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Research article

Serum ornithine to arginine ratio as a novel diagnostic test for rheumatoid arthritis in women

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ABSTRACT

Finding specific serum biomarkers linked to rheumatoid arthritis (RA) can help us understand the course of the disease, and the prognosis. Here, we aimed to explore the potential use of arginine, ornithine, tryptophan, citrulline, serotonin and several other biochemical markers for early diagnosis of rheumatoid arthritis (RA). We examined serum samples from 30 controls and 60 RA patients to achieve this goal. According to our findings, there was a statistically significant difference in the serum levels of ornithine and arginine between RA patients and controls, with higher ornithine and lower arginine in RA patients compared to controls. Furthermore, we found that only patients with high disease activity index had considerably greater levels of serotonin. Additionally, we found that using arginine alone can predict RA disease with 96.7% sensitivity and 80.8 % specificity, while ornithine can predict RA disease with 100% sensitivity and 66.7% specificity. Interestingly, the ornithine to arginine ratio (OR/AR) could identify people with RA disease with 100% sensitivity and 83.3% specificity and this clear discrimination is not affected by the disease index or duration. Hence, RA patients can be distinguished using the ornithine to arginine ratio as a biomarker, which has higher specificity than each analyte alone. Our results can undoubtedly serve as a foundation for additional research and multicenter studies in the future to support accurate therapeutic management strategies for RA patients.

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1. Introduction

Rheumatoid Arthritis (RA) is a complex autoimmune disease affecting joints and potentially other organs, with factors like genetics, age, and environmental exposures contributing to its development [1–5]. Rheumatoid arthritis is a highly prevalent chronic disease currently affecting approximately 1% of world's population [6]. Patient with RA typically have destruction of joint cartilage and bone accompanied by joint stiffness, hyperplasia, microvascular injury, swelling and pain [7]. In addition, RA can cause subcutaneous nodular lesions and can result in inflammation of lungs, pericardium, pleura and sclera [8,9].

Moreover, nearly two thirds of patient with RA develop cachexia and sarcopenia with loss of skeletal muscle mass, degradation of proteins and energy expenditure [10]. This could be due to perturbation in amino acid metabolism which drive the body into a state of negative energy balance leading to skeletal muscles atrophy, loss of muscles strength and reduced physical activity [11].

The diagnosis of the disease remains challenging due to its multifaceted nature, with current methods like Rheumatoid Factor (RF) and Anti-Citrullinated Protein Antibody (ACPA) tests having limitations in sensitivity and specificity [2]. Therefore, there is a pressing need for novel diagnostic markers that can accurately and reliably detect RA at an early stage [12,13].

Among the unique findings in RA patients, serum arginase activity was significantly increased in patients with rheumatoid arthritis (RA) compared to healthy controls and other autoimmune diseases [14,15]. Leading to alterations in amino acid metabolism [16]. Increased arginase activity contribute to consumption of L-arginine and production of L-ornithine needed for the synthesis of proline and polyamines [17,18]. Ornithine and Arginine are two key amino acids involved in the urea cycle and nitric oxide synthesis. Changes in their levels may reflect the underlying inflammatory processes occurring in RA. Therefore, we hypothesized that serum Ornithine to Arginine (OR/AR) ratio, could serve as a simple potential diagnostic marker for RA, reflecting inflammatory processes in the patients. This ratio may offer a novel approach to diagnosing RA, as changes in OR/AR levels could indicate disease presence and progression, providing a reliable tool for early detection and management of RA.

Given that rheumatoid arthritis predominantly affects women, with a female-to-male ratio of approximately 3:1, and considering the potential hormonal influences on amino acid metabolism and disease pathogenesis, we focused our initial investigation on female participants to minimize confounding variables related to sex differences in metabolic profiles. This approach allows for a more homogeneous study population and reduces variability that might obscure the biomarker signals we aimed to detect. Future studies should include male participants to validate the generalizability of our findings across both sexes.

2. Materials and methods

The present study is a descriptive cross-sectional study. Patients were recruited in Assiut university in the period from August 2021 to February 2023. The protocol of the study was reviewed and approved before starting data collection via Medical Ethical Committee Faculty of Medicine, Assiut University (IRB:17300354). Written informed consent was obtained from all patients for participation in the study and publication of the results.

2.1. Subjects

Sixty female rheumatoid arthritis (RA) patients (n = 60) diagnosed according to American College of Rheumatology/European league against rheumatism (ACR/EULAR) classification criteria [19] were enrolled in this study. They aged from 24 to 70 years old. Thirty female healthy controls (n = 30) who are age matched with the studied group were also enrolled in the study. Inclusion criteria for RA patients were: (1) female patients aged 18-70 years; (2) diagnosis according to the ACR/EULAR classification criteria; and (3) willing to provide informed consent. For the control group, the inclusion criteria were: (1) age-matched healthy females without history of any autoimmune disease; and (2) willing to provide informed consent.

