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Comparative Expression of *ADAMTS2*, *CA1*, and *OLAH* in Immune Cells of Rheumatoid Arthritis Patients: Real-time PCR Study on *In-silico* analysis of Treated *vs.* Newly Diagnosed Patients

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Abstract

Background: Rheumatoid Arthritis (RA) is a chronic inflammatory joint disease. Current treatments often have limited efficacy and cause side effects due to their nonspecific action, while early diagnosis is challenging. This study combined bioinformatics and experimental methods to identify key genes and pathways involved in RA, aiming to discover novel therapeutic targets and diagnostic biomarkers.

Methods: RNA-seq data from immune cells of RA patients and healthy donors (GSE117769) were analyzed with DESeq to identify Differentially Expressed Genes (DEGs). Affected pathways were explored using EnrichR, and druggable genes were identified through DGIdb and a literature review. Expression of candidate genes was validated in additional RA blood and synovium microarray datasets (GSE45291, GSE82107, GSE77298) using the GEO2R tool. Finally, RT-qPCR was used to measure the expression of selected genes in Peripheral blood Mononuclear Cells (PBMCs) from newly-diagnosed and chronic RA patients and controls, with associations to clinical features and diagnostic accuracy assessed. Synovial fluid of RA patients were stained with Giemsa.

Results: Combined *in-silico* and experimental analysis demonstrated significant upregulation of *CA1*, *OLAH*, and *ADAMTS2* in the PBMCs of RA patients. However, only *ADAMTS2* showed high expression in the synovial tissue of these patients. While *OLAH* and *ADAMTS2* were predominantly overexpressed in newly-diagnosed cases, *CA1* levels were consistently elevated in both early and chronic stages of RA.

Conclusion: This study identified *CA1*, *OLAH*, and *ADAMTS2* as being upregulated in RA, with *ADAMTS2* showing promise as a therapeutic target, suggesting it may also have potential as a candidate for diagnosis and treatment.

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Introduction

Prolonged inflammation of the synovial joints is a hallmark of Rheumatoid Arthritis (RA), a chronic, progressive autoimmune disease characterized by persistent joint inflammation and swelling. Between 0.5 and

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1% of the global population is affected by this disease [1]. This illness is characterized by joint tenderness, pain, and swelling [1], which hinders diarthrodial joints' normal function and leads to severe physical impairment, mental anguish, and financial hardship [2,3]. The effective management of RA requires a comprehensive, multidisciplinary approach that focuses on symptom control, joint preservation, and improving overall patient quality of life. The primary pharmacological strategy involves the administration of Disease-Modifying Antirheumatic Drugs (DMARDs), often in combination with adjunctive therapies. DMARDs function to attenuate disease progression and maintain joint integrity and function. These agents are broadly categorized into two main classes: nonbiologic DMARDs (*e.g.*, methotrexate, sulfasalazine) and biologic DMARDs (*e.g.*, tumor necrosis factor inhibitors, interleukin-6 receptor antagonists). Adjunctive pharmacological agents, employed as supportive therapies, commonly include corticosteroids, Nonsteroidal Anti-Inflammatory Drugs (NSAIDs), immunosuppressive agents, and analgesics. Although these therapeutic interventions are widely utilized, they may demonstrate limited efficacy in specific patient subsets. Furthermore, prolonged use is associated with clinically significant adverse effects, including but not limited to hypertension, dyslipidemia, and insulin resistance [4-6].

The discovery of new gene networks and molecular pathways associated with the etiology of RA holds significant potential for the development of targeted treatment approaches. Recent developments in high-throughput technologies, specifically RNA sequencing (RNA-seq) and microarray analysis, have significantly improved functional genomics research and gene discovery. The removal of probe-based detection limitations, increased sensitivity, a wider dynamic range, and the ability to detect transcripts that have not yet been annotated are just a few of the benefits that RNA-seq has over microarrays, in particular. The availability of transcriptomic data generated by these techniques is increasing, thanks to open repositories such as the Gene Expression Omnibus (GEO). These datasets, when paired with advanced bioinformatics techniques, allow for extensive, integrative analyses that allow for the methodical discovery of new biomarkers and possible treatment targets for RA and other multifactorial [7].

