Peripheral Blood Gene Expression Signatures Identify Methotrexate Responders in Patients with Rheumatoid Arthritis

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Abstract

Background: Methotrexate (MTX) and biologic therapies have remarkably improved outcomes in patients with rheumatoid arthritis (RA). However, choices of therapeutic regimens in an individual patient remain empiric, resulting in a risk of delayed control of inflammation consequent joint damage. In previous studies we have shown that a large number of protein-coding genes are dysregulated in RA and that the expression levels of the majority of these dysregulated genes return to normal in patients who have control of disease after initiating MTX monotherapy. Furthermore, in vitro responses to MTX are rapid, suggesting that changes in expression could precede clinical outcome, and have prognostic value. The aim of the current study was to analyze whole blood gene expression for correlates of clinical responses to MTX, with the long-term objective of developing prognostic biomarkers.

Methods: RA patients (N=32) and healthy controls (HC; N=8) were from the Investigation of Remission in Rheumatoid Arthritis (IRRA) cohort at Pennsylvania State M.S. Hershey Medical Center. The RA group included 16 patients with active disease and 16 with controlled disease; 8 patients in each group were currently being treated with MTX. Whole blood RNA profiling was carried out in the Penn State College of Medicine Genomics core laboratory. Transcript data were analyzed using clustering algorithms and pathways analysis software to determine relatedness of groups.

Results: Patients with active RA showed patterns of gene dysregulation that were farther from HC values than RA patients with controlled disease, whether or not MTX was a current medication. Comparison of the four RA groups determined by activity levels and MTX use showed over-expression of genes in alpha/beta and gamma interferon pathways, especially in active RA patients not currently taking MTX, suggesting contributions to maintenance of active disease.

Conclusion: Levels of genes that are expressed in peripheral blood of RA patients correlate with disease activity and also with MTX treatment status. Longitudinal studies to determine how early in the treatment course changes in gene expression levels are detectable will be of interest to determine the potential for development of prognostic biomarkers to guide therapeutic decisions.

Background

Patients with rheumatoid arthritis (RA) have benefitted from significant advancements in therapeutics over the past two decades. This is due primarily to the use of methotrexate (MTX) as an anchor drug and to the addition of biologic therapies, especially those targeting TNF alpha as well as newer agents that block other signaling pathways [1]. Nevertheless, not all patients respond to these regimens, and up to half of patients who are started on MTX require addition of another agent. Even the mode and dose of MTX are not well-standardized, with different results observed in individual patients [2]. Reliable prediction of which patient might require higher levels of MTX or an additional disease-modifying anti-rheumatic drug (DMARD) is at present not possible. A result of ongoing inflammation during this therapeutic delay is that significant and irreversible joint damage can take place. Substantial data

indicate that even a few months lost in starting efficacious drugs may result in progressive joint damage, with long-lasting effects on overall outcome [3]. For these reasons, approaches that facilitate delivery of early and effective therapies to an individual patient are an unmet need. This concept of matching therapy to each patient fits with the NIH initiative for precision or personalized medicine. Furthermore, development of a predictive algorithm for non-response to MTX in RA is of particular interest [4, 5].

Many tools are validated to measure disease activity in RA. Clinical measures that correlate with remission or low disease activity include DAS28 scores as well as ones based on clinical evaluation alone, such as the Clinical Disease Activity Index (CDAI) and RAPID 3 [6–8]. Multiplexed blood tests correlate with disease activity in patients with RA and remission status [9, 10]. However, some blood tests have not shown utility. Changes in the levels of the serum autoantibodies, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs or anti-CCP), for example, do not reflect treatment responses [11].

While available clinical and laboratory tests show correlations with disease activity, prediction of a future therapeutic response to MTX or biologics using blood tests has been more elusive [5]. The advantages of a predictive blood test would include the ease of doing this with either the primary care provider or even by just laboratory testing between scheduled rheumatology visits, sparing time in the rheumatologist's office for a clinical evaluation. This information could be utilized to achieve more timely changes in therpay which would predict better outcomes for the patient. A prognostic biomarker might be used to determine what dose of MTX to use, which is something that remains empiric and variable, despite decades of use [2]. Furthermore, it could suggest the need to add another disease-modifying drug (DMARD). In patients who are doing well on MTX, this type of approach could lead to avoidance of unnecessary therapy to optimize care. Tight control algorithms that have been advocated for the treatment of RA patients require frequent clinical monitoring, which is not always feasible, at least in the US health system. The alternative possibility of "observing" patients with blood testing much more readily and frequently than at clinic appointments could serve as a type of rheumatologist-extender.

