Assessment of Galectin-9 in Rheumatoid Arthritis and Its Correlation with Disease Activity

Norhan Mostafa Mohamed Abdelal[*1], Sohair Kamel Sayed [1] Mona Hussein Abd Elsamea[2] Abeer Ahmed Mokhtar[1]

[1]Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt.

[2]Department of Physical Medicine, Rheumatology and Rehabilitation, Faculty of Medicine, Assiut University, Assiut, Egypt.

***Corresponding Author:** Norhan Mostafa Mohamed Abdelal*

***E-mail:** Norhanmostafa9998@gmail.com*

Abstract:

Introduction: Rheumatoid arthritis (RA) is the most common rheumatic inflammatory disease that causes the development of articular damage, lifelong impairment, and extra-articular symptoms [1]. Anti-cyclic citrullinated antibody (anti-CCP) and rheumatoid factor (RF) are the two main diagnostic markers for RA. Still, due to the presence of seronegative RA, we need other markers for diagnosing seronegative patients. Our study revealed that RA patients have higher serum levels galectin-9 (Gal-9), especially seronegative patients.

Patients and Methods: There were 90 participants in this study: 35 age and sex-matched controls and 55 RA patients, measuring serum GAL-9 in all subjects using the ELISA technique.

Result and discussion: RA patients had considerably higher serum GAL-9 than the control group (86.39 ± 47.65 pg/mL vs. 43.89 ± 21.33 pg/mL, respectively, $p < 0.001$). GAL-9 at the cut-off point of ≥51.0, 76.4% sensitivity, 71.4% specificity, total accuracy of 74.4% and $p < 0.001$). Seronegative RA patients had considerably higher serum GAL-9 levels than the control group ($P < 0.001$). For the diagnosis of seronegative RA, at a cut-off point of ≥53.1, 71.8% sensitivity, 74.3% specificity, total accuracy of 72.9% and $p < 0.001$. Furthermore, our study discovered that GAL-9 was considerably higher in patients with high disease activity compared to those with mild and moderate disease activity $p<0.001$ after classifying the patient group into mild, moderate, and severe groups based on disease activity as determined by the DAS-28 ESR score. In conclusion, Galectin-9 can be used as a sensitive marker for diagnosing RA, especially in seronegative patients, and it also helps in distinguishing RA disease activity.

Keywords: Rheumatoid arthritis; Galectin-9; Galectins; Disease activity score DAS28; Rheumatoid factor; Anti-CCP.

Introduction:

As a long-term autoimmune disease, RA produces inflammation and hyperplasia of the synovium and the deterioration of cartilage and bone [1]. Leukocytes, including B cells, T cells, dendritic cells, neutrophils, and macrophages, invade the joints, a crucial feature of RA. It also results in extra-synovial symptoms and harms other organs and joints[2].

Galectin-9, a lectin normally expressed in immune system macrophages, T cells, fibroblasts, and endothelial cells, is crucial for many functions, such as controlling inflammation and immunological responses. These functions are linked to a particular concentration modification in the GAL-9 level 3 to achieve a clear effect. In normal conditions, the GAL-9 concentration determines these actions on activated T-cells. Gal-9 induces T-cells (CD8+ and CD4+) to undergo apoptosis at greater concentrations, while at lower quantities, it stimulates activated T-cells to produce more cytokines.

T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) is a receptor expressed on activated T-cells, where GAL-9 interacts and triggers a cascade of actions that cause T-cells to undergo apoptosis[4].

Various theories on the role of GAL-9 in RA are given. One of them may be linked to polymorphisms in the GAL-9 gene [5] makes GAL-9 cause rheumatoid inflammation mainly through granulocytes by cytokine production, survival, and migration. Furthermore, it causes intracellular citrullinated autoantigens of granulocyte proteins by increasing the synthesis of Peptidyl arginine deiminases 4 (PAD-4) [6].

