Letter to the Editor: Prerequisites to Define and to Validate Therapy-Predicitive Blood Biomarkers

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In the 'Validation Study of Existing Gene Expression Signatures for Anti-TNF Treatment in Patients with Rheumatoid Arthritis' Toonen et al. (1) recently reported on the validation of published transcriptional biomarkers potentially predictive for the success of anti-tumour necrosis factor (anti-TNF) treatment in rheumatoid arthritis (RA). They used a Dutch cohort of RA patients (n = 42), with responders and non-responders classified according to the EULAR DAS28 response criteria (2). Gene expression was validated using Affymetrix GeneChip Exon 1.0 ST arrays.

For comparison, Toonen et al. considered gene sets from a total of 14 studies (3-16); however, data from 9 studies were excluded, since either the first analysis was performed after treatment initiation or the material was taken from a different site (i.e., synovial tissue). Toonen et al. then investigated the specificities and sensitivities of the gene sets derived from the remaining 5 studies (5, 7-9, 14) for identifying responders/non-responders in their cohort.

This is undoubtedly an interesting approach, providing further insight in the interesting field of biomarkers for therapy prediction and individualized medicine. However, some points may require consideration in order to put the results into the right general framework of biomarker development.

Toonen et al. (1) do not explicitly state whether and which dose of methotrexate (MTX) the RA patients received in addition to anti-TNF (the only indirect indication is given in their Table 1). Also, dosing and regimen of anti-TNF therapy and/or the concurrent steroid treatment was not presented. We believe that this information would be helpful in order to allow a reasonable comparison with the other studies. In addition, it would be very interesting to know whether there were any differences between the patients treated with either adalimumab (n = 15) or infliximab (n = 27).

Secondly, there are several differences among the 6 different studies (see Table 1), for example:

- usage of different cell populations; i.e., **a)** PBMC consisting of blood mononuclear cells; **b)** monocytes isolated by negative selection; and **c)** PAX whole blood preparations with variable percentage of mononuclear cells, granulocytes and potentially reticulocytes;
- different forms of therapy (i.e., anti-TNF monotherapy, combination therapy with anti-TNF and MTX, MTX monotherapy; concurrent steroid therapy) and different types of anti-TNF monoclonal antibodies;
- usage of different response criteria, e.g. the EULAR criteria (2) and the revised ACR criteria (17) to clinically separate responders from non-responders;
- different time periods between the initiation of therapy and resampling (range 12 to 26 weeks);
- different array platforms, as well as normalization and bioinformatic analyses; and
- presence/absence of qPCR validation of the transcription results.

At first glance, the different data sets appear totally incomparable, since none of the studies show the same clinical, therapeutic or technical features. In particular, the PAXgene samples from whole blood must be assumed to contain considerable contaminations of granulocytes and reticulocytes, as well as highly variable percentages of different mononuclear cells; this is reflected in a suboptimal performance of the respective gene sets in the validation study of Toonen et al., in particular concerning their specificity (see their Fig. 1 and Table 2; (1)).

This leaves 3 studies relying on either PBMC (with highly variable percentages of monocytes, T-cells, B-cells, and, importantly, dendritic cells) or on isolated monocytes (purity of 83-90%; 14). Again, studies based on PBMC versus isolated monocytes appear hardly comparable. In addition, in the study of Stuhlmüller et al. the biomarkers predictive for the therapeutic success were derived from anti-TNF monotherapy (14), whereas the markers defined by Lequerre et al. (5) and validated in the study of Toonen et al. (1) were derived from anti-TNF and MTX combination therapy. Notably, in the study of Stuhlmüller et al. (14) the biomarker gene sets were identified for anti-TNF monotherapy, but did not discriminate between responders and non-responders in the cohort treated with anti-TNF combination therapy or, for that matter, with MTX monotherapy. In addition, the sensitivity/specificity claimed for the prediction of responder/non-responders to anti-TNF monotherapy was based on a single gene (CD11c), and not on the 3 genes analyzed in the study of Toonen et al. It is thus not very surprising that there was only limited overlap between the candidate genes in the studies of Toonen and Stuhlmüller et

al. (1, 14) and thus limited specificity and sensitivity of the gene set from ref. 14 for the patients studied in ref 1.

On the other hand, it may not be very surprising that comparable results were obtained only in the two studies using the same cell source (PBMC), the same form of treatment (anti-TNF and MTX; similar anti-TNF antibodies; for the question of differences between the antibodies see above), the same response criteria; the same first sampling (day 0) and similar resampling time points (12 and 16 weeks, respectively), and only different array platforms/bioinformatics.

