Article

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Cross-tissue Gene Expression Analysis of Methotrexate Response in Rheumatoid Arthritis

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Background: Methotrexate (MTX) is the main rheumatoid arthritis (RA) therapy. However, the molecular pathways directly modulated by MTX and transcriptomic features that distinguish clinical responders from non-responders remain unclear. This study aimed to define MTX-associated transcriptional programs and baseline synovial gene expression pathways associated with a clinical response to MTX-treated RA through cross-tissue transcriptomic integration.
Methods: Peripheral blood gene expression profiles collected before and after MTX treatment were obtained from the Gene Expression Omnibus (GSE35455) database. Differential gene expression analysis was used to identify upregulated and downregulated genes, and functional enrichment analyses were conducted for each gene set. Clinical response–associated transcriptional signatures were independently evaluated on a dataset of baseline synovial tissue from MTX-treated patients with RA (GSE45867). Gene set enrichment analysis was used to identify pathways associated with the MTX response.
Results: In the GSE35455 dataset, MTX treatment was associated with significant differential expression of 730 genes, which were predominantly downregulated. MTX-downregulated genes showed limited enrichment in classical Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathways but demonstrated significant suppression of inflammatory signaling modules, including TNF-α signaling via NF-κB. In contrast, MTX-upregulated genes were enriched for metabolic stress responses, including Hypoxia and Glycolysis pathways, and innate immune response modules. Disease and phenotype enrichment analyses of the upregulated genes highlighted immune-related disease annotations and lymphoid tissue abnormalities. In synovial tissue (GSE45867), MTX responders exhibited strong baseline enrichment of interferon-stimulated gene programs, cytokine-regulated JAK-STAT signaling pathways, and immune activation signatures.
Conclusions: MTX induces broad immunomodulatory transcriptional effects characterized by the suppression of inflammatory signaling networks, and baseline activation of interferon- and cytokine-responsive programs in the synovial tissue. This cross-tissue integration provides mechanistic insights into MTX response heterogeneity in RA.

Keywords: gene set enrichment analysis; interferon response; methotrexate; rheumatoid arthritis; transcriptomics

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation, progressive joint destruction, and systemic immune dysregulation. Despite substantial advances in targeted biological and small-molecule therapies, methotrexate (MTX) remains a first-line RA treatment in international treatment guidelines [1,2]. MTX is effective in a substantial proportion of patients, improves long-term clinical outcomes, and enhances the efficacy of biological therapies. However, only 30–40% of patients achieve an adequate clinical response to MTX monotherapy, underscoring the marked inter-individual variability in treatment outcomes [3].

Although MTX has been used to treat RA for several decades, its precise molecular mechanism of action remains incompletely defined. Methotrexate exerts pleiotropic immunomodulatory effects, including purine and pyrimidine metabolism inhibition, extracellular adenosine accumulation, pro-inflammatory cytokine production suppression, and T cell and macrophage function modulation [4]. However, downstream transcriptional programs induced by MTX *in vivo*, particularly in human immune cells, remain poorly characterized. Moreover, the molecular features that distinguish MTX responders from non-responders at baseline are not well understood, which limits the development of predictive biomarkers and personalized treatment strategies.

High-throughput transcriptomic profiling has emerged as a powerful approach for investigating drug mechanisms and identifying molecular signatures associated with therapeutic responses in RA [5,6]. Gene expression changes following MTX treatment in the peripheral blood or synovial tissue were previously established; however, many analyses have focused on single tissues, limited gene sets, or candidate pathways, and yielded inconsistent results [7]. RA is a systemic disease in which

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peripheral immune alterations and local synovial pathologies are closely interconnected. Integrative analyses that bridge systemic- and tissue-level transcriptional responses may provide a more comprehensive understanding of MTX action and response heterogeneity.

Gene set–based and network-level analytical approaches, such as functional enrichment analysis and gene set enrichment analysis (GSEA), enable the identification of coordinated biological pathways that may not be apparent in individual gene-level analyses [8,9]. These approaches are particularly well-suited for studying complex immunological drugs, such as MTX, the effects of which are distributed across interconnected signaling networks rather than isolated molecular targets. However, cross-tissue transcriptomic studies integrating MTX-induced changes in the peripheral blood with baseline synovial gene expression predictors of clinical responses are limited.