Second, as Tim-3 negatively regulates the Th1 response. It is hypothesised that interrupting this negative feedback loop may result in autoimmune illnesses. Low surface Tim-3 levels from a single nucleotide polymorphism (SNP) in the Tim-3 coding region 7 allow Th1 and Th17 cells to evade Gal-9-induced apoptosis, leading to chronic inflammation [8].

We therefore hypothesized that GAL-9 might be involved in the pathogenesis of RA. attempting to quantify RA patients' serum GAL-9 levels and evaluate the results concerning clinical markers.

Subject and methods:

In a case-control study, 55 patients of various sexes participated. Patients from January 2023 to December 2023 who visited the Assiut University Hospital's rheumatology clinic. Patients were diagnosed with RA using the classification criteria recommended by the ACR/EULAR, and 35 apparently healthy subjects were taken as controls.

DAS28-ESR score was used in the assessment of disease activity [9].

So we divided the patients and controls into:

Group I: 55 RA patients subdivided into:

a. **Group IA:** 7 RA patients with low disease activity.
b. **Group IB:** 24 RA patient with moderate disease activity.
c. **Group IC:** 24 RA patient with severe disease activity.

Group II: 35 Healthy control.

Another division of RA patients into two groups

a. **Group I:** 40 seropositive RA patients (RF and/or anti-CCP positive).
b. **Group II:** 15 seronegative RA patients (negative RF and anti-CCP).

Exclusion criteria:

1. The patient's age is <20 years old.
2. Patient with evidence of any concomitant inflammatory disease, acute infection, or malignancy

All patients and controls were subjected to:

1. Complete history taking.
2. General and local examinations were performed, and findings were recorded for patients.
3. Laboratory investigations:

Of the 9 milliliters of venous blood drawn, 2 milliliters were drawn into an EDTA-containing tube for a complete blood count (CBC) using a cell counter (Ruby CELLDYN, Abbott, USA). To test the ESR using the Westgren tube [10]. A plain tube was used to collect the remaining 5 ml of serum, which was then separated and kept at -80 °C until needed to measure RF, anti-CCP, CRP, and GAL-9.

The Rapid latex slide agglutination test for the qualitative screening and semi-quantitative determination of CRP and RF measured C-reactive protein (CRP) and RF. The normal range for CPR is less than 6 mg/L; for RF, it is less than 8 IU/mL.

Anti-CCP: was done on Cobas E 411, using Anti-CCP detect kit Lot.no. 58006401. Made in Germany. Negative results are defined as less than 7 U/ml.

Serum GAL-9 was measured using the Elabscience ® Human GAL (Galectin-9) Enzyme-linked immunosorbent assay (ELISA) kit. Was purchased from Elabscience, United States
Catalog No: E-EL-H1059
Lot: NM27RZH02755 96T

Ethical Considerations:

The Assiut University Faculty of Medicine's Ethics Committee approved the experiment under approval number

17101835. Before being included in the trial, patients gave their written consent.

Statistical Analysis:

Data were analyzed using IBM SPSS (Statistical Package for Social Science, version 22). The qualitative data are presented as numbers (percentages) and compared by the Chi-square or Fisher's Exact tests. Quantitative data are presented as mean±standard deviation (±SD) or median (range) and compared by ANOVA or Kruskal-Wallis tests. The correlation between different variables was done using the Pearson correlation test. The receiver operating characteristic (ROC) curve analysis was used to find the best cut-off values to validate the prediction of RA using GAL-9 biomarkers. The p-value was set to be significant at the level of 0.05.

Results

Every group under study was matched for both sex and age, and there were no significant variations between them. However, a female sex predominance was noted among the patients under study; of the 55 patients, 52 (94.5%) were female and 3 (5.5%) were male, resulting in a female: male ratio of 17.5%. Patients with RA were 43.38 ± 10.95 years old on average.

No significant variations were found when CBC, liver, and renal functions were measured in both the patient and control groups.

Among the groups under study, RA patients had significantly higher ESR and increased levels of CRP, RF, anti-CCP, and Gal-9" than the control group. **Table (1)**.