The goal of this study was to identify differentially expressed genes associated with the pathophysiology of RA to identify potential therapeutic genes for the disease by analyzing RNA sequencing data from leukocyte samples downloaded from the GEO database. By conducting comprehensive bioinformatics analysis and verifying *ADAMTS2*, *CA1*, and *OLAH* genes using Real-Time PCR in samples from people with early-stage and chronic RA, the study seeks to discover new biomarkers and molecular targets.

Carbonic anhydrase 1 (CA1) is a zinc metalloenzyme belonging to a large family of carbonic anhydrase isoforms, which catalyze the reversible hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) and protons (H^+) [8]. CA1, may influence the migration of Fibroblast-Like Synoviocytes (FLS) within inflamed joints in RA, thus contributing to disease progression [9]. Supporting this, transgenic mouse models overexpressing *CA* genes exhibit exacerbated RA symptoms and enhanced joint destruction [10].

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) is a family of multidomain extracellular protease enzymes. It has been shown that the inflammatory cytokine IL-1α—known to be pivotal in cartilage and bone homeostasis and pathology—can upregulate *ADAMTS-2* and *ADAMTS-3* expression in osteoblast-like cell lines. This raises the possibility that pro-inflammatory cytokines such as IL-1α may similarly induce *ADAMTS2* expression in RA, contributing to joint degradation [11].

Oleoyl-ACP hydrolase (OLAH) has critical role in fatty acid biosynthesis. Fatty acids are increasingly recognized for their modulatory effects on immune responses, influencing inflammation in diverse ways. For example, short-chain fatty acids have been shown to suppress the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, thereby exerting anti-inflammatory effects. In contrast, medium-chain fatty acids can promote inflammation through activation of Toll-Like Receptor 2 (TLR2) [12].

Materials and Methods

Identification of druggable targets in RA via integrated bioinformatics

Following a comprehensive analysis of the GEO database, dataset GSE117769 was selected, which includes RNA-seq data from white blood cells of 50 RA patients and 50 matched healthy controls, balanced for age, gender, and ethnicity. The dataset encompasses expression profiles of 36,765 genes. Normalization of the count data was conducted using the DESeq2 package, which models gene expression counts via a negative binomial distribution and accounts for library size differences. Differentially Expressed Genes (DEGs) were identified based on cut-off thresholds of log fold change (logFC) >2 and adjusted $p<0.05$, with p-values corrected using the Benjamini-Hochberg False Discovery Rate (FDR) method. Enrichment analysis of associated biological pathways was performed using the EnrichR database. To identify potential druggable targets, genes were analyzed via the Drug-Gene Interaction Database (DGIdb 3.0) [13]. Molecular functions and biological processes of candidate genes were further explored using the GeneCards database. Finally, validation of candidate gene expression was performed using the GSE45291 dataset, which contains whole blood transcriptomic profiles from 513 RA patients and

20 healthy controls obtained *via* microarray analysis. Differential gene expression analysis was performed using GEO2R, which applies the Robust Multi-array Average (RMA) method for background correction and normalization [14].

Validation of target gene expression in RA synovial tissue using GEO data

To investigate gene expression profiles of candidate genes within the synovial tissue microenvironment, two publicly available Gene Expression Omnibus (GEO) datasets: GSE82107 and GSE77298 were analyzed. Dataset GSE82107 comprises synovial biopsy samples from 10 Osteoarthritis (OA) patients and 7 healthy controls, while GSE77298 includes samples from 16 RA patients and 7 healthy controls. Both datasets were generated using the Affymetrix Human Genome U133 Plus 2.0 Array platform [15]. Differential gene expression analysis was performed using GEO2R, significantly dysregulated genes were selected with an adjusted $p<0.05$ using the Benjamini-Hochberg method. Enrichment analysis of associated biological pathways was conducted using the EnrichR database to gain insight into the functional implications of the identified genes.

Participant selection and grouping in RA study

Blood samples were collected from 60 male and female individuals aged 30-60 diagnosed with RA according to the American College of Rheumatology 2010 criteria (ACR 2010). The patients were divided into two groups: Group 1 included 20 newly diagnosed, treatment-naïve RA patients who had not yet received medications such as hydroxychloroquine, methotrexate, or corticosteroids, while Group 2 consisted of 20 chronic RA patients who had been undergoing treatment with these medications for at least six months. Additionally, a control group (Group 3) of 20 healthy individuals without any history of inflammatory or autoimmune diseases in the past three months was included. Exclusion criteria for the study ruled out participants with other autoimmune or inflammatory diseases, alcohol use, pregnancy or recent childbirth, and any malignancies or cancers.

