Molecular Biomarkers of Anti-TNF Response in Patients with Rheumatoid Arthritis

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Abstract

Background

Advances in immunotherapy by blocking TNF have remarkably improved treatment outcomes for rheumatoid arthritis (RA) patients. Although treatment specifically targets TNF-α, the downstream mechanisms of immune suppression are not completely understood, and the reason for the reduced efficacy in a significant fraction of patients remains unclear. Hence this study was designed to detect biomarkers and expression signatures of response to TNF inhibition.

Methods

In this study, we included 39 female patients diagnosed with RA who were non-responders to methotrexate treatment. The blood samples were collected before anti-TNF treatment initiation, and three months into treatment. The clinical evaluations were performed based on European League Against Rheumatism (EULAR) and classified 23 patients as responders and 16 as non-responders after three months following the initiation of anti-TNF treatment. We investigated differences in gene expression in peripheral blood mononuclear cells (PBMCs), the proportion of cell types and cell phenotypes in peripheral blood using flow cytometry, the level of proteins in serum, as well as clinical and demographic factors.

Results

We performed analyses to identify differences between responders and non-responders at both time points (before and after treatment initiation) as well as to detect the changes induced during the treatment using transcriptomics, flow cytometry and proteomics data. The gene expression analysis before treatment revealed notably a higher expression of EPPK1 and BCL6-AS1 in future responders. We further detected suppression of genes and proteins during treatment, most notably a suppression of expression of the gene, T-cell inhibitor CHI3L1 and its protein YKL-40 measured from flow cytometry. We identified an increase in the proportion of T- and B cells, whereas the proportion of granulocytes was suppressed during treatment in responders. Finally, our machine learning models mainly based on transcriptomics data showed high predictive utility (ROC AUC ± SEM: 0.81 ± 0.17) in classifying response before anti-TNF treatment initiation.

Conclusions

Our comprehensive analyses resulted in several useful insights regarding the transcriptional and translational regulations of anti-TNF treatment in RA patients. The study reports first transcriptomics analysis using RNA sequencing of isolated PBMCs from anti-TNF naïve and anti-TNF treated RA patients to study biomarkers and predict anti-TNF response.

Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation in symmetric joints that without effective treatment leads to additional pain and eventually bone destruction. RA is one of the most common autoimmune diseases which affects approximately 0.5 -1 % of the world's population[1]. Currently there is no cure for RA, but several disease modifying anti-rheumatic drugs (DMARDs) are used to treat patients with the disease. During a successful treatment course, inflammation in the joints decreases, resulting in disease remission or low disease activity [2, 3]. Methotrexate (MTX) is the DMARD recommended as the first line treatment of early RA, however at least 30 % of patients do not respond to the treatment where significant disease activity remains [4, 5]. The patients who do not respond to first line treatment are recommended for additional treatment, in most cases with drugs that inhibit tumor necrosis factor (TNFi). TNF is a pro-inflammatory cytokine secreted mainly by monocytes and macrophages, but also by other immune and non-immune cells, including fibroblasts and endothelial cells, involved in systemic inflammation.

The anti-TNF treatment is recommended due to its efficacy in blocking synovial inflammation, and preventing radiological progression[6]. Anti-TNF therapy has been used to treat RA for two decades, but in many contexts one third of treated patients do not respond or have poor responses[7]. The ability to predict which patients will respond to any treatment is limited. Prediction of efficacy of treatments at baseline would help patients to start effective treatment and hence reduce delay in effective treatment of the disease and decrease numbers of adverse effects. This could also help to reduce the cost of starting different biologic therapies and most importantly improve effective health care.

RA affects women more frequently than men, and the response rate for various RA therapies including DMARDs and anti-TNF has in some studies been reported to be lower in women compared to men [8–10]. Sex differences in immune responses are influenced by both the age and sex. Moreover, sex chromosome genes and sex hormones contribute to the difference in immune responses in males and females [11–14].

In the current study, we performed analyses to identify biomarkers of anti-TNF response, as well as signatures of response, in a Swedish cohort of RA patients. The study cohort is well-characterized and allowed us to investigate gene expression differences in peripheral blood mononuclear cells (PBMCs), the proportion of different cell types and cell phenotypes in peripheral blood using flow cytometry, the level of several proteins in serum, as well as clinical and demographic factors.

The PBMCs are mainly composed of different cell types (monocytes, T cells, B cells, NK cells, and dendritic cells) that are important for immune responses and inflammation. Gene expression in PBMCs is a good source to study pathophysiological mechanisms and has emerged as a potential source for the identification of biomarkers [15, 16]. Transcriptomics studies have been used to identify potential biomarkers that might help to predict treatment response [17]. Our study reports the first transcriptomics analysis using RNA sequencing of isolated PBMCs from RA patients with the aim to find biomarkers and predict anti-TNF response. Further, we studied the association of data from flow cytometry, from protein

measurements and from clinical information with anti-TNF response and employed machine learning models to predict anti-TNF response.

Materials And Methods

Patient Cohort

In this study, patient samples are obtained from the COMBINE cohort which includes 239 patient samples [18]. This includes individuals treated with methotrexate (89 patients), treated with anti-TNF drugs (after failing to respond to methotrexate treatment, 59 patients), or treated with second biologic agent (after failing to respond to anti-TNF treatment, 31 patients) and healthy controls, 60 samples). Healthy controls were recruited from the Swedish Blood Centre service in Uppsala with age and sex as closely matched with patient groups as possible. The patients who did not respond adequately to methotrexate treatment according to the local physician's judgement were prescribed for anti-TNF treatment in combination with methotrexate. Patients included in this study donated blood at the clinic before starting anti-TNF therapy, and at the follow up visit after approximately three months. Clinical assessments and routine blood sampling were made at both visits, and are used to calculate the disease activity score based on 28 joints, DAS28 CRP [19]. Of the 59 patients who started anti-TNF therapy, three patients dropped out prior to the scheduled three month visit and therefore lack clinical assessment. Previous studies have indicated that males and females respond differently to RA treatments with biologic agents, therefore the low number of male non-responders in our study cohort indicates a very low power to detect whether males and females potentially have different biomarkers or different treatment effects. In order to avoid larger heterogeneity, we therefore decided to include only female patients (n = 39) in our study (Additional file 1: Fig. S1). The clinical and demographic variables at baseline are shown in Table 1.

Table 1
Demographic characteristics of female rheumatoid arthritis patients treated with anti-TNF.