The sample size was calculated according to G*Power 3 software [20]. Exclusion criteria included: (1) patients with other suspected or known rheumatological disease such as SLE, psoriatic arthritis, multiple sclerosis; (2) patients with other known causes of arthritis; (3) patients with chronic liver or renal disease; (4) those who underwent joint replacement; (5) pregnant or lactating women; (6) patients with malignancies; and (7) patients with serious infections or other severe comorbidities that could interfere with the study assessments." Detailed baseline characteristics of study participants are provided in Supplementary Table S1.

2.2. General examination

A full history was obtained and through clinical examinations were performed after patients' consents. Personal history including disease duration (months), duration of morning stiffness (hours), presence of pain and/or swelling of the shoulders, elbows, wrists, metacarpopharyngeal (MCP) joints and proximal interpharyngeal (PIP) joints of the upper limbs and hips, knees and ankles of the lower limbs, extra articular manifestations, presence or absence of diabetes mellitus or hypertension, current therapy in forms of disease modifying anti-rheumatic drugs (DMARD), systemic steroid and or Non-steroidal anti-inflammatory drugs.

Sample Collection: Five ml of venous blood was taken from each patient and divided as follows: Three ml of venous blood in heparin tubes, with the tube inverted gently to mix with the anticoagulant, left at room temperature for 15 min, then centrifuged at high speed (3000 rpm) for 10 min. The plasma was separated and preserved at −70 °C for analysis of amino acids. Two ml of venous blood in plain tubes for serotonin level estimation by ELISA (enzyme-linked immunosorbent assay)

2.3. Special assessment

All patients were subjected to disease activity calculation score DAS28-CRP [21], which is widely used as an indicator of RA disease activity and response to treatment. The joints included in DAS28 score are bilateral joints of each of proximal interphalangeal joints, metacarpophalangeal joints, wrists, elbows, shoulders and knees. When looking at these joints, both the number of joints with tenderness upon touching and swelling are counted.

2.4. Routine laboratory analysis

Erythrocyte sedimentation rate (ESR) was done using Westergren method, C-reactive protein (CRP), Rheumatoid factor (RF), serum uric acid, serum albumin, serum creatinine and serum urea were analyzed by clinical chemistry Analyzer (BS-240 Mindray).

2.5. Quantitative assay of the amino acids

Plasma assessment for free amino acids was analyzed with Sykam Automatic Amino Acid Analyzer S433 provided by Sykam GmbH, Germany. We used a commercially available amino acid calibration standard solution (Sigma-Aldrich, USA) containing all proteinogenic amino acids at concentrations of 2.5 μmol/mL each. The calibration curve was recorded in the range of 0-500 μmol/L with six calibration points (0, 10, 50, 100, 250, and 500 μmol/L). While the mixture provided by the company may not include all amino acids—such as ornithine or citrulline—we supplemented the analysis using individual amino acid standards (e.g., L-ornithine, cat. no. O2375 and L-citrulline, cat. no. C7629; and L-tryptophan, cat. no. T0254; all from Sigma-Aldrich).

Plasma samples preparation was done by acidic protein precipitation using 10% sulfosalicylic acid and sample dilution buffer at a ratio of 1:1. After centrifugation at 12,000g for 10 min at 4 °C, the supernatant was filtered through a 0.22 μm membrane filter. Sample analysis was done by the ion exchange separation method using high-performance liquid chromatography with post-column ninhydrin derivatization. The column temperature was maintained at 50 °C, and the flow rate was 0.45 mL/min. Each sample was analyzed in triplicate, and the coefficient of variation (CV%) for all amino acids was <5%. Serotonin was determined by ELISA Kit supplied by Sun Red Co., Ltd., with a detection range of 5-1000 ng/mL and intra-assay and inter-assay CV% of <10%.

Our selection of specific amino acids (ornithine, arginine, citrulline, and tryptophan) was based on their established involvement in inflammatory processes and the urea cycle, which has been implicated in RA pathogenesis [16,22]. Specifically, we focused on the ornithine-arginine metabolic pathway given the previous evidence of altered arginase activity in RA [23].

We selected serotonin for measurement based on several previous studies suggesting its potential involvement in inflammatory processes and pain modulation in RA [24,25]. Serotonin has been implicated in immune system regulation and inflammatory responses, with evidence suggesting altered levels in various autoimmune conditions.

2.6. Statistical analysis

Data were verified, coded by the researcher, and analyzed using IBM-SPSS 24.0 (IBM-SPSS Inc., Chicago, IL, USA). Chi-square/Fisher's exact test was used to compare the difference in distribution of frequencies among different groups as appropriate. Shapiro-Wilk test was used to test for normality of continuous variables and independent sample *t*-test and Mann-Whitney test were used to calculate the mean/median differences between groups (parametric and non-parametric). For continuous variables with more than two categories, ANOVA test was calculated to test the mean differences, post-hoc test was calculated using Bonferroni corrections for pairwise comparisons between the two study groups. ROC curve was depicted to explore the diagnostic performance of the biomarkers for disease prediction, analyzed as area under the curve (AUC), standard error (SE) and 95% CI. In all experiments a significant difference was considered when a p-value is less than 0.05.