The aim of the present study was to define the transcriptional programs associated with MTX treatment and clinical response in RA through integrative cross-tissue transcriptomic analysis. Specifically, we sought to: (i) characterize gene expression changes induced by MTX treatment in peripheral blood; (ii) identify biological pathways and network modules enriched among MTX-regulated genes; and (iii) determine baseline synovial tissue gene expression pathways associated with the clinical response to MTX therapy. By integrating systemic and tissue-specific transcriptomic data using functional enrichment and gene set–based approaches, this study aims to provide mechanistic insight into MTX action and identify molecular signatures that may inform the development of candidate molecular signatures for future biomarker-guided therapeutic strategies for RA.

Materials and Methods

Study Datasets and Design

Integrative transcriptomic analysis was conducted to characterize the molecular signatures associated with MTX treatment and clinical response in RA. Gene expression data were obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/geo2r/). MTX-associated transcriptional changes were analyzed using the GSE35455 dataset, which comprises paired peripheral blood gene expression profiles from 58 MTX-treated patients with RA, with samples collected before and after treatment. This paired study design enabled the direct assessment of transcriptional programs modulated by MTX exposure.

To independently evaluate the transcriptional signatures associated with clinical response, baseline synovial tissue gene expression profiles were analyzed using the GSE45867 dataset [10]. This dataset comprised paired synovial biopsy samples obtained from the affected knees of patients with early RA before and 12 weeks after therapy initiation. The patients received either tocilizumab (n = 12) or MTX (n = 8). Only the MTX-treated subgroup (n = 8) was considered. The clinical response was assessed according to the European League Against Rheumatism (EULAR) DAS28-CRP criteria, with a good response defined as a change in DAS28 $\geq$1.2 between the baseline and week 12 [2].

Differential Expression and Functional Enrichment Analysis

Differential gene expression analysis for the GSE35455 dataset was performed using GEO2R, an online analysis tool [11]. Paired comparisons were conducted between pre- and post-MTX treatment samples. Genes represented by multiple microarray probes were summarized by selecting the probe with the mean adjusted *p* value for downstream analyses, whereas probes annotated to multiple genes were assigned to the primary gene symbol. Genes with an absolute $\log_2$ fold change $\geq$0.5 and an adjusted p value < 0.05 were considered differentially expressed. Adjusted *p* values were calculated to control for multiple testing using the false discovery rate (FDR) approach. Identified differentially expressed genes (DEGs) were stratified into upregulated and downregulated gene sets for subsequent direction-specific analyses [8].

To interpret the biological relevance of MTX-associated transcriptional changes, functional enrichment analyses were performed separately for upregulated and downregulated DEGs. Enrichment analyses were conducted within a network pharmacology framework using Enrichr (https://maayanlab.cloud/Enrichr/) [12], with multiple complementary knowledge bases, including Gene Ontology (GO) biological process annotations, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and curated gene set collections, such as the MSigDB Hallmark gene sets. The statistical significance of the enriched terms was assessed using adjusted *p* values to control for multiple tests.

Gene Set Enrichment Analysis of Synovial Tissue Transcriptomes

In dataset GSE45867, GSEA was performed on baseline synovial tissue transcriptomes using R (version 4.5.2; R Foundation for Statistical Computing, Vienna, Austria). Differential expression analysis comparing MTX responders and non-responders was conducted using the limma package (version 3.66.0; Bioconductor, Melbourne, Victoria, Australia; https://bioconductor.org/packages/limma/), and genes were ranked based on signed moderated *t*-statistics [13]. Pre-ranked GSEA was performed using the fgsea package (version 1.36.0; Bioconductor, Melbourne, Victoria, Australia; https://bioconductor.org/packages/fgsea/) to assess the coordinated enrichment of predefined gene sets from the MSigDB Hallmark collection (Subramanian