	RA (n = 39)
Age, median (range)	57 (19–76)
Swedish (%)	34 (82.9)
Current smoker (%)	11 (28.2%)
HLA-DR shared epitope positive (%)	26 (66.6%)
ACPA positive (%)	29 (74.3%)
Bone erosions (%)	18 (46.1%)
DAS28, median (range)	4.79 (2.49–7.48)
28-joint swollen joint count, median (range)	6 (1–25)
28-joint tender joint count, median (range)	8 (1–28)
Prednisolone treatment (%)	23 (58.9%)
Anti-TNF Drugs	16
Remicade	8
Enbrel	11
Humira	2
Simponi	2
Cimzia	2 (0.5–59)
CRP, median (range)	50 (5-100)
Patient global health assessment, median (range)	0.75 (0-2.6)
Physical function (HAQ), median (range)	45 (11–82)
Health professional global health assessment, median (range)	

Table 1 : HAQ = Health Assessment questionnaire disability index; ACPA = anti-citrullinated peptide antibody; DAS28 = Disease Activity Score in 28 joints; CRP = C-reactive protein.

Response measures

We used European League Against Rheumatism (EULAR) response criteria to classify patient response to treatment [20, 21]. In our analysis we consider Good and Moderate EULAR responders as “responders”, and compare these to the EULAR “non- responders”.

RNA sequencing

The RNA was extracted from PBMCs, freshly isolated using CPT tubes (BD Biosciences) and sequenced as previously described [18]. Of the 39 female RA patients, we obtained high quality RNA-seq data from 28 patients (Responders:10; Moderate-responders:9; Non-responders:9) at baseline and 32 patients (Responders:11; Moderate-responders:9; Non-responders:12) after treatment that leads to a total of 25 paired RNA-seq samples (both anti-TNF naïve and treated) (Additional file 1: Fig. S1). The sequencing reads were trimmed using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), trimmed FASTQ files were aligned to the GRCh38 reference genome using Star v.2.5.3a, and gene counts were generated using the function –quantMode GeneCounts [22].

Flow cytometry

We measured the concentration of leukocytes, neutrophils, eosinophils, basophils and monocytes per liter of peripheral blood using XE Sysmex flow cytometry-based analysis. In addition, other immune cell phenotypes were measured by Gallios flow cytometry. Stainings were performed in combinations of antibody panels, one panel focusing on T cell stainings of PBMC, another on B cell stainings of PBMC, a third on NK and monocyte stainings of PBMC and a fourth on staining whole lysed blood, where granulocytes were identified by size and granularity (forward and side scattering properties). PBMCs were isolated by density gradient centrifugation and whole blood lysed using Serotec Erythrolyse buffer. Cells were stained freshly using the following antibodies (clones): CD45RA (B56), TcRgd (B1), HLA-DR (L43), CD4 (OKT4), CD138 (ID4 or DL-101), CD19 (HIB19), NKp44 (P44-8), CD16 (3G8), CD69 (FN50), CD28 (CD28.2), CD45 (HI30), IL21R (2G1-K12), TREM-1 (TREM-26) all from Biolegend, CD3 (UCHT1) and NKG2A (Z199.1) from Becman Coulter, IgD (IA6-2), CD14 (Mphi 9), CD27 (M-T271), CD56 (BI59) from Beckton Dickinson, NKG2D (1D11) from eBioscience. Only the HLA-DR staining was controlled using an isotype control antibody from Biolegend, while the staining of NKG2A, NKG2D, IL21R and TREM-1 were controlled by absence of added antibody (FMO, fluorescence minus one). The data was analyzed using FlowJo (TreeStar Inc, Ashland, OR, USA). In total, we included 422 flow cytometry variables in our analysis. For the correlation analysis, we used the Pearson method.

Protein measurements

The protein measurements were performed as previously described [18]. A log transformation of protein levels was applied for association analysis to negate highly skewed protein levels. We considered only proteins with a minimum of at least 8 different non-zero values, resulting in the analysis of 51 proteins, where 32 proteins were measured with two separate technologies (HumanMAP assay (Myriad RBM) and VectraDA (Crescendo**))**.

Statistical analysis

Gene expression analysis was performed using the DESeq2 Bioconductor package [23]. In the two cross sectional analyses, we adjusted for current smoking (whether patient was smoking when they started treatment), presence of HLA-DRB1 shared epitope (SE) alleles, presence of anti-citrullinated protein

antibody (ACPA), presence of bone erosions (only available at baseline), and whether the patient was prescribed prednisolone when blood was drawn. To account for the potential differences in cell type composition of PBMCs, we also included cell type proportions of the samples (proportion of B cells, T cells, NK cells and monocytes of total PBMCs). Gene expression analysis was performed using the model:

gene expression ~ response + bone erosion + shared epitope + current_smoker + ACPA + prednisolone + prop.B cells + prop.T cells + prop.NK cells + prop.Monocytes

In the longitudinal analysis we investigated changes in gene expression during treatment, and thus used only paired samples (25 patients). To find the effect of treatment on gene expression, we adjusted the model for prednisolone prescription:

gene expression ~ patid + prednisolone + visit

To detect the effect of anti-TNF treatment in responders and non-responders separately, we used a model with interaction terms as described in DESeq2[23].

gene expression ~ response + patid:response + visit:response

Gene set enrichment analysis was performed using the preranked gene set enrichment method using the R package fgsea [24]. We included 1329 canonical pathways (8904 genes) curated from the following online databases BioCarta, KEGG, Matrisome Project, Pathway Interaction Database, Reactome, SigmaAldrich, Signal Transduction KE, Signalling Gateway and SuperArray SABiosciences, collected by MSigDB. We considered pathways with a FDR threshold (FDR < 0.01 or FDR < 0.1) for gene expression contrasts based on the number of significant gene sets.

For protein expression and flow cytometry data, we analyzed the association to EULAR response at two time points using logistic regression. We used the below model for the protein data analysis:

response ~ measurement + ethnicity + erosion + current smoker + shared epitope + ACPA + prednisolone

For the longitudinal analysis of protein, flow cytometry, we used a mixed linear model (lme from nlme R package) :

measurement ~ visit:response + prednisolone + patid,

where patid is a random effect. We computed the estimated marginal means (EMMs) for two contrasts from this model: for the treatment effect in responders and for the treatment effect in non-responders.

Prediction model

We built three classification models of the anti-TNF response data: a linear model (with L1 and L2 regularization, using the glmnet R library), a non-linear model (using the randomForest library in R), and a

kernel-based method (SVM with an RBF kernel, using the smvRadial library in R). We used 10 repeats of 5-fold cross-validation, where in each repeat 5 randomly sampled steps of hyperparameter estimation were employed. We built separate predictive models for clinical variables, gene expression, flow cytometry and for protein measurements, where the covariates mentioned above were included as additional features. For the models based on clinical data, we included CRP, ESR, Swollen joints, Tender joints, Pain (VAS mm), Physical function (HAQ), Patient assessment of global status, Health professional assessment of global status, DAS28, DAS28-CRP, Eosinophils (10*9 /l) and Monocytes (10*9 /l). The prediction models were built on measurements of samples from naive to anti-TNF treated patients (before treatment) and also on measurements taken after three months of anti-TNF therapy (treated). For the gene expression data, we removed genes with all zero counts and ncRNAs (miRNA, snoRNA, piRNA, tRNAs, rRNA and siRNA) that resulted in dataset of 22,628 transcripts (only protein coding and long non-coding transcripts are included) and used transcript per million (TPM) normalized read counts in the model.

