variable disease course. IFN β s are disease-modifying therapies that slow progression and reduce relapse rates in subjects with relapsing MS. Given the observed heterogeneity in the response to therapy, and the link between relapses and loss of physical and cognitive function, there is a great need for pretreatment biomarkers that will allow clinicians to predict response to any given therapy. A recent Stanford study, which showed elevated pretreatment serum levels of IL-17F in IFN β PR, motivated us to validate their preliminary finding in a large independent cohort of subjects with MS from a well-controlled clinical study.

Within the limits of expected interlaboratory variability, we were able to replicate the findings of the Stanford study. Using replicate serum samples from 25 of 26 patients tested in the original study, we observed a correlation coefficient between the serum IL-17F levels measured at the Stanford Core Laboratory and at Panomics of 0.77. We were also able to reproduce their PR classification in 4/6 IL-17F^{high} patients with RRMS and correctly repeat the classification of 6/8 IL-17F^{low} patients.

To ensure the robustness of the validation study, we took steps to ensure that sample quality was maintained and analytical methods were comparable. Because the serum samples from the validation cohort ($n = 118$) were derived from numerous clinical sites ($n = 38$), had been stored at -80°C for up to 14 years, and had undergone at least 2 freeze thaw cycles, we performed a comprehensive assessment of sample quality. To control for interlaboratory and methodologic variation, we used assay formats that were nearly identical to those in the original Stanford study and ran both the replication and the validation cohort simultaneously at a single independent laboratory (Panomics). Comparison of serum inflammation markers in the pretreatment relapsing MS samples to HVs showed broad upregulation of inflammation-related proteins in the MS sera, suggesting lack of degradation of serum proteins from long-term storage. In addition, the validation cohort showed upregulation of serum proteins known to be associated with IFN response. Serum CXCL10 (IP-

Figure 3 Serum interleukin (IL)-17F concentrations

Serum IL-17F concentrations from the Procarta multiplex assay: validation cohort ($n = 235$) pretreatment (805-Pre) and post-treatment (805-3MN); replication cohort (Stanford Study, $n = 25$); healthy volunteers (HVs) ($n = 30$); and patients with systemic lupus erythematosus (SLE) ($n = 20$). (A) y-axis scale 0–50,000 pg/mL, (B) y-axis scale 0–100 pg/mL. Panel B is an expanded scale of the data in panel A.

10) was significantly upregulated in the 3-month samples, and a statistically significant dose response of IFN signature genes was observed between the 30 μ g and 60 μ g dose subgroups in the validation samples for several serum proteins, including CXCL10, sVCAM, BAFF, and B2M.

Despite data supportive of acceptable sample quality, qualification of methods through analysis of the replication samples, and highly conservative classifications of GR and PR, we were unable to validate the findings of the Stanford study in the larger vali-

dation cohort. We did not find statistically significant differences in IL-17F concentrations between GR and PR in the validation cohort nor observe distinct IL-17F^{high} and IL-17F^{low} populations in the PR as compared to GR, controls, or other sample types. This was the case for IL-17F concentrations in pretreatment samples, 3-month samples, and for the fold-change of 3-month vs pretreatment for both classifications of GR and PR. Using the results from the validation cohort, we were also unable to validate the recent finding that IL-7 is upregulated in pretreatment samples of relapsing patients with MS, suggesting a Th1 disease type.¹⁹ IL-7 levels were not predictive of GR and PR status (p values 0.069 and 0.48 for clinical and clinical + MRI subset classifications, respectively) and the IL-7/IL-17F ratio was also not informative (data not shown).