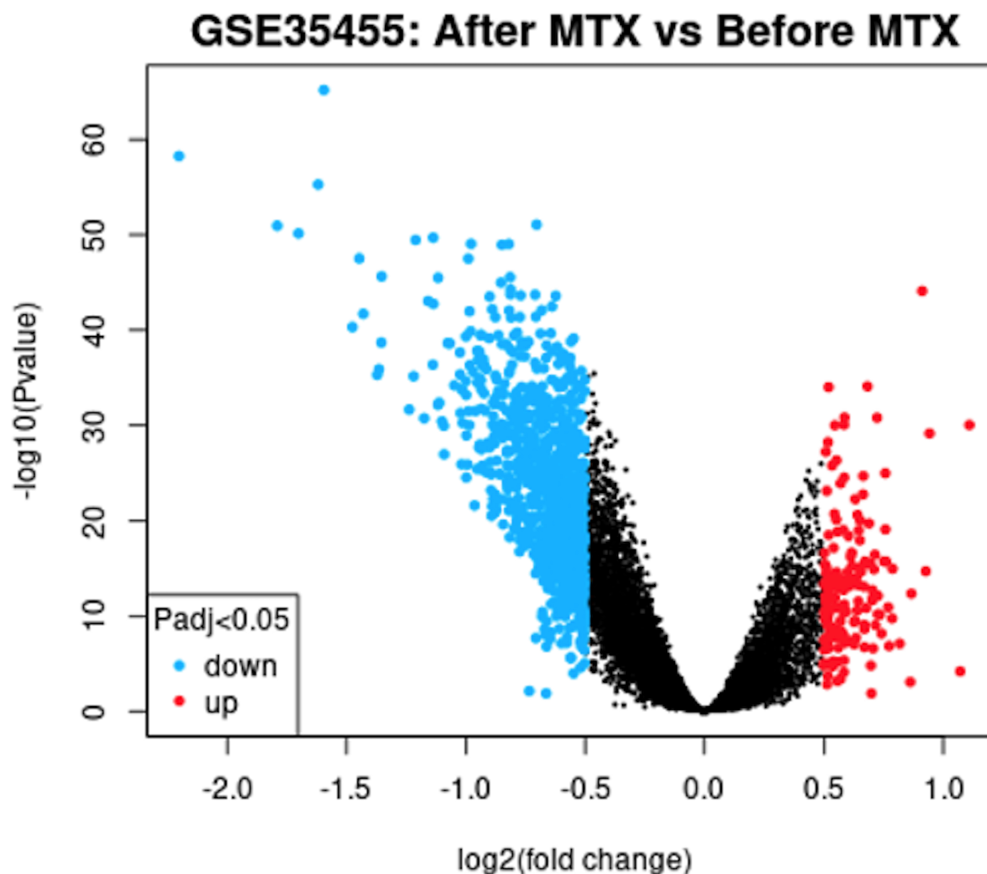

Fig. 1. Differential gene expression following methotrexate treatment in GSE35455. Volcano plot of differential gene expression comparing samples collected after methotrexate treatment with samples collected before treatment in dataset GSE35455. The x-axis indicates $\log_2$ fold change, and the y-axis indicates $-\log_{10}(p$ value). Genes meeting the significance threshold after multiple testing correction (adjusted $p < 0.05$) are highlighted, with downregulated genes shown in blue and upregulated genes shown in red. Genes not meeting significance criteria are shown in black.

et al. [9], 2005). Enrichment scores were normalized to account for differences in gene set size, yielding normalized enrichment scores (NES). Statistical significance was determined using permutation-based testing, and multiple hypothesis testing was controlled using the Benjamini–Hochberg FDR procedure. Pathways with FDR-adjusted p values < 0.05 were considered statistically significant. Enrichment plots and heatmaps were generated in R using the ggplot2 (version 4.0.1; CRAN, Vienna, Austria; https://cran.r-project.org/package=ggplot2) and pheatmap (version 1.0.13; CRAN, Vienna, Austria; https://cran.r-project.org/package=pheatmap) packages.

Results

Transcriptional Pathways Modulated by MTX Treatment in the GSE35455 Dataset

To characterize the transcriptional programs directly modulated by MTX treatment, the GSE35455 dataset, which captures gene expression changes following MTX exposure, was analyzed. After quality control and filtering, differential expression analysis identified 730 genes that were significantly altered by the treatment, including 106 upregulated and 624 downregulated genes (Fig. 1, **Supplementary Table 1**).