Results

Association of baseline gene expression signatures and biomarkers with response to anti-TNF treatment

In order to decrease the heterogeneity between analyzed groups for all the presented analyses, we included only female patients diagnosed with RA in our study (18 responders and 10 non-responders). The gene expression analysis was performed for the baseline data between the group of future responders compared to the group of non-responders. The analysis identified 59 differentially expressed genes and most of these genes achieved significance (FDR $\leq$ 0.1) due to a single outlier sample showing different gene expression profile (Additional file 1: Fig. S2A-B). Therefore, we decided to exclude this patient sample (both anti-TNF naïve and treated) from the subsequent analyses. The further analysis (without the outlier sample) yielded 192 differentially expressed genes (FDR $\leq$ 0.1), including 103 genes with higher expression in the group of responders and 89 genes with higher expression in the group of non-responders. The top differentially expressed genes are represented in Fig. 1A and listed in Table 2 (also see Additional file 2: Table S1).

Many differentially expressed genes showed significant differences between responders and non-responders. However, this was found to be due to outlier expression in one or more patient samples. In order to assess possible heterogeneity and to detect the genes with stable association to response, we employed a leave-one-out (LOO) approach, where we removed one patient sample in each iteration and repeated the association analysis. The genes are considered in each iteration if the gene meets statistical significance with a false discovery rate less than 0.1. After performing the LOO approach, only two genes, Epiplakin (EPPK1) and FosB Proto-Oncogene, AP-1 Transcription Factor Subunit (FOSB) with higher expression in future responders showed significance in all possible 28 iterations (FDR < 0.1 across all 28 LOO iterations, Table 2). And additional five genes, EGR1, EGR2, BCL6-AS1, IGLV10-54 and IGKV1D-39 showed significance (FDR < 0.1) in 27 LOO iterations. The genes EGR1, EGR2 and BCL6-AS1 were expressed higher in future responders, whereas immunoglobulin light chain genes IGLV10-54 and IKV1D-

39 were expressed lower in future responders. We plotted the normalized expression counts of these seven differentially expressed genes and found only genes EPPK1 and BCL6-AS1 showed clear difference in expression between responders and non-responders (Fig. 1B) whereas FOSB, EGR1 and EGR2 did not show clear differences (Additional file 1: Fig. S2C). Also we noticed 6 immunoglobulin light chain genes (IGLV10-54, IGKV1D-39, IGKV3-20, IGLV3-1, IGKV1-17, IGKV2-24) and 1 heavy chain gene (IGHV5-10-1) showing lower expression in group of future responders compared to group of non-responders, however these genes did not pass the LOO analysis (FDR < 0.1) (Fig. 1A). The number of differentially expressed genes that showed significance across the 28 LOO iterations varied from 15 to 1617, indicating that the statistical analyses in DESeq2 were sensitive to outlier samples.

Table 2
Top differentially expressed genes in PBMCs of female future anti-TNF responders and non-responders before treatment inititation.

Genes	Description	log2FoldChange	p-value	Iteration count
FOSB	FosB Proto-Oncogene, AP-1 Transcription Factor Subunit	3.88	6.25E-09	28
EPPK1	Epiplakin 1	1.89	6.06E-07	28
EGR2	Early Growth Response 2	3.98	1.63E-07	27
BCL6-AS1	BCL6 Antisense 1	2.14	2.95E-07	27
EGR1	Early Growth Response 1	3.68	5.91E-06	27
IGLV10-54	Immunoglobulin Lambda Variable 10–54	-2.73	5.46E-06	27
IGKV1D-39	Immunoglobulin Kappa Variable 1D-39	-2.68	2.13E-05	27
PDIA4	Protein Disulfide Isomerase Family A Member 4	-0.45	5.59E-06	26
HSP90B1	Heat shock protein 90 kDa beta member 1	-0.60	1.17E-05	26
FAM46C	family with sequence similarity 46, member C	-1.03	1.42E-05	26
KDM6B	Lysine Demethylase 6B	0.61	1.92E-05	26
FBXO7	F-Box Protein 7	-0.37	2.57E-05	26
PSAT1	Phosphoserine Aminotransferase 1	-0.78	2.49E-05	26
CDC20	Cell Division Cycle 20	-1.95	8.70E-06	25
NDC80	NDC80 Kinetochore Complex Component	-0.80	1.46E-05	25
CHEK1	Checkpoint Kinase 1	-0.80	1.91E-05	25
ITM2C	Integral Membrane Protein 2C	-0.89	4.86E-05	25

Genes	Description	log2FoldChange	p-value	Iteration count
SOGA1	Suppressor Of Glucose, Autophagy associated 1	0.66	5.61E-05	25
TXNDC15	Thioredoxin Domain Containing 15	-0.42	6.42E-05	25
IGLV3-1	Immunoglobulin Lambda Variable 3 – 1	-1.80	8.19E-05	25
MTCO2P12	MT-CO2 Pseudogene 12	2.76	7.17E-04	25

Table 2: The column represents genes, description of genes, log2 fold change, p-value and iteration count. Iteration count is the number of leave-one-out iterations where the gene remained significant .

To understand the characteristics of responders and non-responders, we performed gene set enrichment analysis (GSEA) and identified a total of 127 regulated pathways (FDR ≤ 0.01, Additional file 2: Table S2). We found positive and negative enrichment characteristics for responders compared to non-responders. Notably, response to therapy was preferentially characterized by higher expression of genes involved in graft versus host disease, antigen processing and presentation, and neutrophil degranulation. Future non-responders were characterized by higher expression of genes involved in cell cycle pathways, mainly - cell cycle mitotic activity, cell cycle checkpoints and also for DNA replication and protein translation. The top 15 most upregulated and downregulated pathways are represented in Additional file 1: Fig. S3A; Additional file 2: Table S2).

We further studied the association of immune phenotypes measured by flow cytometry and also the association of blood plasma protein levels to clinically defined response before anti-TNF treatment. Neither flow cytometry measurements nor protein measurements showed any significant difference between responders and non-responders at baseline (data not shown).