We have shown in previous studies that a large number of protein-coding genes are dysregulated in RA and that the expression levels of the majority of these dysregulated genes return to normal in patients who have control of disease after initiating MTX monotherapy [12]. Furthermore, in our previous studies using cell lines treated in vitro with therapeutically-relevant doses of MTX, changes in gene expression levels were seen within 24 to 48 hours [12, 13]. These findings suggested to us that we should be able to detect profiles in RA patients early in the course of treatment with MTX that predict responsiveness to this DMARD.

The objective of the present study was to examine peripheral blood gene expression in a cohort of RA patients with or without ongoing MTX therapy and with either active or inactive disease, and to compare them to a HC group. The long-term goal is to identify gene expression biomarkers predictive of responses to therapies, especially to MTX. The findings suggest that a good response to MTX is associated with levels of gene expression that are closer to those of HC. In addition, gene classification analyses show

overexpression of genes in the Types I and II interferon pathways in active RA, suggesting other potentially useful therapeutic targets or approaches for these patients.

Methods

Study design

This is an observational study conducted with the Penn State Investigation of Remission in Rheumatoid Arthritis (IRRA) cohort, which has been described previously [14].

Study population

RA patients (N=32) from the IRRA cohort were seen in outpatient clinics at Penn State M.S. Hershey Medical Center. Healthy controls (HC; N=8) were recruited from local volunteers. All individuals were enrolled from June 1, 2015 through June 1, 2017 and were over 18 years of age at the time of enrollment. Demographics, smoking status, periodontal status and self-reported outcomes were obtained through patient questionnaires. Disease history and medication use were obtained by electronic medical record review. A Research Electronic Data Capture (REDCap) database was used to store all data.

Patients with RA were classified in two ways. The first classifier was whether disease was considered active or controlled. The controlled designation used scores for disease activity measures, either DAS-28 ESR, DAS-28 CRP (score < 2.6 for either), or Clinical Disease Activity Index (CDAI; score < 2.8). A total of 16 patients in each group, active or controlled, were selected for study. The second classifier was whether the RA patient was being currently treated with MTX. At the time of sample collection, the patient was classified as currently on MTX or not currently on MTX. Within each of the groups, active or controlled, 8 individuals were currently on MTX and 8 were not.

Blood sample preparation and analysis

Peripheral blood samples from RA patients and HC subjects were drawn into PAXgene® Blood RNA Tube (BD Biosciences, San Jose CA) tubes and stored at – 80 degrees C in the Penn State Institute for Personalized Medicine biorepository. RNA prepared from these stored tubes by standard procedures in Genome Science Core Facility was quantitated using a Nanodrop 2000c spectrophotometer (Thermo Fisher, Waltham MA) and RNA quality was determined using an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara CA). RNA sequencing was carried out in the genomics core using an Illumina HiSeq 2500 (Illumina, San Diego CA), as described previously [15].

Statistical analyses – Values are expressed as mean (standard deviation). Patient groups were compared using a two-tailed student's T-test for continuous data and Fisher's exact test for discontinuous variables. P values < 0.05 were considered significant. Figures and statistical analyses were done using Graph Pad Prism version 8.1 (Graph Pad Software, Inc., La Jolla CA). Clustering analyses were done using Heatmapper [16]. Gene ontologies were investigated using the PANTHER classification system [17] and the reactome.org pathway browser (https://reactome.org/PathwayBrowser

Results

Characteristics of study groups

The group of 32 RA patients had an average age of 55.24 (14.8) years which was comparable to the HC group value of 46.8 (16.8) (P=0.12). For the RA patients, average disease duration was 9.6 (8.5) years, 87% were seropositive (for RF and/or CCP) and 78% were female. All of the RA patients were non-Hispanic; 3 were African American. The HC group consisted of 88% females and all were non-Hispanic Caucasians.