Table (1): Laboratory markers among the studied groups.

Laboratory markers	RA (n=55)	Controls (n=35)	P-value
ESR (mm/h)			**<0.001**
- Mean ± SD	32.71 ± 20.38	4.43 ± 1.4	
- Median (range)	30.0 (10.0 – 86.0)	4 (3.0 – 8.0)	
CRP (mg/L)			**<0.001**
- Mean ± SD	19.29 ± 13.79	4.87 ± 3.61	
- Median (range)	16.0 (2.0 – 59.7)	4.0 (0.1 – 20.0)	
Rheumatoid factor			**<0.001**
- Mean± SD	65±168	5±1	
- Median (range)	7(3-1022)	5 (2 -7)	
Anti-CCP (IU/ml)			**<0.001**
- Mean ± SD	204.16 ± 189.41	4 ± 2	
- Median (range)	121.6 (7.0 – 500.0)	(0 – 6)	
Serum Galactin-9 (pg/ml)			**<0.001**
- Mean ± SD	86.39 ± 47.65	43.89 ± 21.33	
- Median (range)	79.7 (10.0 –210.0)	44.7 (6.7 – 100.0)	

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor; Anti-CCP: Anti–cyclic citrullinated peptide. Data are presented as mean ± SD and median (range). Significance defined by $p < 0.05$.

The ROC curve analysis showed that at cut off ≥51.0, 76.4% sensitivity 71.4% specificity, 74.4% total accuracy and $p<0.001$) for diagnosing RA (figure 1, table 2) and for diagnosing seronegative RA patients, at GAL-9 cut off ≥53.1, 71.8% sensitivity, 74.3% specificity, total accuracy of 72.9% and p<0.001) (figure 2, table 2).

Table (2): The serum GAL-9 biomarker's best cut-off, sensitivity, and specificity for diagnosing RA and seronegative individuals.

	Cut off	95%CI	Sensi-tivity	Speci-ficity	PPV	NPV	Accuracy	AUC	P-value
RA	≥51.0	0.698 – 0.883	76.4%	71.4%	80.8%	65.8%	74.4%	0.790	**<0.001**
Seronegative RA	≥53.1	0.631-0.863	71.8%	74.3%	75.7%	70.3%	72.9	0.747	**<0.001**

PPV stands for positive predictive value, NPV for negative predictive value, AUC for area under the curve, and CI for confidence interval.

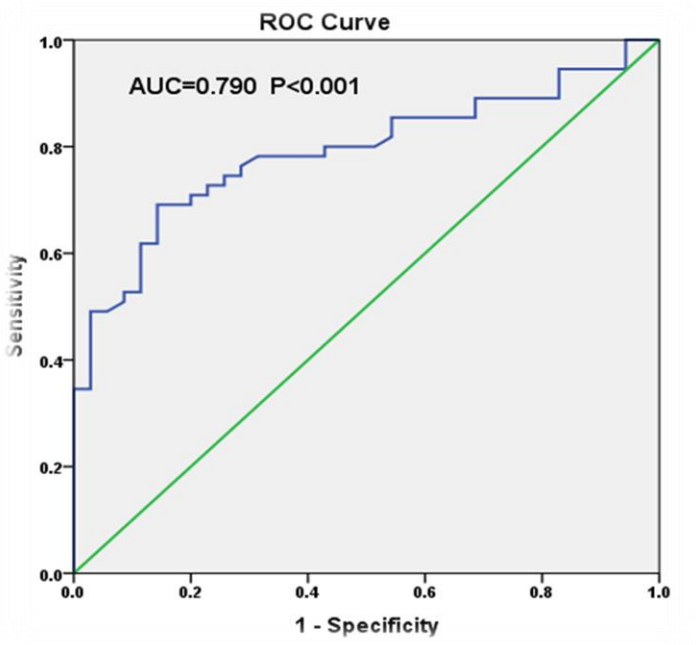

Figure (1): RA detection using ROC curves. The reference line (green) and GAL-9 (blue). P-value = <0.001, area under the curve = 0.790 (0.698 to 0.883).