Association of gene expression signatures and response upon 3 months of anti-TNF treatment

With the aim to identify gene expression signatures associated with response upon therapy with TNF-blockade, we performed differential expression analysis in PBMC between groups of responders and non-responders after 3 months of the treatment. This analysis identified significant differences in expression for 19 genes (FDR ≤ 0.1) in PBMC (Additional file 1: Fig. S4A). We investigated the effects of outliers on differential gene expression among the follow up samples using a LOO approach. None of the previously found 19 genes remained significantly differentially expressed in all 32 iterations. However, three genes, BRDOS (Additional file 1: Fig. S4B), C2orf42 and HBA2 showed significance (FDR ≤ 0.1) in 31 iterations. All three genes are expressed lower in the group of responders compared to the group of non-responders. Additionally, five genes, EPHB3, MKS1, NCK1-AS1 (Additional file 1: Fig. S4B), SLC25A39, FBXO7 showed

significance in 30 iterations. The list of differentially expressed genes and number of iterations where each gene showed significance are presented in Additional file 2: Table S3.

The gene set enrichment analysis was performed using differentially expressed genes, sorted based on log2 fold change and the analysis identified 27 regulated pathways. Interestingly, all the 27 pathways that showed significance had a lower expression in the group of responders. The regulated pathways were predominantly enriched for metabolism of RNA, metabolism of proteins and metabolism of amino acids and derivatives (Additional file 1: Fig. S3B; Additional file 2: Table S4) suggesting overall downregulation of biosynthesis in PBMCs of patients who have responded to anti-TNF treatment.

Further, our association analysis of immune phenotypes and plasma protein levels to clinically defined response of anti-TNF treatment did not show any significant association between responders and non-responders after three months (data not shown).

Effect on gene expression in PBMCs during anti-TNF treatment

Since biological and technical variations between individuals may significantly affect the analyses, paired samples of PBMC from the same patient before and after treatment is the most preferable approach for addressing changes related to the treatment. We analyzed the effect of anti-TNF treatment on gene expression using paired PBMC samples from all 25 RA patients, not considering response. The analysis identified 25 genes that showed significant treatment effect on gene expression (FDR $\leq$ 0.1). The expression of 14 genes were suppressed and 11 genes showed a slight increase in expression compared to baseline with 3 months of treatment with TNF blockade (Additional file 2: Table S5). The transcription factor BHLHE40 that controls cytokine production by T cells and Chitinase-6-like protein, CHI3L1were suppressed during treatment whereas the B cell novel protein 1 (alias FAM129C) and Tetratricopeptide Repeat Domain 21A (TTC21A) were induced by treatment (Fig. 2A). The pathway analysis of differentially expressed genes did not show any enrichment of gene sets (FDR $\leq$ 0.01), but when using a less conservative threshold of FDR $\leq$ 0.1, we detected the regulation of 114 gene sets during treatment across all patients (Additional file 2: Table S6). Induced genes are significantly enriched for genes involved in the RNA and protein metabolism, and interferon signaling, whereas suppressed genes are predominantly enriched for genes involved in neutrophil degranulation, hemostasis and signaling by GPCR (Fig. 2B).

Effect of anti-TNF treatment on gene expression in PBMCs in relation to treatment response

The trajectory of gene expression changes may correlate with measured clinical outcomes. Therefore, we investigated the transcriptional changes in paired samples of responders (n = 17) and non-responders (n = 8) to anti-TNF treatment separately. Our analysis identified five genes that were significantly regulated in responders, whereas no significant regulation was identified in non-responders. Of the five regulated genes in responders, C-X-C Motif Chemokine Receptor 2 (CXCR2), Myeloperoxidase (MPO), Myeloid Associated Differentiation Marker (MYADM), TNF Alpha Induced Protein 6 (TNFAIP6) were suppressed by treatment, whereas gene Low Affinity Immunoglobulin Gamma Fc Region Receptor II-B (FCGR2B) was

induced during anti-TNF treatment. The gene expression plots for all five genes using normalized counts shows a clear difference in responders before and after anti-TNF treatment (Fig. 3A). We observed regulation of these genes in the same direction for non-responders, but with less significance (Additional file 1: Fig. S5).

Further, we performed gene set enrichment analysis of differentially expressed genes in responders and found 78 pathways that are significantly enriched (Additional file 2: Table S7). In responders, we noticed induction of pathways involved in regulation of cell cycle mitotic and protein metabolism, whereas genes involved in extracellular matrix organization, neutrophil degranulation, signaling by GPCR, signaling by interleukins, hemostasis and immune responses such as Toll like receptor cascades were downregulated (Fig. 3B; Additional file 2: Table S7).

Changes in cell phenotypes during anti-TNF treatment

Using a linear model we studied the changes in 422 immune phenotypes measured by flow cytometry during treatment with anti-TNF. When analyzing the effect of treatment in responders and non-responders, we observed differences in seven cell phenotypes in responders and no significant differences in non-responders (Additional file 2: Table S8). After treatment in responders, we detected a strong suppression of the proportion of granulocytes among leukocytes (defined as CD45 + cells), as well as a decrease in overall concentration of neutrophils in whole blood. The proportion of T cells (defined as CD3 + cells) and B cells (CD3-CD19+) among leukocytes was instead up-regulated during anti-TNF treatment among responders, along with the proportion of NKG2A + NKp44 + NK cells out of all $NKp44^{+}$ NK cells (Fig. 3C). We compared beta coefficient values of responders and non-responders for the selected 58 cell phenotypes (p-value < 0.05) and we observed a positive correlation between response groups ($r = 0.46$, p value = 0.0008) indicating similar directionality of regulation for the most cell phenotypes.

Effect of anti-TNF treatment on levels of different proteins blood plasma

To identify the proteins that are regulated during treatment in responders and non-responders separately, we performed longitudinal analysis on paired patient samples. In responders, we identified regulation of 12 proteins in plasma compared to one protein in non-responders. Proteins were predominantly downregulated by anti-TNF treatment in responders, including CRP, IL-6, MMP-1, MMP-3, SAA, TNF-RI, VEGF, TNFR2, MIG, MIP-1 beta and YKL-40 (Fig. 3D). Interestingly, we observed downregulation of protein matrix metalloproteinase-3 (MMP-3) measured using two different methods. The protein adiponectin is induced during anti-TNF treatment among non-responders, as well as with less significance in responders (FDR: 0.11). The list of proteins that were regulated during anti-TNF treatment is shown in Additional file 2: Table S9.

Classifier performance for anti-TNF response data

We investigated the utility of machine learning algorithms to predict anti-TNF response using clinical data, flow cytometry measurements, protein measurements and transcriptomic data. At baseline, the model based on transcriptomics data predicted response fairly accurately with linear model (ROC AUC ± SEM : 0.81 ± 0.17) (Fig. 4) whereas the models based on clinical data, flow cytometry data and protein data showed limited predictive utility. The kernel method at baseline predicted response with ROC AUC: 0.73 ± 0.17 for clinical data, ROC AUC: 0.72 ± 0.18 for flow cytometry and ROC AUC: 0.72 ± 0.15 for protein data (Fig. 4). We further studied the classifier performance of the models based on all four data types at three months (after treatment) and we observed limited classifier utility of models based on flow cytometry, protein as well as transcriptomics data. In contrast, the linear model based on clinical data showed good classifier performance with ROC AUC: 0.85 ± 0.15 at three months. For the FACS data, we found maximum classifier utility of ROC AUC: 0.68 ± 0.17 using linear model, whereas the models based on proteins and transcriptomics data showed maximum classifier utility using non-linear method with ROC AUC: 0.73 ± 0.15 and ROC AUC: 0.72 ± 0.18 respectively.