Also, the authors should consider the possibility of obtaining false positive results, for example because of multiple testing, since the study of Stuhlmüller et al. (14) failed in identifying predictive biomarkers for the combination therapy with anti-TNF and MTX. However, the latter study may have missed gene expression information due to the primary focus on monocytes. In addition, a meta analysis of the predictive data obtained in the studies of Toonen and Lequerre (1, 5) will be useful and informative.

Finally, we would like to mention three technical/clinical concerns with the study of Toonen et al (1). First, although we understand that true positives could be defined as true anti-TNF non-responders identified as non-responders on the basis of their continuing disease, the purpose of the biomarkers is to actually predict response to anti-TNF therapy and thus true positives should perhaps be defined as true anti-TNF responders identified as responders (as also done in most of the published studies on this topic). Thus, the sensitivities/specificities presented by Toonen et al. in Table 2 should possibly be interpreted in an inverted fashion. Secondly, although Fig. 1 clearly depicts the performance of the biomarkers derived from the different published studies in the Dutch cohort, it does not yet provide a discriminative threshold level for the expression of one or more molecular markers, required for the prediction of future responders to anti-TNF therapy in a truly individual-oriented fashion (14). Thirdly, sensitivities/specificities were only given for the discrimination of good responders and non-responders (excluding RA patients with a moderate response); it may be expected that inclusion of the moderate responders, necessary to provide all RA patients with a reliable therapy recommendation in clinical practice, will influence the performance of the predictors.

Taken together, however, the analyses of Toonen et al. provide important information in the much needed biomarker-based approach to provide our patients with the best possible treatment.

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Sample source	Isolation	Trial	Medication (Resp. / non-Resp.)	α-TNF antibody	Patient cohorts	Steroids (mg)	Criteria of Response (Resp. / Nonresp.)	Sampling / resampling (day / week)	Microarray platform	Scanning, Normalisat. Bioinformatics, Statistics	q-PCR Validation (gene no.)	Sensitivity / specificity (initial study)	Sensitivity / specificity (Toonen cohort)	Reference
Blood	PBMC	Own observational study	anti-TNF + MTX (18/24)	Infliximab Adalimumab	1	?	DAS28	0 / 16	Affymetrix Exon Microarray 1.0	Affym.Expression Console Partek, RMA normalis. Pearson distance, K-means				Toonen et al. PLOS One 2012
Blood	PBMC	Own observational study	anti-TNF + MTX (16/17)	Infliximab	2	≤15mg	DAS28	0 / 12	Custom cDNA Nylonmembr. array [α33P]dCTP label	XdotsReader R-software, leave-one-out SAM, t-Test Fisher's exact test	+ (20/8)	80 / 100	71 / 61	Lequerré et al.
Blood	Monocytes	Abbott DE011 & Abbott PREMIER Own observational study	anti-TNF (15/12) anti-TNF + MTX (7/9) MTX (22/12)	Adalimumab	2	≤10mg	ACR	0 / **26**	Affymetrix U133A Microarray	MAS 5.0 BioRetis Pearson distance , Mann-Whitney U-test	+ / (1)	100 / 92	79 / 56	Stuhlmüller et al.
Blood	PAXgene	Own observational study	anti-TNF (43/25)	Infliximab	2	?	DAS28	0 / 14	Agilent cDNA 4x44 K Microarray	Agilent Scanner software Quantile Normalisation Leave-one-out U & Z-Test		100 / 96	67 / 33	Tanino et al.
Blood	PAXgene	Own observational study	anti-TNF + MTX (8/10)	Infliximab	1	yes (?)	ACR DAS28	0 / **22**	Custom cDNA Microarray	ImaGene software global Lowess Norm. Wilcoxon/Kruskal–Wallis, Welch t-Test	+ (1)		71 / 28	Sekiguchi et al.
Blood	PAXgene	Own observational study	anti-TNF + MTX (37/7)	Infliximab	1	≤10mg	DAS28	0 / 14	Ilumina Human-6 v.1 Microarray	Bead Studio Software Quantile Normalisation k-Nearest Neighbours U & Z-Test		94 / 86	92 / 17	Julia et al.

Table-1 : Validation of predictive biomarkers

The table shows the comparisons and the differences highlighted in colour between the 5 studies selected for the validation of therapy predictive biomarkers by Toonen et al. **(1)**