Enrichment of MTX-downregulated Genes

GO biological process enrichment analysis of the 624 MTX-downregulated genes identified a limited number of significantly enriched terms after correction for multiple testing. This enrichment analysis demonstrated the significant (adjusted $p = 0.0226$) negative regulation of the receptor signaling pathway via Janus kinase–signal transducer and activator of transcription (JAK–STAT) (GO:0046426), indicating cytokine-mediated signaling following MTX treatment. The remaining enriched GO biological processes did not retain significance after adjustment. The top 10 GO terms are shown in **Supplementary Table 2**.

Hallmark Pathway Enrichment of Downregulated Genes

Hallmark gene set enrichment analysis of MTX-downregulated genes revealed the significant suppression

Table 1. Enriched MSigDB Hallmark pathways among 624 methotrexatedownregulated genes in GSE35455.

Term	Adjusted *p*-value	Odds ratio	Genes
TNFα Signaling via NF-κB	0.0028	3.1325	*PPP1R15A*; *CXCL6*; *DUSP2*; *CEBPB*; *PFKFB3*; *ATP2B1*; *F3*; *SLC2A6*; *ICAM1*; *NR4A2*; *SOCS3*; *KLF6*; *NR4A3*; *TNIP1*; *TNIP2*; *BCL3*; *IL12B*; *FJX1*
Estrogen Response Late	0.0135	2.7449	*UNC13B*; *DUSP2*; *PDZK1*; *ASCL1*; *PRLR*; *OLFM1*; *SLC9A3R1*; *GALE*; *IMPA2*; *CDH1*; *PERP*; *PLXNB1*; *CKB*; *S100A9*; *PTGES*; *LTF*
Apical Junction	0.0253	2.555	*COL17A1*; *CDSN*; *NEXN*; *KRT31*; *GTF2F1*; *ICAM1*; *CLDN6*; *CLDN11*; *TRO*; *ADAM15*; *CDH1*; *CERCAM*; *SPEG*; *NF2*; *PFN1*

Table 2. Enriched MSigDB Hallmark pathways among 106 methotrexate-upregulated genes in GSE35455.

Term	Adjusted *p*-value	Odds ratio	Genes
Hypoxia	0.0028	7.2176	*HSPA5*; *CITED2*; *PDK3*; *CXCR4*; *IRS2*; *PYGM*; *GAPDH*
Glycolysis	0.0100	6.0928	*TPST1*; *HSPA5*; *CITED2*; *PDK3*; *CXCR4*; *IRS2*

of Tumor Necrosis Factor α (TNFα) signaling via Nuclear Factor (NF) κB (adjusted p = 0.0028), highlighting the modulation of a central inflammatory pathway by MTX (Table 1). Additional enrichment was observed for the Estrogen Response Late and Apical Junction gene sets, suggesting broader transcriptional and structural remodeling following treatment.

Enrichment of MTX-upregulated Genes

Analysis of the 106 MTX-upregulated genes revealed the enrichment of a distinct set of pathways. Hallmark gene set enrichment analysis identified significant enrichment of hypoxia (adjusted p = 0.0028) and glycolysis-related (adjusted p = 0.010) genes, indicating the activation of metabolic and stress response programs following MTX exposure (Table 2). The leading edge genes contributing to these pathways include *HSPA5*, *CITED2*, *PDK3*, *CXCR4*, and *IRS2*.

KEGG pathway analysis of the upregulated genes identified enrichment of pathways annotated as *Salmonella* infection (including *CYFIP2*, *TUBA1B*, *HSP90AA1*, *TLR5*, *GAPDH*, *ACTB*, and *STX10*) and pathogenic *Escherichia coli* infection (including *CYFIP2*, *CLDN10*, *TUBA1B*, *TLR5*, *GAPDH*, and *ACTB*; adjusted p = 0.0472; Table 3). These pathways likely reflect the activation of innate immunity and stress response modules rather than true infectious processes, which are common features of inflammatory transcriptional signatures.