Discussion

This study represents a comprehensive analysis of transcriptomics, proteomics and flow cytometry data analysis of female RA patients treated with anti-TNF. Here, we used data from multiple data types to infer differences between groups of responders and non-responders before and after treatment. Our analysis was directed towards finding transcriptional and translational regulations during anti-TNF treatment using paired samples collected at two time points. We identified genes that are differentially regulated between responders and non-responders both at baseline and after anti-TNF treatment. We further reported changes in gene expression, protein measurements and cell phenotypes during the course of anti-TNF treatment. Interestingly, our integrated studies revealed that CHI3L1 transcript and its protein product YKL-40 were suppressed upon anti-TNF treatment. The anti-TNF treatment in responders also resulted in an increased proportion of B cells, T cells and NK cells, whereas the proportion of granulocytes was strongly suppressed in responders.

The identification of potential biomarkers with prognostic value for response to a given therapy is challenging as RA is a very heterogeneous disease by its clinical characteristics and pathological processes. Previously, various attempts have been made to find biomarkers for anti-TNF treatment, but with limited success [25–28]. This may be due to the high clinical heterogeneity in RA patient samples and strong confounding (covariates) effects. For example, variation in cell subsets is one of the strong covariates of gene expression [29]. For the current study, we used a well characterized cohort which allowed us to adjust for the important covariates, such as clinical measurements and proportions of T cells, B cells, NK cells and monocytes measured from flow cytometry. For RA, the response measure is calculated based on changes in DAS28 scores as defined by the EULAR response criteria [30]. In addition, RNA sequencing experiments can often generate outlier read counts in one or more RNA samples. The presence of these outliers in the data considerably limits the power of differential testing and therefore we performed extensive transcriptomics analysis using leave-one-out approach to address possible heterogeneity and find genes with a stable association to response. To the best of our knowledge, this is

the first study that integrates RNA sequencing of PBMCs, broad flow cytometry measurements, and measurements of protein levels in serum and plasma to find biomarkers in RA patients treated with TNF inhibitors.

In the analysis using baseline samples, we report two genes EPPK1 and BCL6-AS1 that showed stronger association with response groups. The cell adhesion gene epiplakin 1, EPPK1 and BCL6-AS1 showed higher expression in future responders compared to future non-responders. The infiltration of inflammatory cells into the synovial lining is achieved by deregulation of cell adhesion molecules and the studies have previously reported the association of cell adhesion genes and suggested a role of these molecules in the pathogenesis of rheumatoid arthritis and hemophilic arthropathy [31, 32]. We also found the expression of the EPPK1 gene to be positively correlated with the proportion of NK cells and proportion of monocytes. The gene BCL6-AS1, a long non-coding RNA was shown to be correlated with the BCL6 translocation zone that promotes chromosomal breaks in immunoglobulin heavy chain (IgH) switch regions through convergent transcription [33, 34]. We found the expression of BCL6-AS1 to be positively correlated (r2: 0.89) with the expression of BCL6 gene which is important for the formation of both Tfh cells and germinal B cells that enhances humoral responses [35–37]. Importantly, the differences we observed in genes, EPPK1(log2 fold change:1.95, FDR:5.5E-03) and BCL6-AS1(log2 fold change:1.51, FDR:8.16E-02) were maintained between responders and non-responders following the adjustment to CRP measurements in the gene expression model. Interestingly, future non-responders showed an increase in cell cycle pathways mainly cell cycle mitotic activity, cell cycle checkpoints. This may partly explain the hyperproliferation of cells that leads to the accumulation of pro-inflammatory cytokines in the inflamed joints [38, 39].

Our analysis of differences in gene expression patterns between responders and non-responders in samples obtained at 3 months after anti-TNF treatment showed significant differences in two genes, BRD3OS was expressed higher in non-responders as compared to responders and NCK-AS1 was expressed higher in responders as compared to non-responders. Interestingly, pathway analysis suggested downregulation of pathways involved in metabolism of RNA, metabolism of amino acids and derivatives and metabolism of proteins in responders. This inactivation of pathways involved in metabolism among responders corroborates with the previous findings that proliferation and rapid activation of immune and stromal cells requires a metabolically highly active state. Such a high metabolic state induces overproduction of enzymes that lead to degradation of cartilage and bones and the production of cytokines that promote immune cell infiltration [40–42].

The longitudinal studies using paired samples of PBMCs from the same patient before and after treatment removes inter-individual variations, and therefore provides better possibility to detect changes induced by treatment. We analyzed the treatment effects in RA patients using two different approaches, i) changes in gene expression patterns in samples from all RA patients not considering response status; ii) treatment effect separately in responders and non-responders in order to understand to which extent anti-TNF treatment regulates gene expression differently in responders and non-responders. In the first approach, our study revealed twenty-five genes that were differentially expressed in all RA patients,

whereas in the second approach we found five genes that were differentially expressed in responders and no genes that were differentially expressed in non-responders. In both these approaches, we found four common genes CXCR2, MPO and MYADM that were downregulated, whereas FCGR2B was upregulated upon treatment. We also observed that the TNFAIP6 gene (TNF Alpha Induced Protein 6, also called tumor necrosis factor stimulated gene-6, TSG-6) was suppressed by anti-TNF treatment in responders. Previous studies have shown that expression of TSG-6 has a strong correlation with disease severity and is a potential biomarker of inflammation [43–45]. Higher expression of TSG-6 has been found in synovial fluid of patients with osteoarthritis and rheumatoid arthritis and the protein coded by this gene is secreted by cells of articular joints. TSG-6 has been reported to play a key role in remodeling of extracellular matrix, regulation of leukocyte migration and stimulation of cell proliferation during inflammation [46, 47]. These previous findings correspond well with our pathway analysis, which suggests that treatment with anti-TNF results in downregulation of genes involved in extracellular matrix organization and signaling by interleukins, and an upregulation of genes involved in regulation of mitotic cell cycle.

Interestingly, the differentially expressed genes that are regulated upon treatment in responders showed regulation in the same direction for non-responders. Thus, the differences that we observed in responders and not in non-responders could be due to the result of different powers of the analysis in the two groups. Extended future studies with large sample size are warranted to validate these results.