The two RA groups defined according to disease activity measures as either active or controlled were comparable in age and duration of disease (Table 1). The active group included 15 females (94%) which was higher than the inactive group, with 10 females (63%), a difference of borderline significance (P=0.08). Current smokers also were more prevalent in the active than the inactive group (37.5% vs 6.25%; P=0.08). These findings are consistent with other data indicating that females and smokers generally have less optimal responses to RA therapies [18-20]. Three patients in the controlled group were in spontaneous drug-free remission [21]. Mean values for the autoantibodies RF and CCP were not significantly different between the two groups, while ESR and CRP were significantly higher in the active patients. The active and controlled designations were based on standard indices, DAS-ESR, DAS-CRP or CDAI, and these were all significantly higher in the active group (Table 1). Each of the two groups included equal numbers of patients (8 in each group) who were either currently on MTX or were not taking MTX. None of the disease features were significantly different within the MTX+/- subsets except for disease duration, which was longer in controlled patients who were not on MTX (12.4 years) than who were currently taking MTX (5.3 years; P=0.020; Table 2).

Gene expression clustering was carried out, comparing the 16 active RA patients, including those currently on MTX as well as those not on MTX, to 8 HC using a t-test, and 40 genes were identified for which P<0.020. These 40 genes were entered into the Heatmapper software and used to generate a heatmap which clearly distinguished HC from RA patients (Figure 1). Analysis of the 40 genes using the Panther Gene Ontology tool showed that the most common molecular function classifications were catalytic activity (40%) and binding (30%). The most common biologic processes were cellular/process (28%), metabolic process (25%) and localization (12.5%).

In previous studies we have recognized that pro-inflammatory pathways (such as NF-kappaB) are activated and anti-inflammatory pathways (p53, lincRNA-p21) are repressed in cells from patients with RA and that patients who are treated with MTX show normalization of some of these gene sets [12, 22]. We also took into consideration, as others have noted, that changes in gene expression in patients are likely related to both disease activity and to effects of drug exposure [23]. Therefore, the patient data were examined in terms of both MTX treatment status and disease activity to look for changes reflecting patterns of expression that are more similar to those seen in HC. Genes were identified that were either significantly over-expressed or under-expressed in RA-active patients compared to the HC group. Significance in this context was defined as P<0.001 and Q<0.05, where Q corrects for false discovery rate

for multiple testing. A set of 379 genes, normalized to corresponding levels in HC, was significantly under-expressed in the RA active group and another set of 41 genes was significantly over-expressed in this group compared to the HC group. Analyses to compare expression levels of these sets of genes in the RA patients on the basis of whether disease was controlled or not controlled, regardless of MTX treatment status, identified three patterns (Figure 2). The first two patterns show gene expression levels in active RA patients that were either downregulated (Figure 2A) or upregulated (Figure 2B) compared to HC . In both of these gene sets, the group of RA patients with controlled disease in general showed levels of gene expression that were closer to those observed in HC. A third group of genes (Figure 2C) did not show any relationship with disease activity.

To address the effects of current treatment with MTX, further analyses were done comparing the dysregulated gene set in four groups of RA patients: (1) on MTX and active (+MTX active), (2) not on MTX and active (-MTX active), (3) on MTX and controlled (+MTX cont) and (4) not on MTX and controlled (-MTX cont) (Figure 3). In this comparison, the active RA patients shared similarities of dysregulated gene expression, regardless of whether MTX was currently being used or not. The active patients also had a greater representation of dysregulated genes, while patients with controlled disease exhibited gene expression levels that were closer to those in HC, regardless of MTX status.

Ontologic analysis of genes that were dysregulated RA patients was carried out for the 4 groups that were defined by disease activity and MTX status using Reactome Pathway Analysis. This revealed a gradient in the numbers of dysregulated genes in the 4 RA groups, related to both activity and MTX treatment (Figure 4). The RA groups with active disease showed a significant over-representation of pathways related to antigen presentation and signaling in interferon (IFN) pathways, including both alpha/beta and gamma subtypes. Active RA patients not on MTX had the highest expression levels for each of these pathways, followed by patients on MTX who had active disease, then by MTX-treated patients with controlled disease, and finally by patients who were not on MTX and had controlled disease.