Disease and Phenotype Associations of MTX-upregulated Genes

Disease association analysis of MTX-upregulated genes revealed significant enrichment of immune-related disease annotations, including lymphoma, immune system cancer, non-Hodgkin's lymphoma, and lymphatic system cancer (adjusted p = 0.0372; odds ratio = 54.6346), driven primarily by *CXCR4* and *ACTB*. Consistently, Mammalian Phenotype enrichment analysis identified abnormal lymph node B-cell domain morphology (MP:0002344; adjusted p = 0.0370; odds ratio = 57.9145) involving *PTEN*, *CXCR4*, and *TNFSF13B*. Several disease/phenotype terms were annotated to identical leading genes and therefore produce identical enrichment statistics; the full enrichment output is provided in **Supplementary Table 3**.

Synovial Transcriptional Pathways Associated With the MTX Response

GSEA was performed on baseline synovial tissue transcriptomes from the GSE45867 dataset to identify Hallmark pathways associated with the MTX response. Across the Hallmark gene set collection, a restricted subset of pathways showed significant enrichment in MTX responders compared with that in non-responders (Table 4; **Supplementary Tables 4,5**).

The highest enrichment was observed for the interferon response pathways. The interferon gamma response pathway showed the strongest enrichment (NES = 2.61, adjusted p = 1.29×10^{-21}), followed by the interferon alpha response pathway (NES = 2.46, adjusted p = 1.31×10^{-11}). The leading-edge subsets for these pathways comprised multiple interferon-stimulated genes, including *CXCL9*, *STAT1*, *JAK2*, *IRF1*, *GBP1*, *IFIT3*, *ISG15*, *OAS1*, and *MX1* (Table 4). A heatmap demonstrated coordinated upregulation of interferon-stimulated genes in MTX responders at the baseline (Fig. 2A).

Several cytokine-regulated signaling pathways were also significantly enriched. The interleukin-6 (IL 6)–JAK–STAT3 (NES = 2.28, adjusted p = 1.60×10^{-8}) and IL 2–STAT5 signaling pathways were enriched (NES = 2.02, adjusted p = 6.67×10^{-9}). Enrichment of TNFα signaling via

Table 3. Enriched KEGG pathways among 106 methotrexate-upregulated genes in GSE35455.

Term	Adjusted *p*-value	Odds ratio	Genes
Salmonella infection	0.0472	5.7419	*CYFIP2*; *TUBA1B*; *HSP90AA1*; *TLR5*; *GAPDH*; *ACTB*; *STX10*
Pathogenic Escherichia coli infection	0.0472	6.1894	*CYFIP2*; *CLDN10*; *TUBA1B*; *TLR5*; *GAPDH*; *ACTB*

Table 4. Hallmark pathways are significantly enriched in the baseline synovial tissue of methotrexate responders (GSE45867).

Pathway (MSigDB Hallmark)	NES	Adjusted *p* value	Gene set size	Representative leading-edge genes
Hallmark Interferon Gamma Response	2.61	1.29×10^{-21}	200	*CXCL9*, *STAT1*, *JAK2*, *IRF1*, *GBP1*
Hallmark Interferon Alpha Response	2.46	1.31×10^{-11}	200	*IFIT3*, *ISG15*, *OAS1*, *STAT1*, *MX1*
Hallmark Allograft Rejection	2.30	3.93×10^{-14}	200	*CXCL9*, *FAS*, *JAK2*, *STAT1*, *PTPRC*
Hallmark IL6 JAK STAT3 Signaling	2.28	1.60×10^{-8}	87	*STAT3*, *SOCS3*, *IL6R*, *CEBPD*
Hallmark IL2 STAT5 Signaling	2.02	6.67×10^{-9}	200	*IL2RA*, *STAT5A*, *PTPRC*, *CDK6*
Hallmark TNFα Signaling Via NF-κB	1.56	0.0011	200	*TNFAIP8*, *CD274*, *SKAP2*, *B4GALT1*
Hallmark Epithelial-Mesenchymal Transition	1.76	1.19×10^{-5}	200	*CTNNA1*, *ADAM9*, *VCAN*, *SPP1*