Table 1
Basic Characterstics and Analyses of Rheumatoid Arthritis Patients vs Control.

Parameter (Mean ± SD)	Control (n = 30)	Rheumatoid Arthritis (n = 60)	p-Value
Age [years]	49.7 ± 9.2	49.1 ± 14.3	0.832
ESR (mm/h)	9.0 ± 2.3	36.0 ± 2.4	<0.0001
Creatinine (mg/dL)	0.6 ± 0.2	0.7 ± 0.1	0.080
Urea (mg/dL)	37.3 ± 4.8	42.2 ± 15.1	0.025
Uric acid (mg/dL)	4.1 ± 0.8	3.4 ± 0.9	0.001
Serotonin (mg/L)	36.9 ± 6.6	36.5 ± 13.1	0.862
Citrulline (mg/L)	25.4 ± 7.7	27.8 ± 4.3	0.070
Tryptophan (mg/L)	49.2 ± 12.1	43.7 ± 13.7	0.058
Ornithine (mg/L)	75.9 ± 17.3	95.7 ± 15.4	<0.0001
Arginine (mg/L)	106.3 ± 18.1	67.5 ± 18.0	<0.0001
OR/AR ratio	0.7 ± 0.2	1.5 ± 0.4	<0.0001
GABR ratio	1.1 ± 0.2	0.6 ± 0.2	<0.0001

3. Results

3.1. Basic characterstics and analyses of Rheumatoid arthritis and control groups

In a comparative study between a control group and a group of patients with Rheumatoid Arthritis, several parameters were measured and analyzed and presented in Table 1. The age of the participants in both groups was comparable, with a mean age of 49.7 ± 9.2 years in the control group and 49.1 ± 14.3 years in the RA group ($p = 0.832$).

Significant differences were observed in several parameters between the two groups. The Erythrocyte Sedimentation Rate (ESR) was significantly higher in the RA group 36.0 ± 2.4 mm/h compared to the control group 9.0 ± 2.3 mm/h, ($p < 0.0001$). Similarly, the levels of Ornithine was higher in the RA group 95.7 ± 15.4 mg/L in vs. 75.9 ± 17.3 mg/L in the control group, ($p < 0.0001$). Arginine was lower in RA group (67.5 ± 18.0 mg/L) compared to the control group 106.3 ± 18.1 mg/L, ($p < 0.0001$). The ratio between ornithine to arginine (OR/AR) was significanlty higher in RA group 1.5 ± 0.4 vs. 0.7 ± 0.2 in the control group, ($p < 0.0001$), and global arginine bioavailability (GABR) ratio which is calculated as (Arginine/[Citrulline + Ornithine]) was lower in the RA group 0.6 ± 0.2 vs. 1.1 ± 0.2 in the control group, ($p < 0.0001$).

On the other hand, Urea levels were slightly higher in the Rheumatoid Arthritis group (42.2 ± 15.1 mg/dL) compared to the control group (37.3 ± 4.8 mg/dL), and this difference was statistically significant ($p = 0.025$). Uric acid levels were lower in the Rheumatoid Arthritis group (3.4 ± 0.9 mg/dL) compared to the control group (4.1 ± 0.8 mg/dL), and this difference was also statistically significant ($p = 0.001$).

However, no significant differences were observed in the levels of Creatinine ($p = 0.080$), Serotonin ($p = 0.862$), Citrulline ($p = 0.070$), and Tryptophan ($p = 0.058$) between the two groups.

Values are presented as mean ± standard deviation. P-values were calculated using independent sample *t*-test for normally distributed variables and Mann-Whitney *U* test for non-normally distributed variables. ESR: erythrocyte sedimentation rate; OR/AR: ornithine to arginine ratio; GABR: global arginine bioavailability ratio calculated as Arginine/[Citrulline + Ornithine].

3.2. Biomarkers concentrations according to the Disease Activity Score

To investigate whether the studied parameters could predict the severity of rheumatoid arthritis, participants of the RA group were divided into four groups based on their Disease Activity Score (DAS). We used the conventional Disease Activity Score using 28 joint counts and CRP values as reported before [21]. Remission is defined as DAS score of less than 2.6, low disease activity patients have a score of more than or equal to 2.6 and less than or equal to 3.2, moderate disease activity have a score of more than 3.2 and less than or equal to 5.1, and high disease activity have a score of more than 5.1. Remission is defined as DAS score of less than 2.6, low disease activity patients have a score of more than or equal to 2.6 and less than or equal to 3.2, moderate disease activity have a score of more than 3.2 and less than or equal to 5.1), and high disease activity have a score of more than 5.1.