We studied changes in cell phenotypes in paired blood samples and observed changes in seven cell populations in responders to anti-TNF treatment, indicating a marked change in major cell type proportions during treatment (Fig. 3C). The proportions of B cells and T cells among leukocytes were increased during treatment in responders whereas there was a strong reduction in the proportions of granulocytes. There was also a decrease in overall concentration of neutrophils in whole blood in responders as well as in non-responders. The reduction of neutrophils seen by flow cytometry was also associated with significant reduction in peripheral blood neutrophil count seen by routine blood analysis, leading to 19% of patients becoming neutropenic by a clinical judgement. This finding corroborates a recent study which showed that patients treated with anti-TNF in combination with methotrexate decreased the blood neutrophil counts regardless of their clinical response to therapy [48, 49]. Additionally, our results also corroborate with earlier studies showing an induction of B cells and NK cells in whole blood following anti-TNF treatment [50]. Along with these changes we also see an upregulation of the proportion of activated NK cells (NKp44+) in responders that express the inhibitory protein NKG2A.

The longitudinal analysis of protein data showed changes in plasma protein levels differently in responders as compared to non-responders. The expression of the protein YKL-40 was reduced upon treatment (beta: -0.26) and the gene encoding for YKL-40, *CHI3L1*, was also down-regulated (Fig. 2A, Fig. 3D). In the analysis including only responders, the gene CHI3L1 showed strong suppression (log2 fold change: -1.07) upon treatment, however with lower statistical significance (p-value < 1.73E-04; FDR < 0.21). The CHI3L1 gene may negatively regulate T cell activation and may also inhibit Th1 differentiation via the IFNγ-STAT1 pathway [51]. We also showed that treatment with TNF inhibitors leads to a larger proportion of T cells among responders (beta: 8.16).

Interestingly, we also found two proteins, soluble TNFR2 and adiponectin which plasma levels are increased after treatment both in responders and non-responders. The TNFR2 protein is predominantly produced by certain T cells, mainly regulatory T cells, endothelial cells, thymocytes, mesenchymal stem cells and cells from the nervous system [52]. Various studies have pointed out that the increase in TNFR2 signaling may lead to the activation and proliferation of Tregs and promote tissue regeneration [53–55]. The protein adiponectin is considered as a pro-inflammatory mediator, and a similar induction of adiponectin as seen here for TNF-blockade has been shown previously after treatment with anti-IL-6R, Tocilizumab [56]. Importantly, it has been shown that adiponectin has a multifaceted role in RA with both pro-inflammatory and anti-inflammatory functions [57]. The studies on immune phenotypes and protein levels both before and after treatment did not show any significant differences between responders and non-responders. As for other findings in the present study, this lack of differences may be due to both insufficient power in our analysis and to an insufficient choice of protein markers that we investigated. Further studies on proteins as biomarkers for response therapies, including TNF-inhibition, in RA are thus warranted.

We studied clinical variables, cell phenotypes, protein measurements and transcriptomics data to assess their ability to predict response to anti-TNF treatment. We found high predictive utility for response using different data types and algorithms before treatment. The linear model based on transcriptomics data at baseline found a suggestive good predictability using the presently applied gene expression classifier. We also illustrated that models with transcriptomics data alone predict response with higher accuracy compared to models with clinical data alone. Interestingly, we also found clinical variables add less further utility to the transcriptomics based predictive models at baseline (data not shown). Previously, a similar outcome was observed in a study where models using transcriptomics data alone predicted fibrous cap thickness response to statin treatment with better accuracy compared to the models with clinical data or transcriptomics plus clinical variables [58]. We caution that our prediction model findings need to be validated in independent cohorts, before its potential use in clinical settings.

Overall, we used a well-characterized cohort of RA patients and reported gene expression, flow cytometry and proteomics profiling in response to anti-TNF treatment. We identified gene expression differences in response groups at baseline and more significant regulations during the treatment. The anti-TNF treatment causes a major regulation of cell subsets that corroborates with the previous reports, which are also mirrored in the protein analysis. We also report strong decrease in expression of genes, proteins and cell subsets in responders upon treatment. Also, our study highlights machine learning predictive utility of the anti-TNF treatment response using different biological measurements at baseline. The limitation of our study is that we have done profiling of only female RA patients and that relatively few patients were included. Our findings also need to be validated in independent cohorts and to be extended in larger studies with more patient samples.

Conclusion

In summary, our findings resulted in several insights regarding the relationships between a set of biomarkers and response to anti-TNF therapy in female RA patients. Our integrative and multi-omics approach identified gene expression signatures, changes in protein concentration as well as cell phenotypes in responders. Our machine learning model based on transcriptomics data showed high predictive utility in stratifying patients and/or predict response before anti-TNF treatment initiation. We envision that machine learning algorithms based on multi-omics data will help to support rheumatologist's decision towards personalized treatment of RA patients.

Abbreviations

ACPA: Anti-citrullinated peptide

AUC: Area under curve

CRP: C-reactive protein

DAS28: Disease activity score in 28 joints

DMARDs: Disease modifying anti-rheumatic drugs

EULAR: European League Against Rheumatism

FDR: False discovery rate

GSEA: Gene set enrichment analysis

HAQ: Health Assessment questionnaire disability index

LOO: Leave one out approach

MTX: Methotrexate

NK cells: Natural Killer cells

PBMCs: Peripheral blood mononuclear cells

RA: Rheumatoid arthritis

ROC: Receiver operating characteristic

SEM: Standard error of the mean

Th1: T helper1

TNF: Tumor necrosis factor

Tregs: Regulatory T cells

Declarations

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Availability of data and materials

The datasets may contain personal details of the participants and cannot be uploaded at the public domain and will be available by request only from the authors.

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NY, BB, AC, LB, LP and LK conceived the study. MMU, PS and NV performed wet lab experiments. NY, BB, AC, LF and LP contributed to the data preparation. NY, BB and MM performed the data analysis. NY, BB,

MM, DZ, LP and LK interpreted the data. NY prepared figures. NY, BB, MM, SJ LB, LP, LK wrote the manuscript. All authors read and approved the final version of the manuscript.