It is not unusual for findings based on small studies to not validate when evaluated using larger cohorts.^{20–21} As the Stanford study included 26 subjects (GR = 12, PR = 14) who were not treated under a clinical trial protocol, it is possible that selection bias led to a population of PR with increased IL-17F levels. In the validation cohort, we used definitions of PR that selected for subjects with 3 or more relapses over 3 years and sustained increases in disability as measured by a 1 or more point increase in EDSS sustained for 6 months, positing that if pretreatment IL-17F concentrations correlated with relapses, and relapses are associated with cumulative disability, then pretreatment IL-17F concentrations would also be predictive of a combined score including relapses and sustained increases in EDSS. But since relapses did not require rapid clinical confirmation in the original C94-805 study, there is the possibility of bias with respect to the definition of a relapse. It is possible that the uniform clinical review of relapses in the Stanford study, with the requirement for steroid treatment, and high median pretreatment relapse rates in PR (2 and 1.3, Stanford and validation cohorts, respectively), could have selected for subjects with significant inflammatory disease. While this could have led to selection bias between the study populations, this would not explain the absence of a significant IL-17F signal in both pretreatment and post-treatment samples in the MRI subset of the validation cohort, where neuroinflammatory activity was specifically excluded or required for GR and PR, respectively, through the assessment of new and enlarging T2 lesions.

Other differences in the study cohorts that could have affected outcomes include differences in the duration of therapy (2 vs 3 years), a single vs multicenter study, slightly lower EDSS scores at baseline (2.5 vs 3.6), and different therapeutic regimens of

Figure 4 Pretreatment serum interleukin (IL)-17F levels in validation cohort

Serum IL-17F concentrations in the validation cohort: (A) pretreatment concentrations for good responders (GR) (n = 54) and poor responders (PR) (n = 64), and (B) pretreatment concentrations for GR (n = 11) and PR (n = 19) in the MRI subset.

IFN β (3 independent vs monotherapy), for the Stanford vs the current study, respectively. In addition, the clinical trial context of the validation cohort allowed for uniform assessment, exclusion of subjects who developed NABs against IFN β , and monitoring of patient compliance.

Because IL-17F exists as both a disulfide-bonded F/F homodimer and an intermediate affinity A/F heterodimer, we also quantified IL-17F/F levels using an ELISA to determine if homodimer levels could predict IFN β response in the validation cohort. As a comparator, we also measured levels in HVs and in a set of fresh samples from patients with MS. For the validation and HV samples, 97.0% and 83.3%, respectively, were below the limit of detection (15.6 pg/mL), suggesting that F/F homodimers are present in serum at lower concentrations than A/F heterodimers. This was also seen in the replicate samples from the Stanford study (data not shown). The majority of IL-17A concentrations in the validation cohort measured using the Human Inflammation Map were also below the limit of detection (8.5 pg/mL).

With the increasing number of approved disease-modifying therapies for RRMS, pretreatment biomarkers are needed to realize the goal of personalized medicine. Given the evidence that immune dysfunction plays a pivotal role in pathogenesis, the search for predictive biomarkers continues to focus on mediators of immune function. Although it appears that differences in study cohorts and not measurement techniques account for our inability to validate the finding of the Stanford study that elevated serum IL-17F levels are potential predictors of poor response to IFN β , differences in the IL-17F measurements between the IL-17F/F ELISA and the Panomics multiplex assay, which measured both IL-17F/F and IL-17A/F, point to the importance of characterizing the sensitivity and specificity of assays used to measure serum IL-17F.²² The results of the current investiga-

tion underscore the importance of using representative datasets from large well-characterized patient cohorts for biomarker validation.

AUTHOR CONTRIBUTIONS

S. Bushnell: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, acquisition of data, study supervision, obtaining funding. Z. Zhao: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, statistical analysis. C. Stebbins: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, acquisition of data. D. Cadavid: drafting/revising the manuscript, study concept or design, analysis or interpretation of data. A. Buko: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, study supervision. E. Whalley: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, study supervision. J. Davis: drafting/revising the manuscript, study concept or design, analysis or interpretation of data. E. Versage: drafting/revising the manuscript, study concept or design, study supervision. J. Richert: drafting/revising the manuscript, study concept or design, analysis or interpretation of data. R. Axtell: study concept or design, analysis or interpretation of data, study supervision. L. Steinman: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, study supervision. R. Medori: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patients, statistical analysis.

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DISCLOSURE

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