Results for the comparison is presented in Table 2, and significance was calculated by one-way ANOVA test. The age of onset, disease duration, and age of the participants did not significantly differ across the groups ($p > 0.05$). The Erythrocyte Sedimentation Rate (ESR) showed a significant increase with increasing DAS score ($p = 0.005$), indicating higher inflammation levels in patients with more active disease.

Interestingly, Uric acid and Serotonin levels showed significant differences across the groups ($p < 0.0001$ for both). Patients in the high DAS score group had the highest levels of Uric acid (4.2 ± 1.2 mg/dL) and Serotonin (53.0 ± 9.2 mg/L). This suggests a potential role of these metabolites in disease activity.

Citrulline and Tryptophan levels also varied significantly across the groups ($p = 0.049$ and $p = 0.032$, respectively), although the pattern did not strictly follow the DAS score. No significant differences were observed in the levels of creatinine, urea, ornithine, arginine, OR/AR ratio, and GABR ratio across the groups ($p > 0.05$ for all), suggesting that these parameters are not associated with

Table 2
Comparison of Parameters According to Rheumatoid Arthritis DAS score.

Parameter (Mean ± SD)	Remission (n = 17)	Low (n = 7)	Moderate (n = 24)	High (n = 12)	p-Value
Age [years]	49.2 ± 16.3	51.9 ± 13.8	49.0 ± 14.0	47.8 ± 14.1	0.952
Disease Duration [months]	66.8 ± 79.1	104.3 ± 102.5	55.2 ± 55.4	94.0 ± 93.2	0.336
RA onset age	43.6 ± 13.3	43.2 ± 8.4	44.4 ± 13.3	40.0 ± 15.2	0.826
ESR (mm/h)	22.1 ± 14.3	27.1 ± 13.2	41.5 ± 26.5	50.1 ± 25.1	0.005
Creatinine (mg/dL)	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.127
Urea (mg/dL)	38.1 ± 9.8	39.7 ± 4.9	43.4 ± 20.9	47.3 ± 10.4	0.412
Uric acid (mg/dL)	3.5 ± 0.8	3.7 ± 0.9	2.9 ± 0.4	4.2 ± 1.2	<0.0001
Serotonin (mg/L)	29.0 ± 9.8	30.0 ± 4.6	35.4 ± 11.4	53.0 ± 9.2	<0.0001
Citrulline (mg/L)	29.1 ± 5.0	23.9 ± 2.2	28.2 ± 3.6	27.3 ± 4.7	0.049
Tryptophan (mg/L)	38.2 ± 10.4	44.0 ± 3.5	42.8 ± 15.5	43.7 ± 13.7	0.032
Ornithine (mg/L)	96.4 ± 14.6	87.2 ± 8.9	98.8 ± 16.2	93.3 ± 17.5	0.343
Arginine (mg/L)	68.3 ± 16.3	61.5 ± 25.7	66.8 ± 15.0	71.0 ± 22.1	0.743
OR/AR ratio	1.5 ± 0.4	1.6 ± 0.6	1.5 ± 0.4	1.4 ± 0.3	0.649
GABR ratio	0.6 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.775
DAS score	2.2 ± 0.3	2.8 ± 0.3	4.1 ± 0.7	5.8 ± 0.3	<0.0001

disease activity in Rheumatoid Arthritis.

Interestingly, we observed a non-linear pattern in uric acid levels across disease severity groups, with the moderate disease activity group showing lower levels compared to the other groups. This pattern might be influenced by several factors, including the uneven distribution of patients across groups and potential confounding variables such as medication use, particularly allopurinol or uricosuric agents, which could affect uric acid levels. Additionally, factors such as renal function, dietary intake, and comorbidities might contribute to this observation. The supplementary boxplot (Fig. S1) illustrates the distribution of uric acid values across these groups, showing considerable individual variability within each group. Future studies with larger, more balanced groups are needed to clarify the relationship between uric acid levels and RA disease severity. A notable finding is the non-linear pattern among RA patients, with significantly lower uric acid concentration in the moderate disease activity group (median: 2.8 mg/dL) compared to both the remission (median: 3.4 mg/dL) and low disease activity groups (median: 3.5 mg/dL). The high disease activity group exhibited the highest median uric acid level (4.0 mg/dL) with the greatest variability as evidenced by the wider range.

Boxplot visualization of serum uric acid levels (mg/dL) comparing healthy controls ($n = 30$) with rheumatoid arthritis patients categorized by disease activity according to Remission ($n = 17$, DAS<2.6), Low disease activity ($n = 7$, $2.6 \leq$DAS≤ 3.2), Moderate disease activity ($n = 24$, $3.2 <$DAS≤ 5.1), and High disease activity ($n = 12$, DAS>5.1). Each boxplot displays the median (horizontal line within box), and whiskers extending to minimum and maximum values. Individual data points are overlaid to show the distribution within each group. Statistical significance was determined by ordinary one-way ANOVA comparing the control group to each disease activity group. p-value is written above the comparison lines, where a p-value less than 0.05 is considered significant.