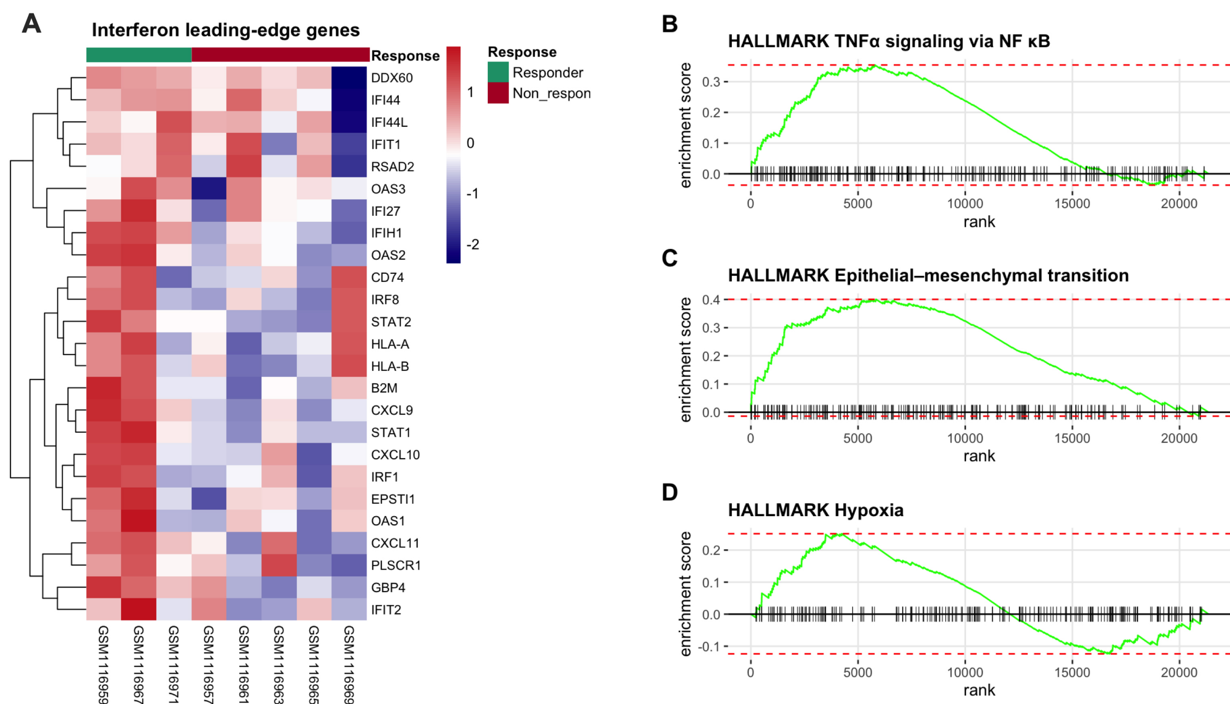

Fig. 2. Synovial transcriptional pathways associated with methotrexate response. Gene set enrichment analysis (GSEA) of baseline synovial tissue transcriptomes from patients with rheumatoid arthritis treated with methotrexate (GSE45867). Hallmark pathways were ranked by normalized enrichment score (NES) using a preranked gene list. (A) Heatmap of representative leading-edge genes from the interferon gamma response pathway identified by GSEA. Expression values were z–score–scaled across samples for each gene. Columns represent individual baseline synovial samples, and rows represent interferon-responsive genes contributing to pathway enrichment. Color intensity indicates relative gene expression (red, higher; blue, lower). (B) Enrichment plot for the TNF-α signaling via the NFκB pathway. (C) Enrichment plot for the Epithelial–mesenchymal transition pathway. (D) Enrichment plot for the Hypoxia pathway. Tick marks indicate positions of pathway genes in the ranked list; the green line represents the running enrichment score. NES and adjusted *p* values are indicated for each pathway.

NF κB was also observed (NES = 1.56, adjusted $p = 0.0011$; Table 4). The leading edge genes contributing to these pathways included *STAT3*, *SOCS3*, *IL6R*, *CEBPD*, *IL2RA*, *STAT5A*, *PTPRC*, *CDK6*, *TNFAIP8*, *CD274*, *SKAP2*, and *B4GALT1*. The enrichment profile for TNFα signaling via NF κB is shown in Fig. 2B.