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Ethics declarations

Ethics approval and consent to participate

The COMBINE biobank was generated after written informed consent from all participants had been obtained according to the declaration of Helsinki and with approval by the Stockholm (number 2010-351-31-2) and Uppsala (2009-013) Regional Ethics Committees

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

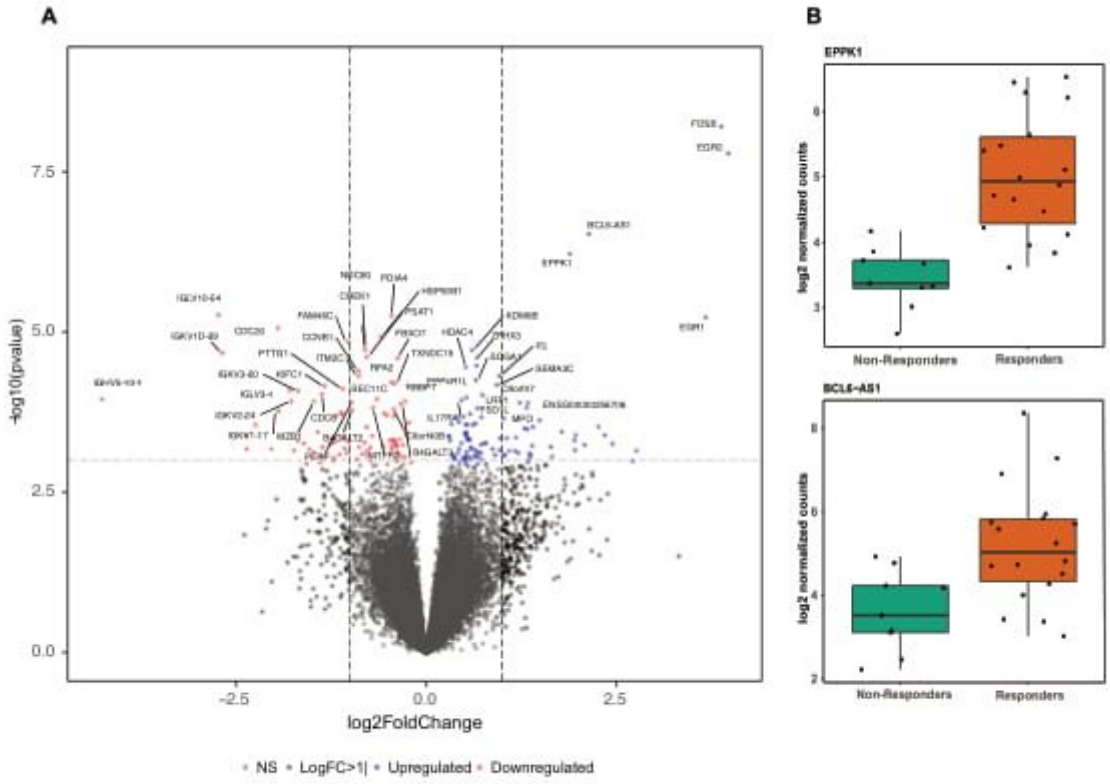

Figure 1

A) Volcano plot representation of differentially expressed genes in PBMCs between future responders and non-responders before anti-TNF treatment. Top regulated genes are marked in blue (upregulated genes) and red (downregulated genes). The vertical lines correspond to log2 fold change of 1 (genes are represented in black) and the horizontal line represents a p-value of 0.001. B) The box plot of normalized log2 expression values for the differentially expressed genes, EPPK1 and BCL6-AS1 in PBMCs before treatment.

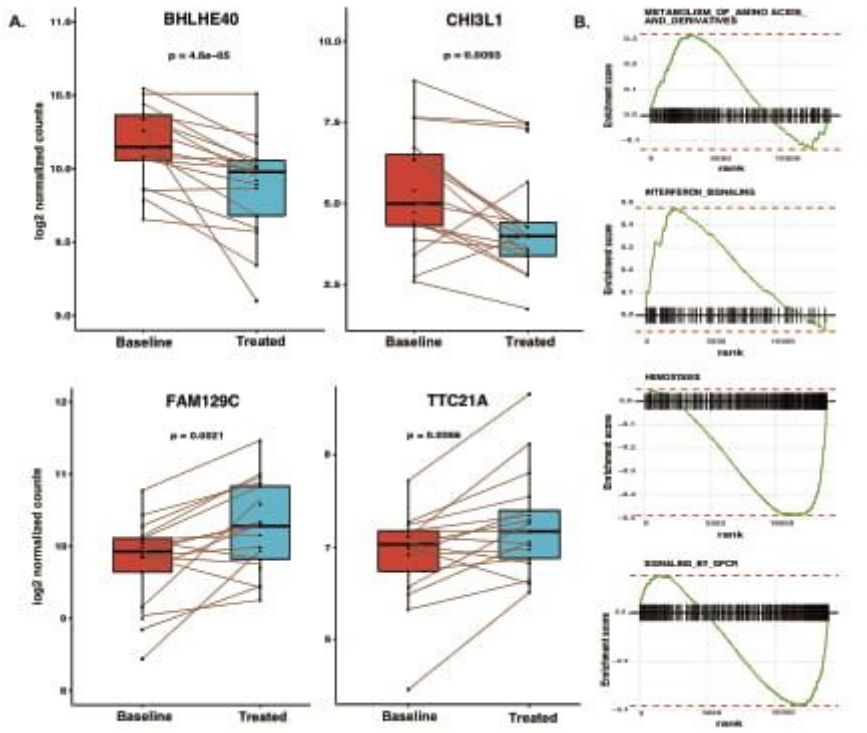

Figure 2

A) The box plots showing the expression levels of differentially expressed genes (all RA patients) in PBMCs for baseline vs. treated patient samples. The expression levels of responders for selected genes are plotted. Abbreviations: BHLHE40, Basic Helix-Loop-Helix Family Member E40; CHI3L1, Chitinase 3 Like 1; FAM129C, Family With Sequence Similarity 129 Member C; TTC21A, Tetratricopeptide Repeat Domain 21A. B).The enrichment plot from GSEA representing functional gene sets enriched between baseline and treated RA patients.