3.3. Post hoc analysis of serotonin across different Disease Activity Score sets

High level of serotonin in patients in the high DAS score group led us investigate if this group differ from other groups, this was achieved using Tukey's Honestly Significant Difference test.

Table 3 presents the results conducted to compare the mean serotonin levels across different Disease Activity Score sets in patients with RA. The mean serotonin level for the Remission set is 28.95, for the low disease activity (Low) set is 30.01, for the moderate disease activity (Moderate) set is 35.41, and for the high disease activity (High) set is 52.99.

The subsets 1 and 2 indicate the homogeneous subsets of DAS sets that do not differ significantly from each other at the 0.05 level. The groups are ordered such that the means increase from the top subset to the bottom. In this case, the Remission, Low, and Moderate DAS sets form one subset, and the High DAS set forms another, indicating a significant difference in the mean serotonin levels between High DAS group with a $p < 0.0001$.

3.4. Biomarker concentrations according to the duration of the Rheumatoid arthritis disease

Table 4 presents a comparison of various parameters between two groups of rheumatoid arthritis patients: those with a disease duration of less than 2 years ($n = 30$) and those with a disease duration of more than 2 years ($n = 30$).

Significant differences were observed in the age of the patients, disease duration, Erythrocyte Sedimentation Rate (ESR), Tryptophan levels, Arginine levels, OR/AR ratio, and GABR ratio. Patients with longer disease duration (>2 years) were significantly older (56.1 ± 14.9 years) compared to those with shorter disease duration ($\leq$2 years) (42.2 ± 10.0 years, $p < 0.0001$), suggesting that age may be a confounding factor when analyzing the relationship between disease duration and biochemical parameters. Similarly, the disease duration was significantly longer in the group with a disease duration of more than 2 years (126.5 ± 76.5 months) compared to the other group (17.4 ± 6.5 months, $p < 0.0001$).

The ESR was significantly lower in the group with a disease duration of more than 2 years (27.1 ± 16.3 mm/h) compared to the other group (45.0 ± 27.4 mm/h, $p = 0.003$). Tryptophan levels were significantly lower in the group with a disease duration of more than 2 years (34.4 ± 9.1 mg/L) compared to the other group (53.1 ± 11.1 mg/L, $p < 0.0001$). Arginine levels were significantly lower in the group with a disease duration of more than 2 years (57.0 ± 16.3 mg/L) compared to the other group (77.7 ± 13.3 mg/L, $p < 0.0001$).

The OR/AR ratio was significantly higher in the group with a disease duration of more than 2 years (1.8 ± 0.4) compared to the other group (1.2 ± 0.2, $p < 0.0001$). The GABR ratio was significantly lower in the group with a disease duration of more than 2 years (0.5 ± 0.3) compared to the other group (0.6 ± 0.1, $p = 0.036$).

No significant differences were observed in the levels of creatinine, urea, uric acid, serotonin, citrulline, ornithine, and DAS score between the two groups. These findings suggest that while some metabolic processes may be altered with the duration of Rheumatoid Arthritis, others remain unaffected.

Table 3
Tukey HSD Test for post hoc Comparison of Differences in Serotonin.

RA Groups	1	2
Remission (n = 17)	28.954647	
Low (n = 7)	30.005571	
Moderate (n = 24)	35.414208	
High (n = 12)		52.996592
p-value		<0.0001

Table 4
Comparison of parameters according to the duration of the disease.

Parameter (Mean ± SD)	RA disease duration		p-Value
	≤2 years (n = 30)	>2 years (n = 30)	
Age [years]	42.2 ± 10.0	56.1 ± 14.9	<0.0001
Disease Duration [months]	17.4 ± 6.5	126.5 ± 76.5	<0.0001
ESR (mm/h)	45.0 ± 27.4	27.1 ± 16.3	0.003
Creatinine (mg/dL)	0.7 ± 0.2	0.7 ± 0.1	0.972
Urea (mg/dL)	44.2 ± 12.0	40 ± 17.8	0.307
Uric acid (mg/dL)	3.5 ± 1.1	3.3 ± 0.7	0.321
Serotonin (mg/L)	37.0 ± 13.8	36.0 ± 12.6	0.767
Citrulline (mg/L)	28.4 ± 3.7	27.1 ± 4.9	0.245
Tryptophan (mg/L)	53.1 ± 11.1	34.4 ± 9.1	<0.0001
Ornithine (mg/L)	94.1 ± 17.1	97.2 ± 13.7	0.437
Arginine (mg/L)	77.7 ± 13.3	57.0 ± 16.3	<0.0001
OR/AR ratio	1.2 ± 0.2	1.8 ± 0.4	<0.0001
GABR ratio	0.6 ± 0.1	0.5 ± 0.3	0.036
DAS score	3.8 ± 1.4	3.7 ± 1.4	0.831

3.5. *Diagnostic accuracy of the biomarkers to predict the diagnosis with Rheumatoid arthritis*

To evaluate the diagnostic performance of the studied parameters, we used the Area Under the Curve (AUC) from Receiver Operating Characteristic (ROC) analysis, Fig. 1. The cut-off values were determined to maximize the sensitivity and specificity of each parameter.