Discussion

Over the past two decades, RA patients have benefitted from development of highly active therapies including biologic agents that block cytokines and cellular signaling pathways. The availability of such treatments along with MTX has resulted in high response rates, so that guideline recommendations are to aim for remission or sustained low disease activity [24]. Despite these advances, significant numbers of RA patients do not respond satisfactorily to available therapies. Delay in initiating early effective intervention has been identified as a major risk factor for a refractory disease course [19]. At least 30% of patients who are initially treated with MTX do not have adequate disease control and will need addition of another agent, usually a biologic DMARD [25]. If it were possible to predict at the outset which patients were likely to require more than MTX, this information could be used to justify addition of a second agent earlier in the disease course, which would improve the likelihood of a good clinical outcome [5, 18].

Tools that assist with monitoring responses to therapy and drive treat-to-target strategies are available for use in clinical practice. The commercially-available Vectra DA diagnostic, for example, incorporates 12 different serum markers into a single reported score that correlates with responses to MTX and other DMARDs [9]. It has been used to assess responses to therapy over time and can confirm maintenance of low activity states. Other measures that incorporate clinical assessments, such as the DAS-28 scores that include acute phase reactant blood tests as well as instruments such as the CDAI or RAPID3, which do not require any laboratory tests, also are useful in driving remission-inducing treatment strategies [8, 26].

However, correlation with clinical status and response to treatment is more straightforward than prediction of the response to an intervention using information obtained pre-treatment or very early in the course of therapy. Developing a risk profile for those RA patients who are likely to have a more difficult treatment course is especially needed to optimize use of available therapeutics [20]. Some elements of such a risk profile, including demographic and environmental variables, clinical measures and laboratory tests have been recognized. One model that was developed for prediction of inadequate response to MTX in the first year of disease included higher disease activity and current smoking as contributing factors [18]. It is also generally accepted that female sex predicts a course that is more refractory to treatment [19, 20]. Patients positive for the HLA-DRB1 shared epitope generally have more severe disease, and appear to also have a poorer response to MTX [20]. However, these and other identified predictors have only modest utility in an individual patient, and are not sufficiently robust to be used for a personalized approach to therapy. Levels of autoantibodies that are routinely measured in clinical practice (RF, CCP), have been shown to not be predictive of response to treatment [11, 20]. Other proposed serum biomarkers also have been shown not to be useful, including VCAM-1, which when measured at pre-treatment baseline did not add to clinical predictors of treatment response to MTX [27] and S100A9 which did not predict responses to etanercept [28].

We have previously shown that a large number of genes that are dysregulated in RA patients return toward levels seen in HC subjects after treatment with MTX [12]. Furthermore, in vitro responses to MTX in cell culture occur very rapidly [12], suggesting that such changes might be detected early in patients who initiate treatment with MTX. Analysis of the patients in the current study used the available data to distinguish between effects of disease activity and MTX treatment. While some similarities were observed in the disease activity groups regardless of MTX status, it was also found that patients who achieved low levels of disease activity with MTX have the greatest changes towards normal. Furthermore, patients in whom MTX treatment had not produced a low activity state showed greater dysregulation of the gene expression profile (Fig. 4). The data collected here are cross-sectional and not longitudinal, but are suggestive that a patient in whom these normalizing trends in gene expression were observed would be predictive of MTX responsiveness and this might be expected to occur early, prior to definitive changes in the clinical variables. A similar approach has been recently reported by others, showing that changes in whole blood gene expression profiles at 4 weeks were predictive of MTX nonresponsiveness [29]. The gene expression classifier in this study was found to be superior to models that were generated with clinical measures. The 4-week timepoint alone had very good predictive capability, while the pretreatment point alone had some limited value in predicting response. Since many patients are kept on MTX as

monotherapy for up to 6 months before considering adding therapies, this type of early test result might be utilized in an individual patient to make changes in therapy that would lead to better outcomes.