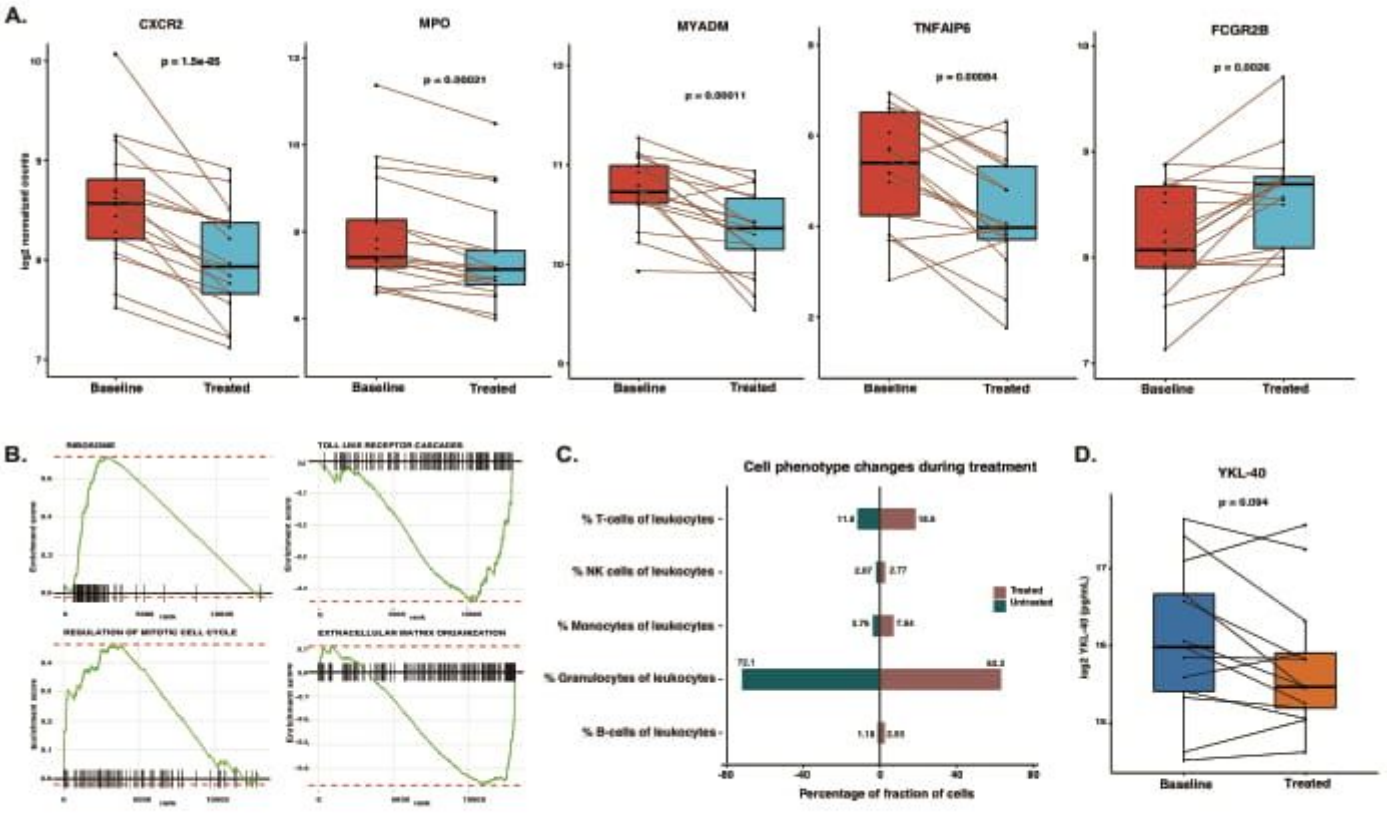

Figure 3

A) The box plots showing the expression levels of genes in responders at baseline vs. treated patient samples. Abbreviations: CXCR2, C-X-C Motif Chemokine Receptor 2; MPO, Myeloperoxidase; MYADM, Myeloid Associated Differentiation Marker; TNFAIP6, TNF Alpha Induced Protein 6; FCGR2B, Fc Fragment Of IgG Receptor IIb. B) The enrichment plot from GSEA representing functional gene sets enriched in PBMCs for baseline vs. treated RA patients for responders. C) The bar plot showing the percentage of granulocytes, B cells, T cells, NK cells and monocytes of peripheral blood leukocytes before and after anti-TNF treatment in responders. D) The box plots showing the YKL-40 protein expression levels in responders before and after anti-TNF treatment.

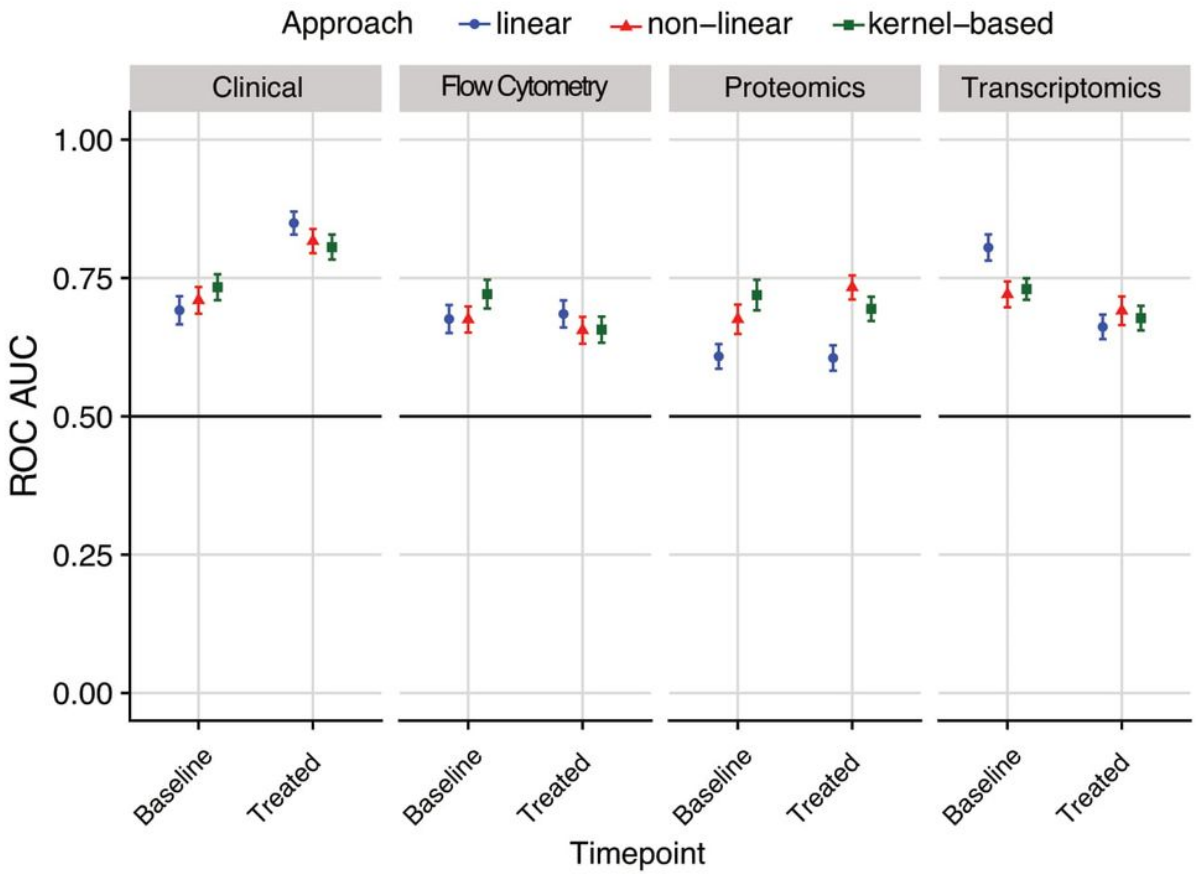

Figure 4

Statistical machine learning models to predict response (evaluated after three months) at baseline and after anti-TNF treatment using clinical variables, flow cytometry measurements, protein measurements and gene expression data. The Y-axis represents the area under the receiver operating characteristic (ROC) curves (AUCs) calculated for estimating the predicted performance.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile2.xlsx
- AdditionalFile1.docx