The OR/AR ratio demonstrated outstanding diagnostic performance with an AUC of 0.954. At a cut-off value of 0.80, both sensitivity and specificity were high at 100.0% and 83.3%, respectively ($p < 0.0001$).

Finally, the GABR ratio also showed excellent diagnostic accuracy with an AUC of 0.936. At a cut-off value of 0.68, the sensitivity was 100.0%, and the specificity was 70.0% ($p < 0.0001$).

These findings suggest that these parameters, particularly the OR/AR ratio, could be useful biomarkers for the diagnosis of the

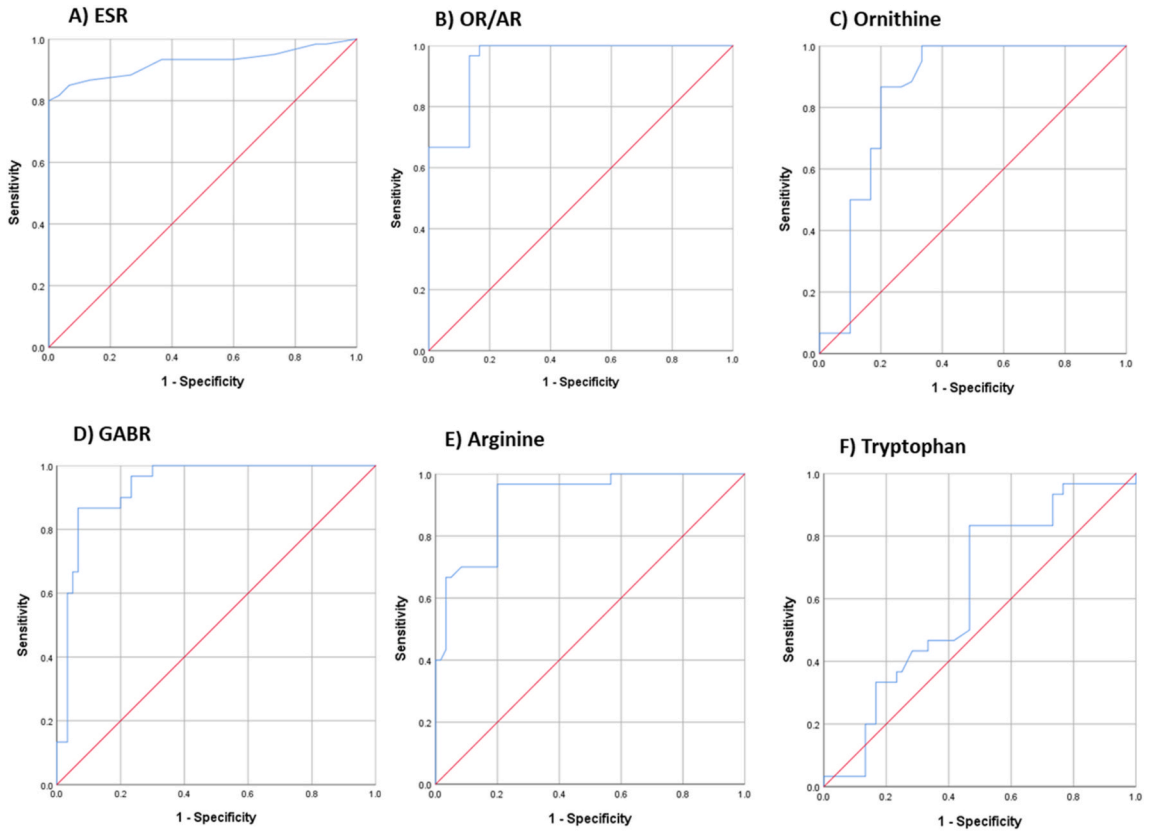

Fig. 1. ROC curve for ESR (A), OR/AR (B), Ornithine (C), GABR (D), Arginine (E) and Tryptophan (F).

disease. However, further studies are needed to validate these results in larger and diverse patient populations.

Table 5 presents an evaluation of various biomarkers for their potential use in diagnosing Rheumatoid Arthritis. The Erythrocyte Sedimentation Rate (ESR) shows a strong diagnostic performance with an Area Under the Curve (AUC) of 0.924. At a cut-off value of 14.50 mm/h, ESR achieves a sensitivity of 80.0% and a specificity of 100.0%, with a statistically significant p-value of less than 0.0001.