Collecting samples and gene expression assay using qRT-PCR

Collecting samples and gene expression assay using qRT-PCR. Ten *ml* of fresh peripheral blood was collected in K3 EDTA tubes to prevent clotting. Peripheral Blood Mononuclear Cells (PBMCs) contain several types of cells such as lymphocytes, monocytes and macrophages. It represents the only site of active gene expression in the blood. PBMCs were isolated using Ficoll density gradient centrifugation. Total RNA was extracted from PBMCs using RNXplus reagent following the manufacturer's instructions. RNA quality and quantity were assessed by measuring the absorbance ratio (A260/A280) with a spectrophotometer and integrity was confirmed by agarose gel electrophoresis. cDNA synthesis was performed using 1 μg of total RNA and the CinnaGen first-strand cDNA synthesis kit according to the manufacturer's protocol. Negative control reactions without reverse transcriptase (NRT) were included to monitor for genomic DNA contamination.

Gene expression of target genes was measured by real-time PCR on the ABI StepOne Plus system using SYBR Green Master Mix. GAPDH was used as the internal control and normalization strategy. Cycling conditions were as follows: initial denaturation at 95°*C* for 10 *min* followed by 40 cycles of 95°*C* for 15 *s*, 60°*C* for 30 *s*, and 72°*C* for 30 *s*. Melt curve analysis verified amplification specificity. Experiments were conducted in triplicate with at least three biological replicates.

Design and preparation of primers

The sequences of the target genes were retrieved from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide). Primers were designed using Gene Runner 6.0 software. To ensure specificity for cDNA and avoid amplification of contaminating genomic DNA, primers were designed to span exon-exon junctions, a widely accepted strategy in qRT-PCR primer design. Primer melting temperature (Tm) and secondary structures were evaluated using the OligoAnalyzer tool (https://www.idtdna.com/pages/tools/*oligoanalyzer)*[1]. Primer sequences provided in (Table 1).

Synovial fluid analysis

Synovial fluid samples were collected sterilely from patients with newly diagnosed rheumatoid arthritis in tubes containing anticoagulant. The samples were examined morphologically under a microscope using Neubauer slides (hemocytometer) and stained with Giemsa stain.

Statistical analysis

Data were analyzed using SPSS 16 and GraphPad Prism 9 with one-way ANOVA, Shapiro-Wilk, Mann-Whitney, and Student's t-tests. Results are presented as Mean±SD from at least two experimental replicates, with significance set at $p<0.05$.

Table 1. The sequences of primers

Gene names	Forward 5′ → 3′	Reverse 5′ → 3′
OLAH	TTGGACGGGACTATGTGTG	CGTGAGCAGGAGCCAAAC
ADAMTS2	TTGGACGGGACTATGTGTG	CGTGAGCAGGAGCCAAAC
CA1	GCATCGCATTCCCAAAGATG	TTAGATGGTACGTTAGAGGTGC
GAPDH	GGGAAGGTGAAGGTCGGAGT	TCCACTTTACCAGAGTTAAAAGCAG

Table 2. Pathways that are regulated by upregulated genes (retrieved from EnrichR database)

Term	Adjusted p-value	Combined score
Nitrogen metabolism	0.176036	165.438216
Fatty acid biosynthesis	0.176036	153.3319168
Arginine biosynthesis	0.176036	117.3871491
Spliceosome	0.176036	38.87726923

Table 4. Comparative analysis of ADAMTS2, CA1, and OLAH levels in microarray data

Gene name	logFC	Average expression	Adjusted p-value
ADAMTS2	0.65855	3.0683	2.45E-16
CA1	0.89861	9.912	0.00036611
OLAH	1.5361	4.2955	1.48E-18

Results

ADAMTS2, CA1 and OLAH genes nominated as candidate targets for RA

Analysis of 36,765 genes revealed that 19 genes were significantly differentially expressed in the RA group, defined by a log fold change (LogFC) >2 and an adjusted p<0.05. However, pathway enrichment analysis of these differentially expressed genes did not yield any statistically significant pathways (Table 2). Subsequent bioinformatics analysis identified three of the upregulated genes as druggable targets (Table 3). To further validate their relevance as potential biomarkers or therapeutic targets, the expression of these genes was assessed in a larger independent microarray dataset. All three candidate genes were significantly upregulated in this dataset as well (Tables 3 and 4).