The allograft rejection pathway was among the most significantly enriched pathways (NES = 2.30, adjusted $p = 3.93 \times 10^{-14}$), with leading edge genes including *CXCL9*, *FAS*, *JAK2*, *STAT1*, and *PTPRC* (Table 4). The complete gene lists for each significantly enriched pathway are provided in **Supplementary Table 5**.

Epithelial-mesenchymal transition also showed significant enrichment (NES = 1.76, adjusted p = 1.19 $\times$ 10^{-5}), with leading edge genes including *CTNNA1*, *ADAM9*, *VCAN*, and *SPP1* (Table 4, Fig. 2C). In contrast, the hypoxia pathway did not show significant enrichment (NES = 1.105, adjusted $p = 3.84 \times 10^{-1}$) and its enrichment curve lacked a concentrated leading edge subset (Fig. 2D, **Supplementary Table 4**). Other Hallmark pathways were not statistically significant after adjusting for multiple tests (**Supplementary Table 4**).

Only a limited number of pathways showed negative NESs, indicating relative enrichment in MTX non-responders; these pathways are listed in **Supplementary Table 4**.

Discussion

This study aimed to elucidate the transcriptional programs associated with MTX treatment and identify baseline synovial gene expression pathways associated with the clinical response in RA through integrative cross-tissue transcriptomic analysis. By combining MTX-induced gene expression changes in peripheral blood with baseline synovial tissue signatures associated with treatment response, this study aimed to address persistent gaps in our understanding of the molecular mechanisms underlying MTX efficacy and interindividual variability in therapeutic outcomes. A key strength of this study is its cross-tissue integrative design, which links MTX-induced systemic transcriptional changes in peripheral blood with baseline molecular states in synovial tissue associated with treatment response.

Analysis of paired peripheral blood transcriptomes revealed that MTX treatment was associated with widespread transcriptional remodeling, with a marked predominance of downregulated genes. Notably, the MTX-downregulated genes demonstrated limited enrichment in the classical GO and KEGG pathways after correction for multiple testing. This finding is consistent with previous studies suggesting that MTX does not act through the selective inhibition of discrete molecular pathways, but rather exerts diffuse, network-level immunomodulatory effects [4,14]. Despite the limited enrichment observed using conventional pathway databases, curated gene set analysis revealed significant suppression of TNF-α signaling via NF-κB, highlighting modulation of a central inflammatory axis implicated in RA pathogenesis. This observation aligns with established evidence that MTX indirectly attenuates pro-inflammatory cytokine signaling through mechanisms such as extracellular adenosine accumulation and downstream inhibition of NF-κB activity [4,15].

The inconsistency between limited GO/KEGG enrichment and clear suppression of curated inflammatory signaling modules underscores the importance of network- and gene set–based analytical approaches when investigating the molecular effects of complex immunomodulatory drugs. Similar patterns have been reported in transcriptomic studies of disease-modifying antirheumatic drugs, where pathway-level effects are more readily captured using curated or regulatory gene sets than ontology-based annotations [6,16,17]. These findings reinforce the concept that MTX primarily acts through the coordinated perturbation of inflammatory signaling networks rather than by targeting isolated molecular pathways.

In contrast to MTX-downregulated genes, MTX-upregulated genes exhibited clear enrichment of metabolic and cellular stress response programs, including the Hallmark Hypoxia and Glycolysis pathways. These results suggested that MTX induces adaptive metabolic reprogramming in circulating immune cells, potentially reflecting responses to folate pathway inhibition, altered mitochondrial function, and bioenergetic stress. Immunometabolic pathways play a central role in RA pathogenesis and treatment response, with activated immune cells exhibiting enhanced glycolytic flux and metabolic plasticity [18,19]. Recent multi-omics studies have established metabolic dysregulation as a core feature of RA pathogenesis [20], and the present findings extend this framework by demonstrating that MTX actively induces metabolic and stress response programs in circulating immune cells, rather than merely reflecting preexisting disease-associated metabolic states.

KEGG pathway enrichment of MTX-upregulated genes identified pathways annotated as bacterial infection, which likely reflect activation of innate immune and cytoskeletal stress modules rather than infectious processes. These annotations are commonly observed in inflammatory transcriptomic datasets, and shared components of host defense, cytoskeletal remodeling, and stress-response pathways [20,21]. Together, these findings indicate that MTX treatment induces a coordinated shift in immune cell metabolic and stress response states, and concurrently suppresses central inflammatory signaling networks.