Ornithine also shows promise as a potential biomarker for RA with an AUC of 0.846. At a cut-off value of 77.10 mg/L, Ornithine achieves a perfect sensitivity of 100.0% and a specificity of 66.7%. The results are statistically significant with a p-value of less than 0.0001.

Arginine demonstrates a strong potential as a biomarker for RA with an AUC of 0.917. A cut-off value of 80.00 mg/L for arginine results in a high sensitivity of 96.7% and a specificity of 80.8%, with a statistically significant p-value of less than 0.0001.

However, Tryptophan, Serotonin, and Citrulline do not show statistically significant results as their p-values are greater than 0.05. Therefore, their diagnostic potential for RA is not determined in this study.

Lastly, the OR/AR ratio and GABR ratio both show excellent AUCs of 0.954 and 0.936 respectively. They both achieve a perfect sensitivity of 100.0% at their respective cut-off values, with specificities of 83.3% and 70.0%. The p-values for both ratios are less than 0.0001, indicating statistically significant results.

In conclusion, the table suggests that ESR, Ornithine, Arginine, OR/AR ratio, and GABR ratio could be potential biomarkers for RA, while Tryptophan, Serotonin, and Citrulline may not be as effective.

4. Discussion

Our investigation extends the research of numerous existing studies that have concentrated on examining the significance of amino acid concentrations in relation to rheumatoid arthritis (RA) [26–28]. This study explored the potential use of arginine, ornithine, tryptophan, citrulline, serotonin and derived ratios as biomarkers for diagnosing RA or predicting the progression of the disease.

Our main finding, which has never been reported before, is the finding that serum ornithine to arginine ratio could be a diagnostic test with an exceptional performance that can provide clear discrimination between RA patients and controls regardless of the severity of the disease. These findings stem from our initial observation that RA patients have low serum arginine and high serum ornithine.

Arginine and ornithine are interconnected in several metabolic processes. In the urea cycle arginine is converted into urea and ornithine through the action of the enzyme arginase [17,18]. Conversion of arginine to urea and ornithine is critical for the disposal of ammonia and maintenance of the body's nitrogen balance [29] and ornithine can be further metabolized into citrulline, collagen, or glutamate [30].

The alterations in the urea cycle, evidenced by increased ornithine and decreased arginine levels in RA patients, may be linked to the disease pathogenesis through several mechanisms. The urea cycle plays a crucial role in nitrogen metabolism and the disposal of ammonia, which could be disrupted in inflammatory conditions like RA. Previous studies have reported alterations in various amino acids in RA patients, supporting our findings. He et al. observed significant changes in multiple amino acids, including decreased arginine and increased ornithine, in RA patients compared to healthy controls [11]. Similarly, studies have shown disturbances in amino acid metabolism in inflammatory conditions that may be relevant to RA pathogenesis [31].

Increased arginase activity, which converts arginine to ornithine and urea, has been observed in RA [14] and may contribute to the imbalance in these amino acids. This increased arginase activity could result from the enhanced expression of arginase enzymes by activated macrophages and neutrophils in the inflamed synovium [23]. Furthermore, the depletion of arginine may impair nitric oxide (NO) production, affecting vascular function and immune responses in RA patients [32].

The altered urea cycle activity may also influence other metabolic pathways relevant to RA pathogenesis. For instance, ornithine serves as a precursor for polyamines and proline, which are essential for cell proliferation and collagen synthesis, respectively [33]. Dysregulation of these pathways could contribute to the synovial hyperplasia and extracellular matrix remodeling observed in RA. Additionally, the interplay between the urea cycle and other amino acid metabolic pathways, such as the kynurenine pathway of tryptophan metabolism, may further influence immune regulation and inflammation in RA [34].RA patients suffer from chronic degradation of the articular cartilage and bone erosion [35]. This continuous degenerative process requires extensive demand for

Table 5
Diagnostic performance of the investigated parameters.

	AUC	Cut-off value	Sensitivity	Specificity	p-value
ESR (mm/h)	0.924	14.50[a]	80.0%	100.0%	<0.0001
Ornithine (mg/L)	0.846	77.10[a]	100.0%	66.7%	<0.0001
Arginine (mg/L)	0.917	80.00[b]	96.7%	80.8%	<0.0001
Tryptophan (mg/L)	0.616	ND	ND	ND	0.073
Serotonin (mg/L)	0.402	ND	ND	ND	0.131
Citrulline (mg/L)	0.611	ND	ND	ND	0.087
OR/AR ratio	0.954	0.80[a]	100.0%	83.3%	<0.0001
GABR ratio	0.936	0.68[b]	100.0%	70.0%	<0.0001

ESE: erythrocyte sedimentation rate; OR/AR: ornithine to arginine ratio, GABR: Global arginine bioavailability ratio =(Arginine/[Citrulline + Ornithine]), ND: not done because p-value is more than 0.05.