Transcriptomic evidence highlights ADAMTS2 upregulation in arthritic synovial tissue

Gene expression in synovial tissue was analyzed using GEO datasets, specifically the GSE77298 and GSE82107 files. In the GSE77298 dataset, 187 genes were significantly upregulated (fold change >2), while 471 genes were downregulated (fold change <-2). In the GSE82107 dataset, 55 genes were significantly upregulated (fold change >2), and 478 genes were downregulated (fold change <-2). Notably, the IL-17 signaling pathway was the main common pathway among the upregulated genes in both datasets, whereas pathways related to cardiac function and heart muscle disease were commonly and significantly enriched among the downregulated genes (Tables 5 and 6). According to both datasets, *ADAMTS2* was significantly overexpressed in synovial biopsies from osteoarthritis and RA patients, while *OLAH* expression was decreased in both conditions. In contrast, the expression of the *CA1* gene showed variability; interestingly, among the different isoforms of the *CA* gene, *CA12* was consistently increased in both tissues (Table 7).

Participant characteristics

The participants included in this investigation were categorized into three distinct groups based on their age range of 30-60 years. It should be noted that the allocation of individuals in each group was done in a way that ensured the matching of both age and sex across all groups. The mean age of the participants in groups 1 to 3 was found to be 49±6.17, 49±6.44, and 49±6.97 years, respectively. This observation was made with a p-value of 0.882. Furthermore, it is worth mentioning that among the total participants, 7 individuals (35%) were males and 13 individuals (65%) were females, with this distribution being consistent across all three groups.

Elevated CA1, ADAMTS2, and OLAH expression in peripheral blood of RA patient

Figure 1 shows that all target genes were overexpressed in PBMCs of RA patients, with varying levels. The *CA1* gene increased over 5-fold in both new and chronic groups compared to controls (p<0.038 and p<0.002). *ADAMTS2* expression was also significantly elevated, especially in new cases with a 2-fold higher increase than chronic patients. In contrast, *OLAH* expression was notably high (over 12-fold) mainly in new RA cases.

Synovial fluid analysis

In image prepared from synovial fluid sample taken from the newly diagnosed RA patient showed an increase in leukocytes, especially neutrophils in the synovial fluid indicating an active inflammatory condition in the joints of these patients (Figure 2).

Discussion

The development of RA involves complex molecular pathways that impair the immune system, leading to persistent inflammation. Joint damage is caused by immune cell migration into the synovium and the proliferation of synovial fibroblasts. The specific molecular mechanisms behind the onset and progression of RA are still poorly understood, although inflammation and immune complexes are acknowledged as major contributors to the disease. Because of this knowledge

Table 3. The properties and expression change of the selected genes in rheumatoid arthritis

Gene symbol	Base mean	log2FC	Padj	Molecular function	Biological process
ADAMTS2	18.6	2.8	0.000000721	Metalloendopeptidase activity	- Extracellular matrix organization - Collagen fibril organization
CA1	7.51	2.03	0.000227134	Carbonate dehydratase activity	One-carbon metabolic process
OLAH	17.81	2.06	0.000000721	Aleoyl-[acyl-carrier-protein] hydrolase activity	Medium-chain fatty acid biosynthetic process

Table 5. Pathways affected by dysregulated genes in the synovial tissue of RA patients (GSE77298 dataset)

Down-regulated genes			Up-regulated genes		
Pathway	**Adjusted p-value**	**Combined score**	**Pathway**	**Adjusted p-value**	**Combined score**
Cardiac muscle contraction	1.21E-12	414.9669	Rheumatoid arthritis	1.21E-9	310.0458
Dilated cardiomyopathy	6.02E-12	342.8089	IL-17 signaling pathway	6.02E-8	178.7869
Hypertrophic cardiomyopathy	1.48E-11	334.25			
Adrenergic signaling in cardiomyocytes	3.55E-13	312.0595	Viral protein interaction with cytokine and cytokine receptor	1.48E-6	161.1051
Calcium signaling pathway	1.57E-10	152.2223			

Table 6. Pathways influenced by dysregulated genes in the synovial tissue of RA patients (GSE82107 dataset)