Pathway analyses in this same previous study highlighted genes involved in the response to Type I interferon and the Type I interferon signaling pathway as being relatively enriched in the nonresponders [29]. This result is similar to that reported in the current study, in which nonresponders to MTX showed higher levels of gene specificities in interferon-related pathways. The Type I IFN pathway and the signature of genes that are involved in IFN responses have been shown to be elevated in patients with RA, though it appears that both the alpha and beta IFN subtypes are represented, while in other autoimmune conditions such as systemic lupus erythematosus (SLE), the alpha type predominates [30]. In general, the Type I IFN signature in patients with RA was found not to be suppressed by MTX [31]. Of interest is the previously reported finding that the ratio of serum activity of the Type I IFNs beta/alpha was predictive of reponse to anti-TNF therapeutics [32]. Another study has reported that an elevated IFN gene expression signature in neutrophils from RA patients, including alpha, beta and gamma subtypes, was correlated with a good response to treatment with TNF inhibitors [33]. Involvement of IFN-gamma in enhancing invasiveness of synovial cells has been recently reported and thus expression of this cytokine might predict a more damaging course [34]. Taken together with these previous results, our findings suggest that the patients in whom IFN-related gene expression remains elevated may require treatment with TNFi or other agents agents such as JAK-STAT inhibitors to achieve remission [35].

The present study is limited by the cross-sectional rather than longitudinal nature of the data, and it is not known how rapidly changes toward normal gene expression patterns occur in patients who are started on MTX. If changes in the signature occur as early as 4 weeks of MTX treatment, as reported by others [29], the finding that gene patterns are not showing normalizing changes could be utilized to indicate likely MTX nonresponse status and accelerate the addition of a second agent to the treatment. If predictive signatures were shown to be present at the baseline, when MTX is initiated, those would be even stronger signals to be utilized in rapid acceleration of therapy in given individuals.

In addition to the cross-sectional design, another limitation of this study is that the dose and mode of MTX administration was not controlled, as treatment was carried out according to each rheumatologist's preference. In addition, the study population was small and predominantly non-Hispanic Caucasian; findings might differ in other racial or ethnic groups.

Conclusions

Gene expression levels measured in whole blood samples from RA patients are correlated with disease activity and treatment with MTX. Patients with clinical improvement generally show patterns that are closer to those of healthy controls. In other studies we have shown that in vitro changes in gene expression occur rapidly after exposure to MTX, and therefore early in vivo changes in treated patients is likely. A prospective study to determine whether such early changes would be of value in predicting MTX responsiveness will be of value to develop biomarkers for clinical practice.

Declarations

Ethics approval and consent to participate: This study was approved by the Pennsylvania State M.S. Hershey Medical Center Institutional Review Board and conducted under Good Clinical Practice guidelines and in concordance with the Helsinki declaration. All subjects provided written informed consent prior to participating.

Consent for publication: Not applicable.

Availability of data and materials: Deposition of RNA-seq data into GEO is complete. Accession number is GSE141529.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

RRJ, TMA and NJO conceived of the study design and analysis and wrote the manuscript, AN contributed to data analysis, DF coordinated patient recruitment and sample collection and YIK supervised collection of RNAseq data in the Genomics Core. All authors contributed to preparation of the manuscript and approved the final version for submission.

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Abbreviations

CPA – anti-citrullinated peptide antibodies

CCP – cyclic citrullinated peptide

CDAI – Clinical disease activity index

DAS – Disease activity score

DMARD – Disease-modifying anti-rheumatic drug

HC – healthy control

IFN – interferon

IRRA – Induction of Remission in Rheumatoid Arthritis (cohort)

MTX – methotrexate

RA – rheumatoid arthritis

RF – rheumatoid factor

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Tables

Table 1: Features of RA patients in Active and Controlled Groups

Feature	Active (N=16)	Controlled (N=16)	P value
Age (years)	56.55 (17.18)*	53.91 (12.46)	NS
Duration (years)	10.42 (10.35)	8.88 (6.40)	NS
Female (%)	15/16 (93.8)	10/16 (62.5)	0.08**
Current Smoker (%)	37.5	6.3	0.08
RF Units	160 (228)	104 (160)	NS
CCP Units	79 (111)	109 (112)	NS
ESR (mm/hr)	30.1 (23.6)	13.6 (13.5)	0.05
CRP (mg/L)	14.2 (8.2)	7.4 (2.7)	0.006
CDAI	31.67 (15.32)	2.71 (1.97)	0.001
DAS-ESR	5.29 (1.06)	1.80 (0.79)	4×10^{-9}
DAS-CRP	5.02 (0.85)	2.0 (0.28)	6.5×10^{-13}

*Values represent Mean (S.D.) or percent, as indicated.