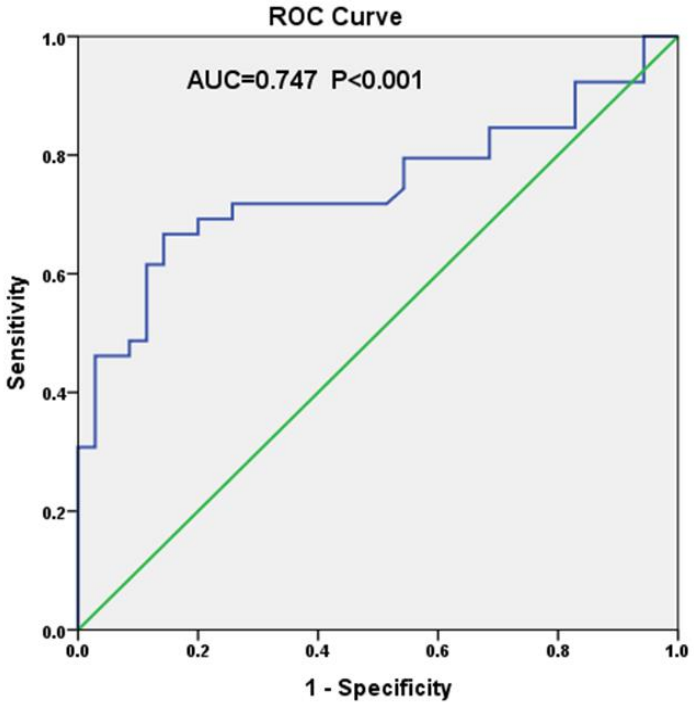

Figure 2: ROC curves showing the reference line (green) and GAL-9 (blue) for detecting seronegative RA. P-value = <0.001, area under the curve = 0.747 (0.631 to 0.863).

Table (3: Seronegative RA patients had substantially higher GAL-9 levels than the control group, and seropositive RA patients had significantly higher GAL-9 levels than the control group. However, the GAL-9 levels of seropositive and seronegative RA did not differ significantly.

Table (3): Comparison of GAL-9 between seropositive, seronegative RA patients and control groups.

	Seronegative RA (n=15)	**Seropositive RA (n=40)**	**Controls (n=35)**	**P-value**
Galactin-9 (pg/ml)				P<0.001
• Mean ± SD	81.13 ± 48.18	99.24 ± 45.23	43.89 ± 21.33	P^1=0.272
• Median (range)	77.7 (10.0 – 210.5)	85.5 (48.3 – 181.6)	44.7 (6.7 – 100.0)	P^2<0.001
				P^3<0.001

Quantitative data are presented as mean ± SD and median (range). Significance defined by $p < 0.05$

P-value: Comparison among all groups.

P-value^1: Comparison between sero-negative and sero-positive RA patients.

P-value^2: Comparison between sero-negative patients and controls.

P-value^3: Comparison between sero-positive patients and controls

ESR, CRP, anti-CCP, and GAL-9 were significantly higher in individuals with high RA disease activity than in those with mild or moderate disease activity. However, there was no apparent distinction in RF between the groups of RA disease activity studied, nor was there a significant difference in ESR, CRP, anti-CCP, or GAL-9 between the groups with mild and moderate disease activity, as shown in Table 4.

Table (4): Comparison of "ESR, CRP", "RF, Anti-CCP", and serum GAL-9 marker according to RA activity score.