Down-regulated genes			Up-regulated genes		
Pathway	**Adjusted p-value**	**Combined score**	**Pathway**	**Adjusted p-value**	**Combined score**
Cardiac muscle contraction	3.18E-07	169.3986	IL-17 signaling pathway	4.70E-04	268.4348
Dilated cardiomyopathy	3.15E-07	168.7028	Malaria	0.013902	194.2256
Hypertrophic cardiomyopathy	3.45E-07	158.5876	African trypanosomiasis	0.053185	115.2962
Adrenergic signaling in cardiomyocytes	4.87E-05	68.85295	Rheumatoid arthritis	0.053185	78.21353

Table 7. Comparative analysis of ADAMTS2, CA1, and OLAH expression levels in the synovial tissue of RA patients

GSE82107	Adjusted p-value	logFC	GSE77298	Adjusted p-value	logFC
ADAMTS2	0.024174	1.175944	ADAMTS2	1.37E-02	1.255252
OLAH	0.151166	-1.00239	OLAH	2.44E-01	-0.62148
CA12	0.063358	0.882862	CA12	3.65E-03	1.407767
CA1	0.444997	0.420611	CA1	7.51E-01	-0.19003

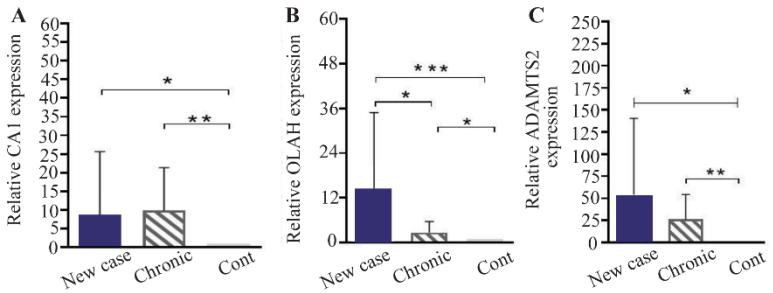

Figure 1. Results of changes in the expression of A) *CA1* B) *OLAH* and C) *ADAMTS2* genes in PBMC cells of new case rheumatoid arthritis patients, chronic patients, and control group.

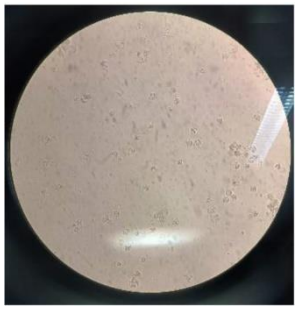

Figure 2. The image prepared from synovial fluid sample taken from newly diagnosed patients with rheumatoid arthritis. Leukocytes in a synovial fluid sample under Neubauer slide (Hemocytometer; mm^3).

gap, current therapies are less effective and frequently fail to prevent joint damage [16]. In the present study, bioinformatics analyses with Real-Time PCR experiments were combined to identify novel target genes for RA management. Integrated *in silico* and experimental data revealed significant upregulation of the enzymes CA1, OLAH, and ADAMTS2 in PBMCs of RA patients. Notably, only ADAMTS2 and another isoform of Carbonic anhydrase gene, *CA12* exhibited high expression in synovial tissue. The ADAMTS family, characterized by disintegrin, metalloproteinase, and thrombospondin motifs, plays a crucial role in remodeling the extracellular matrix through the degradation

of fibrillar collagens [17,18]. While several members of this family, including ADAMTS-4, ADAMTS-5, ADAMTS-7, and ADAMTS-12, have been implicated in the pathogenesis of RA [19], the specific role of ADAMTS2 in RA remains poorly defined. Findings demonstrate that *ADAMTS2* expression is significantly elevated in both the peripheral blood and synovial tissue of RA patients, suggesting its involvement in disease processes. Supporting this, prior research by Alper *et al* revealed that the inflammatory cytokine IL-1α—known to be pivotal in cartilage and bone homeostasis and pathology—can upregulate *ADAMTS-2* and *ADAMTS-3* expression in osteoblast-like cell lines [11]. This raises the possibility that pro-inflammatory cytokines such as IL-1α may similarly induce *ADAMTS2* expression in RA, contributing to joint degradation. Therefore, targeting ADAMTS2 may represent a promising therapeutic strategy to mitigate extracellular matrix breakdown and prevent joint damage in RA patients.