** P Values calculated by t-test or Fisher's exact test.

Table 2: Features of RA patients in MTX treatment subsets

Feature	Active		Controlled	
	+ MTX	- MTX	+ MTX	- MTX
Age (years)	52.38 (18.61)	60.72 (15.68)	56.58 (13.23)	51.26 (11.88)
Duration (years)	7.73 (12.92)	13.12 (6.80)	5.33* (4.35)	12.43* (6.32)
Female (%)	7/8 (88)	2/8 (25)	4/8 (50)	6/8 (75)
Current Smoker (%)	4/8 (50)	2/8 (25)	0/8 (0)	1/8 (12)
RF Units	192.0 (201)	122.67 (164.53)	127.25 (173.32)	81.63 (153.69)
CCP Units	72.60 (121)	87.25 (110.64)	122.10 (116.32)	95.89 (114.32)
ESR (mm/hr)	24.8 (17.9)	34.1 (27.7)	15.8 (15.4)	8.0 (4.6)
CRP (mg/L)	15.1 (9.9)	13.31 (6.41)	6.64 (2.02)	8.17 (3.26)
CDAI	25.12 (7.97)	39.85 (19.51)	4.05 (1.06)	1.90 (2.10)
DAS-ESR	4.77 (0.56)	5.67 (1.20)	1.96 (0.84)	1.38 (0.51)
DAS-CRP	4.74 (0.69)	5.34 (0.95)	2.10 (0.24)	1.87 (0.29)

Each group includes 8 RA patients. See also Table 1 footnote.

All comparisons of +MTX/-MTX subsets within Active or Controlled groups were not significant except for disease duration in the controlled patients, * P = 0.020.