	Mild, n=7 (IA)	**Moderate, n=24 (IB)**	**High, n=24 (IC)**	**P-value**
Rheumatoid factor				**0.104**
Negative	6 (85.7%)	17 (70.8%)	11 (45.8%)	
Positive	1 (14.3%)	7 (29.2%)	13 (54.2%)	
Anti-CCP (IU/ml)				**P<0.001**
Mean ± SD	21.96 ± 30.15	136.31 ± 171.50	325.15 ± 156.19	**P^1=0.207**
Median (range)	7.0 (7.0 – 89.2)	61.9 (7.0 – 500.0)	372.4 (7.0 – 500.0)	**P^2<0.001**
				P^3<0.001
ESR (mm/h)				**P<0.001**
Mean ± SD	16.71 ± 8.12	24.08 ± 10.96	46.00 ± 22.31	**P^1=0.566**
Median (range)	16.0 (10.0 – 33.0)	25.0 (10.0 – 46.0)	44.0 (10.0 – 86.0)	**P^2<0.001**
				P^3<0.001
CRP (mg/L)				**P<0.001**
Mean ± SD	10.53 ± 6.60	11.52 ± 7.70	29.62 ± 13.34	**P^1=0.974**
Median (range)	9.5 (5.5 – 24.0)	8.0 (2.0 – 30.0)	30.0 (8.9 – 59.7)	**P^2<0.001**
				P^3<0.001
Serum Galectin-9 (pg/ml)				**P<0.001**
Mean ± SD	54.26 ± 30.69	61.95 ± 31.62	120.23 ± 44.19	**P^1=0.883**
Median (range)	66.6 (10.3 – 87.5)	65.7 (10.0 – 142.4)	115.9 (48.3–210.5)	**P^2<0.001**
				P^3<0.001

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein. DAS28: Disease activity score. Quantitative data are presented as mean ± SD and median (range). Significance defined by $p < 0.05$.
P-value: Comparison among all groups.
P-value[1]: Comparison between mild and moderate RA activity score.
P-value[2]: Comparison between mild and high RA activity score.
P-value[3]: Comparison between moderate and high RA activity score.

Discussion

Rheumatoid arthritis is a systemic autoimmune disease that affects not only joints but also extra-articular organs. It is defined by a continuous inflammatory process [2].

All RA patients in our study had considerably higher ESR and CRP than the control group (p<0.001). ESR and CRP were also considerably higher in RA patients compared to the control group (p<0.001), according to research by Rukavishnikov et al. (2020 [11] and Shrivastava et al. (2015 [12].

Anti-CCP and RF are helpful indicators for RA [13]. The RA patient group in our study had considerably higher levels of anti-CCP and RF than the control group (P<0.001). Myngbay et al. (2019, 14) supported these findings, who found that RA patients had significantly higher (P<0.001) RF and anti-CCP levels than the control group.

ESR, CRP, and anti-CCP levels were also considerably greater in high disease activity than in moderate and low (P<0.001). When it was determined using RF to determine disease activity, our study discovered no significant difference between the two. Rajni et al., 2022 [15] results matched our study and found that high disease activity had significantly higher ESR and CRP compared to moderate and low disease activity, Rahali et al., 2023 [16] agreed with our study, finding that anti-CCP was significantly higher in high disease activity compared to mild and moderate disease activity (P<0.001), and Bruns et al., 2016[17] who showed that no significant difference between RF and disease activity was found**.** However, Watanabe et al. (2017, [18] showed that RF was significantly higher in high disease activity compared to low disease activity.

One potential novel biomarker for RA diagnosis and disease activity assessment is GAL-9 [19]. According to this thesis, RA patients had considerably higher serum levels of GAL-9 than the control group (P<0.001). Ameen et al. (2023 [5], Fujita et al. (2020 [20], Sun et al. (2021 [21] and Wiersma et al. (2019 [19] all confirmed these findings by comparing the GAL-9 levels of RA patients and the control group. They found that RA patients had significantly higher serum levels of GAL-9 (p=0.017, p=0.001, p=0.026, and p<0.0001), respectively.

The role of GAL-9 in detecting disease activity is significant. Serum GAL-9 levels were considerably higher in high RA disease activity than in mild and moderate disease activity (p<0.001), according to our study that evaluated serum GAL-9 levels in RA disease activity.

Similar findings to ours were reported by Fujita et al. (2020 [20] and Sun et al. (2021 [21] who found that circulating GAL-9 was considerably higher in patients without clinical remission than in those with clinical remission (p = 0.013 and P = 0.001, respectively).