Carbonic anhydrase 1 (CA1) is a zinc metalloenzyme belonging to a large family of carbonic anhydrase isoforms, which catalyze the reversible hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) and protons (H^+) [8]. This reaction is fundamental for maintaining pH homeostasis, a critical aspect of physiological equilibrium [9]. Data demonstrate that CA1 is predominantly expressed in the blood cells of RA patients, whereas other isoforms, such as CA12, show increased expression in synovial tissue. Although both CA1 and CA12 catalyze the same biochemical reaction, they differ significantly in cellular localization and function; CA1 primarily regulates systemic acid-base balance in erythrocytes [20], while CA12, a membrane-bound isozyme expressed at epithelial surfaces, modulates local pH and has been implicated in pathological processes including tumor microenvironment adaptation under hypoxic conditions [21,22]. Emerging evidence suggests that carbonic anhydrases, including CA1, may influence the migration of FLS within inflamed joints in RA, thus contributing to disease progression [9].

Supporting this, transgenic mouse models overexpressing *CA* genes exhibit exacerbated RA symptoms and enhanced joint destruction [10]. Moreover, the study by Strowitzki and colleagues highlighted the role of carbonic anhydrase in regulating intracellular pH in monocytes and macrophages, which in turn modulates inflammatory gene expression. Pharmacological inhibition of carbonic anhydrases disrupts this pH-sensitive inflammatory signaling and impairs CO_2 sensing in macrophages [23].

Notably, acetazolamide, a carbonic anhydrase inhibitor, has been shown to attenuate inflammation and hypoxic pulmonary hypertension through modulation of immune cell activity [24]. Together, these findings, aligned with our results, implicate *CA1* overexpression in PBMC as a contributing factor to inflammatory responses during both acute and chronic phases of RA, likely *via* modulation of immune cell acidity. Therefore, targeting CA1 with specific inhibitors presents a promising therapeutic avenue for mitigating inflammation and joint damage in RA.

Oleoyl-ACP hydrolase (*OLAH*) has emerged as a gene of interest in RA due to its critical role in fatty acid biosynthesis. Fatty acids are increasingly recognized for their modulatory effects on immune responses, influencing inflammation in diverse ways. For example, short-chain fatty acids have been shown to suppress the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, thereby exerting anti-inflammatory effects. In contrast, medium-chain fatty acids can promote inflammation through activation of TLR2 [12]. Furthermore, long-chain fatty acids, including palmitic acid, have been implicated in driving pro-inflammatory signaling by engaging multiple Toll-like receptor complexes such as TLR2/TLR1, TLR2/TLR6, and TLR4 [25]. Given these complex interactions, recent research has increasingly focused on how variations in fatty acid concentration, metabolism, and composition contribute to RA pathogenesis [26]. The involvement of OLAH in fatty acid metabolism thus underscores its potential significance in modulating inflammatory pathways within RA and warrants further investigation. Given that OLAH and ADAMTS2 were predominantly overexpressed in newly diagnosed RA patients, while CA1 levels remained consistently elevated throughout both early and chronic stages, our findings suggest that CA1 and OLAH primarily drive the initiation and propagation of inflammation, whereas ADAMTS2 is mainly involved in tissue remodeling and joint destruction. Collectively, these genes hold promise as biomarkers for early diagnosis and as potential targets for novel therapeutic interventions in RA. Nonetheless, this study is limited by a relatively small sample size, lack of functional assays to establish causality, and a focus on PBMCs rather than comprehensive joint tissue analysis. Future investigations with larger cohorts and mechanistic studies are warranted to validate these targets and elucidate their roles in RA pathophysiology.

Conclusion

This integrative analysis identified *CA1*, *OLAH*, and *ADAMTS2* as key dysregulated genes in RA, with *ADAMTS2* consistently upregulated in both blood and synovial tissue. Their distinct expression patterns highlight their roles in inflammation and joint damage, supporting their potential as diagnostic biomarkers and therapeutic targets. These findings advance molecular insights into RA and lay the groundwork for future translational applications.

Ethics approval

During collecting data, the researchers obtained informed consent from the participants after explaining the purpose and objective of the study. This study was approved by the Ethics Committee of Golestan Univer-

sity of Medical Sciences (IR.GOUMS.REC.1400.421). All procedures performed agreed with the principles of the Declaration of Helsinki and later amendments.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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