A major contribution of this study was the identification of baseline synovial tissue transcriptional signatures associated with the clinical response to MTX. GSEA of baseline synovial transcriptomes revealed strong enrichment of interferon-stimulated gene programs, including interferon gamma and interferon alpha responses, in MTX responders compared with those in non-responders. These findings are consistent with emerging evidence that heightened baseline immune activation, particularly involving interferon signaling, may help identify patients who are more likely to respond to immunomodulatory therapies [6,7]. Notably, Plant *et al.* [7] identified interferon-enriched transcriptional modules in the peripheral blood associated with MTX non-response; however, the present analysis demonstrated that baseline synovial interferon activation is associated with a favorable response, underscoring the tissue-specific nature of interferon signaling and highlighting the added value of cross-tissue transcriptomic integration. Interferon-driven transcriptional states have been associated with the treat-

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ment response in RA and other autoimmune diseases, potentially reflecting an inflammatory milieu that is more amenable to pharmacologic suppression [22].

In addition to interferon signaling, responders exhibited enrichment of cytokine-regulated JAK–STAT pathways, including IL-6–JAK–STAT3 and IL-2–STAT5 signaling. These pathways are central to RA pathophysiology and immune cell differentiation [23], and their enrichment suggests that MTX may be particularly effective in patients whose synovial inflammation is driven by cytokine-dependent signaling networks. The concurrent enrichment of TNF-α signaling via NF-κB at baseline in responders further supports the notion that MTX efficacy is linked to the presence of active inflammatory signaling circuits that can be effectively modulated by treatment. Notably, hypoxia signaling was not enriched in the baseline synovial tissue of responders, despite being induced following MTX treatment in the peripheral blood, highlighting tissue-specific differences in transcriptional programs and emphasizing the value of cross-tissue analyses.

These findings have important clinical implications. Identification of baseline synovial interferon and cytokine signaling signatures associated with MTX response may inform the development of candidate molecular signatures, enable earlier optimization of treatment strategies, and reduce delays in achieving disease control. Furthermore, the immunometabolic adaptations induced by MTX highlight potential opportunities for therapeutic optimization by targeting metabolic pathways. Advances in precision medicine and molecular profiling have increasingly supported the feasibility of integrating these biomarkers into RA management [3,5].

This study had several limitations. The synovial tissue analysis was based on a relatively small number of MTX-treated patients, reflecting the limited availability of paired synovial biopsy datasets. Accordingly, these synovial tissue findings should be regarded as hypotheses and require validation in larger, independent cohorts. Transcriptomic analyses were conducted using bulk microarray data, which did not resolve cell-type-specific responses or post-transcriptional regulation. Additionally, the observational nature of the datasets precludes direct causal inferences regarding MTX mechanisms. Future studies should involve larger independent cohorts and integrate single-cell or spatial transcriptomic approaches to elucidate cell-specific responses. The integration of transcriptomic data with proteomic, metabolomic, and clinical variables may further refine the candidate molecular signatures associated with MTX responses. Ultimately, prospective studies that incorporate molecular stratification at treatment initiation are required to translate these insights into clinically actionable decision-support tools.

Conclusions

Collectively, these findings provide new insight into MTX mechanisms and response heterogeneity in RA. By integrating systemic transcriptional changes induced by MTX with baseline synovial gene expression patterns associated with treatment response, this study moves beyond prior single-tissue analyses and offers a more comprehensive framework for understanding the action of MTX. MTX suppresses central inflammatory signaling networks while inducing metabolic and stress-response programs, and baseline activation of interferon- and cytokine-driven pathways in synovial tissue is associated with a favorable clinical response. These observations support the concept of molecularly stratified treatment strategies and suggest that transcriptomic profiling may help identify patients who are most likely to benefit from MTX therapy.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article.

Author Contributions

RM designed the study, performed the research, analyzed the data, and wrote the manuscript. RM has read and approved the final manuscript, participated sufficiently in the work, and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The author declares no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202638207.87.

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