Figures

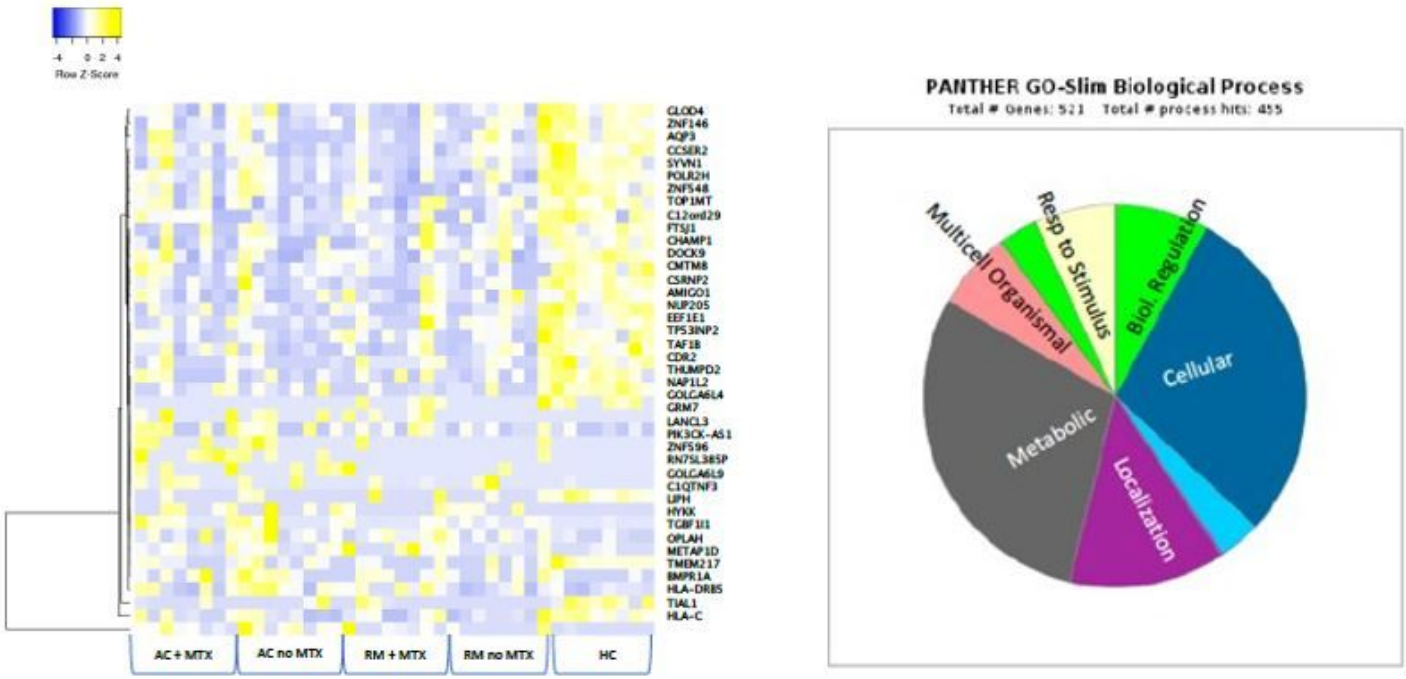

Figure 1

Heat map clustering of four RA groups and HC based on 40 gene specificities that were dysregulated in the group of 16 active RA patients vs HC (P<0.020). Heatmapper used to create clustering. The four RA groups are Active (AC) + MTX or AC no MTX, remission (RM) + MTX and RM no MTX. The 40 genes were also entered into the Panther Gene Ontology tool for molecular function classifications.

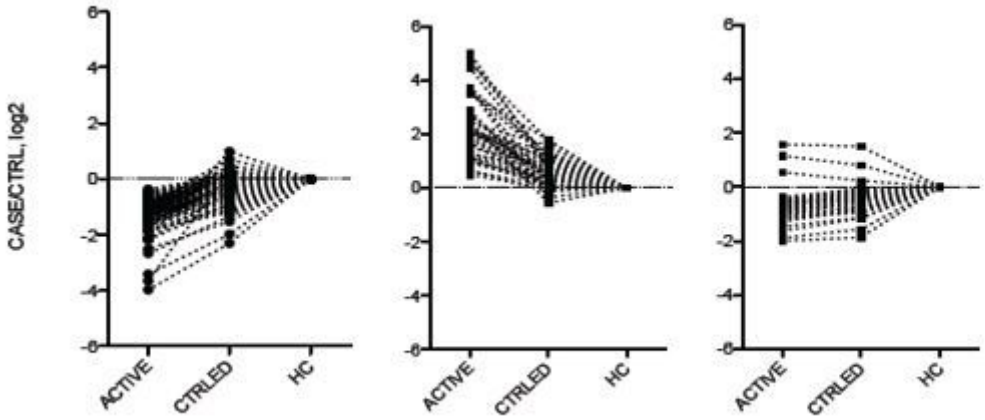

Figure 2

Cross-sectional analysis of whole blood sequencing data for RA patients with active or controlled disease as defined by disease activity scores, compared to healthy controls (HC). One group of genes is downregulated in active patients and closer to HC levels in controlled disease patients (left panel); another group of genes is upregulated and shows more normal levels in controlled patients (middle panel). The third group does not show a relationship to disease activity (right panel).