Disagreed with our results, were Ameen et al., 2023[5] and Vilar et al., 2019 [22.] They found that high disease activity patients had significantly milder serum GAL-9 level (p<0.001), and GAL-9 was significantly increased in remission patients or mild disease activity than in moderate or high disease activity (P<0.001 and p <0.0001, respectively).

The predictive ability of GAL-9 for RA diagnosis was assessed by using the ROC curve. At GAL-9 cut-off≥51.0 pg/mL, 76.4% sensitivity, 71.4% specificity, and total accuracy of 74.4%. Based on these data, we could assume that GAL-9 is a sensitive biomarker for distinguishing RA patients from healthy controls.

Studies agreed with our findings, Ameen et al. (2023 [5], found that with a GAL-9 cut off of 142.7 pg/ml, 53.3%

sensitivity, 88% specificity, and a total accuracy was 63.5%.

Our findings demonstrated a positive correlation between GAL-9 and ESR, CRP, anti-CCP, number of sensitive joints, and DAS-28 score. GAL-9, RF, and the number of swollen joints showed a positive but negligible relation. Like our work, Fujita et al. (2017 [4] revealed a positive association between GAL-9 and ESR, anti-CCP, DAS-28, and a positive but insignificant correlation between GAL-9 and RF. Vilar et al. (2019 [22] found a positive but negligible relation between GAL-9 and RF levels.

A study that disagreed with these results was Ameen et al. (2023 [5]**), who showed** highly significant negative correlations with DAS-28, ESR, CRP, and number of swollen and tender joints. Also, the GAL-9 level had an insignificant negative correlation with Anti-CCP.

These findings could be caused by hereditary or environmental factors and the use of drugs that change the course of the disease.

These results support GAL-9 specificity in discriminating high disease activity from other severity grades. So other markers were examined (to know further the extent of usage of GAL-9 in RA patients).

This thesis indicates the usefulness of GAL-9 in diagnosing seronegative RA after excluding any other inflammatory or malignant condition, by comparing serum GAL-9 levels in seropositive, seronegative, and control groups. GAL-9 levels were greatly elevated in seronegative RA compared to the control group (p= <0.001) and significantly higher in seropositive RA compared to the control group (p= <0.001). Still, there was no significant difference between seronegative and seropositive RA.

The predictive power of the GAL-9 biomarker in diagnosing seronegative RA was assessed through further study. The ROC curve demonstrated the power of GAL-9 in differentiating seronegative RA, which showed 71.8% sensitivity, 74.3% specificity, a total accuracy of 72.9%, and p=<0.001 at a cut-off≥53.1 pg/mL.

We concluded that GAL-9 can diagnose RA, especially seronegative RA, and distinguish RA disease activity (according to DAS28 score).

References:

1. **Doody KM**, **Bottini N**, **Firestein GS**. Epigenetic alterations in rheumatoid arthritis fibroblast-like synoviocytes. *Epigenomics*. 2017;9(4):479-492.
2. **Conforti A**, **Di Cola I**, **Pavlych V**, et al. Beyond the joints, the extra-articular manifestations in rheumatoid arthritis. *Autoimmun Rev*. 2021;20(2):102735.
3. **John S**, **Mishra R**. Galectin-9: From cell biology to complex disease dynamics. *J Biosci*. 2016;41:507-534.
4. **Fujita K**, **Iwama H**, **Oura K**, et al. Cancer therapy due to apoptosis: galectin-9. *Int J Mol Sci*. 2017;18(1):74.
5. **Ameen SG**, **Zidan MA**, **Amer AS**, et al. A study of the association between Galectin-9 gene (LGALS9) polymorphisms and rheumatoid arthritis in Egyptian patients. *Egypt Rheumatol Rehabil*. 2023;50(1):30.
6. **Skapenko A**, **Leipe J**, **Lipsky PE**, et al. The role of the T cell in autoimmune inflammation. *Arthritis Res Ther*. 2005;7 Suppl 2:S4.
7. **Razi B**, **Reykandeh SE**, **Alizadeh S**, et al. TIM family gene polymorphism and susceptibility to rheumatoid arthritis: Systematic review and meta-analysis. *PLoS One*. 2019;14(2):e0211146.
8. **Kandel S**, **Adhikary P**, **Li G**, et al. The TIM3/Gal9 signaling pathway: An emerging target for cancer immunotherapy. *Cancer Lett*. 2021;510:67-78.
9. **Inoue E**, **Yamanaka H**, **Hara M**, et al. Comparison of Disease Activity Score (DAS) 28-erythrocyte sedimentation rate and DAS28-C-reactive protein threshold values. *Ann Rheum Dis*. 2007;66(3):407-409.