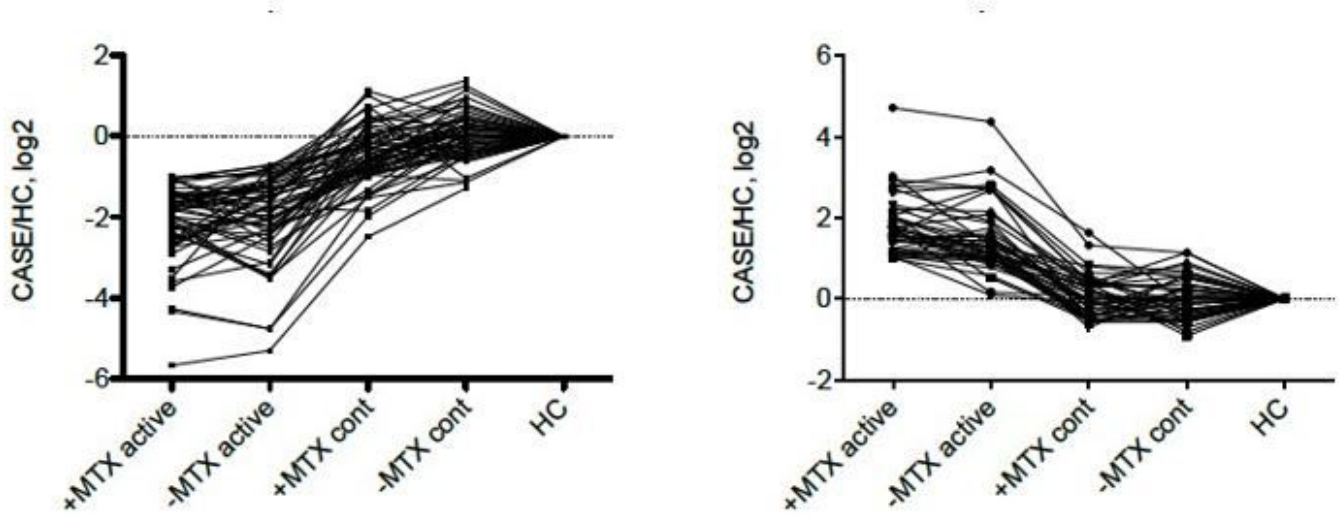

Figure 3

Cross-sectional analysis of whole blood sequencing data for RA patients with active or controlled disease and who are currently taking MTX (+MTX) or who are not currently taking MTX (-MTX). The RA groups are either down- or up-regulated when normalized to corresponding values for each gene in healthy controls (HC), The two active groups show similarities in expression profile, regardless of MTX treatment status. Similarly, patients who have controlled disease, with or without current MTX, show values that are closer to the HC profile.

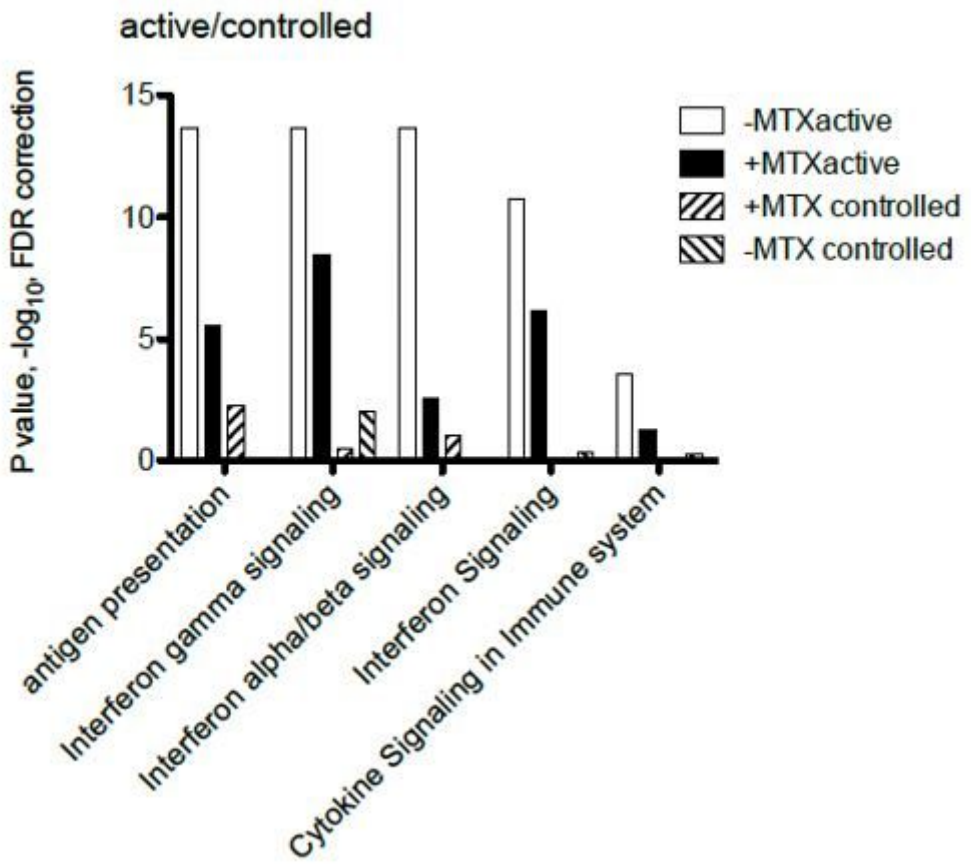

Figure 4

Ontologic analysis of genes that were dysregulated in four groups of RA patients defined by MTX use and disease activity. Highest levels of dysregulated genes were observed in active patients who were not currently taking MTX, followed by active patients who were taking MTX. The controlled groups had lower levels in all categories. Analysis was done using Reactome Pathway Analysis software (https://reactome.org/PathwayBrowser).