10. **Tishkowski K**, **Gupta V**. Erythrocyte Sedimentation Rate. In: StatPearls. StatPearls Publishing; 2024.
11. **Rukavishnikov G**, **Neznanov N**, **Mazurov V**, et al. Depressive symptoms and C-reactive protein, ESR levels in a sample of patients with rheumatoid arthritis: preliminary study results. *Eur Neuropsychopharmacol*. 2020;40:S211.
12. **Shrivastava AK**, **Singh HV**, **Raizada A**, et al. Inflammatory markers in patients with rheumatoid arthritis. *Allergol Immunopathol (Madr)*. 2015;43(1):81-87.
13. **Ding B**, **Padyukov L**, **Lundström E**, et al. Different patterns of associations with anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis in the extended major histocompatibility complex region. *Arthritis Rheum*. 2009;60(1):30-38.
14. **Myngbay A**, **Bexeitov Y**, **Adilbayeva A**, et al. CTHRC1: a new candidate biomarker for improved rheumatoid arthritis diagnosis. *Front Immunol*. 2019;10:1353.
15. **Bagdi R**, **Aswani P**, **Singh VK**, et al. C-reactive protein as a disease activity marker in rheumatoid arthritis. *Int J Health Sci*. 2022;6(S2):10587-10593.
16. **Rahali FZ**, **Tarmidi M**, **Hazime R**, et al. Clinical significance of anti-cyclic citrullinated peptide (anti-CCP) antibodies in rheumatoid arthritis: Literature review. *SN Compr Clin Med*. 2023;5(1):272.
17. **Bruns A**, **Nicaise-Roland P**, **Hayem G**, et al. Prospective cohort study of effects of infliximab on rheumatoid factor, anti-cyclic citrullinated peptide antibodies and antinuclear antibodies in patients with long-standing rheumatoid arthritis. *Joint Bone Spine*. 2009;76(3):248-253.
18. **Watanabe S**, **Gono T**, **Nishina K**, et al. Rheumatoid factor is correlated with disease activity and inflammatory markers in antineutrophil cytoplasmic antibody-associated vasculitis. *BMC Immunol*. 2017;18(1):1-7.
19. **Wiersma VR**, **Clarke A**, **Pouwels SD**, et al. Galectin-9 is a possible promoter of immunopathology in rheumatoid arthritis by activation of peptidyl arginine deiminase 4 (PAD-4) in granulocytes. *Int J Mol Sci*. 2019;20(16):4046.
20. **Fujita Y**, **Asano T**, **Matsuoka N**, et al. Differential regulation and correlation between galectin-9 and anti-CCP antibody (ACPA) in rheumatoid arthritis patients. *Arthritis Res Ther*. 2020;22(1):1-9.
21. **Sun J**, **Sui Y**, **Wang Y**, et al. Galectin-9 expression correlates with therapeutic effect in rheumatoid arthritis. *Sci Rep*. 2021;11(1):5562.
22. **Vilar KD**, **Pereira MC**, **Tavares Dantas A**, et al. Galectin-9 gene (LGALS9) polymorphisms are associated with rheumatoid arthritis in Brazilian patients. *PLoS One*. 2019;14(10):e0223191.