[a] Values in RA patients are higher than the cut-off value.

[b] Values in RA patients are lower than the cut-off value.

ornithine as a source to replenish collagen, the major and specific molecule in the cartilage. Thus, it is probable that elevated levels of ornithine are associated with a compensatory or repair mechanism for the articular cartilage damage.

We found that ornithine concentrations were lowest in patients with low disease activity, which was supported by previous studies which found that serum ornithine level decrease with the alleviation of RA [36], and higher ornithine concentration is associated with increased systemic inflammation [37]. Interestingly, opposite finding were found with arginine, where L-arginine supplementation ameliorates chronic bone loss in RA patients and dampens inflammation [38]. These findings are in line with our finding that RA patients have low serum arginine which agrees with several studies [26,39]. So, it could be concluded that arginine exhibits anti-inflammatory properties while ornithine exhibits pro-inflammatory properties. Ornithine is a precursor of glutamate, and glutamate increase the expression of TNF-a [40,41].

Citrulline is an amino acid formed following posttranslational modification of arginine by peptidyl arginine deiminase (PAD), which is strongly implicated in RA at both the genetic and cellular levels; its inhibitors have demonstrated therapeutic efficacy in inflammatory arthritis [42]. However, our results showed that free serum citrulline is not significantly different between RA patients and controls and is not a useful for diagnosis or follow up of RA disease. Therefore, it is important to remark here that for RA pathogenesis, free citrulline is not as important as citrullination of autoantigens and generation of autoreactive immune cells against the modified protein structure after citrullination [43].

Several studies investigated the role of serotonin in RA with contradictory findings [24,44]. Here we found that increased serotonin is not diagnostic for RA, but it could be as a prognostic factor for development or disease course. Noteworthy, only patients with severe RA disease had significantly higher than controls and other patients. The elevated serotonin levels observed in patients with high disease activity may have important physiological implications. Serotonin is a monoamine neurotransmitter involved in various physiological processes, including immune regulation, inflammation, and pain modulation [45]. In the context of RA, serotonin may contribute to disease pathogenesis through several mechanisms. First, serotonin can modulate immune cell function, influencing the proliferation and activation of lymphocytes, and macrophages, which are key players in RA pathogenesis [46]. Second, serotonin can regulate inflammatory cytokine production, potentially exacerbating the pro-inflammatory environment characteristic of RA [47]. Third, serotonin plays a crucial role in pain perception, and elevated levels may contribute to the increased pain sensitivity observed in RA patients with high disease activity [46]. Furthermore, serotonin has been implicated in bone metabolism, influencing osteoblast and osteoclast function, which may contribute to the bone erosion seen in RA [48]. The correlation between serotonin levels and disease activity observed in our study suggests that serotonin might serve as a potential indicator of disease severity and could represent a therapeutic target for managing RA, particularly in patients with high disease activity.

Our study has several strengths, including the novel finding of OR/AR ratio as a potential diagnostic marker for RA with excellent sensitivity and specificity, the comprehensive analysis of multiple serum biomarkers, and the stratification of patients based on disease activity and duration. However, we acknowledge several limitations. First, our sample size was relatively small, and patients were recruited from a single center, which may limit the generalizability of our findings. Second, our study included only female participants, and therefore, our results may not be applicable to male RA patients. Third, we did not adjust for potential confounding factors such as medications, comorbidities, and dietary intake, which might influence amino acid metabolism. Fourth, the cross-sectional design of our study prevents us from establishing causality between the observed metabolic alterations and RA pathogenesis. Future longitudinal studies with larger, more diverse cohorts and adjustment for potential confounders are needed to validate our findings and explore the underlying mechanisms linking amino acid metabolism to RA.

Our results suggest that serum ornithine/arginine potentially functions as an early biomarker of diagnosis of RA. More studies are needed to gain an understanding of the underlying mechanisms that link disturbance in these amino acids with the initiation of the disease.

5. Conclusions

Our preliminary study showed that serum ornithine/arginine could be used as a biomarker for diagnosing RA with acceptable sensitivity and specificity. Furthermore, our data suggest that serotonin levels may be associated with disease severity, as we observed significantly elevated levels only in patients with high disease activity. However, more extensive studies are needed to establish whether serotonin could serve as a reliable marker for disease severity in RA. Although we included a relatively low number of subjects from a single center, the data can certainly constitute a start point for future extensive research. The potential insight gained from more research on this topic can establish what serum biomarkers can be included in clinical practice as additional therapeutic targets.

CRediT authorship contribution statement

Safwan M. Al-Adwan: Supervision, Software, Project administration. **Talal S. Al-Qaisi:** Writing – review & editing, Writing – original draft, Visualization. **Ghaleb A. Oriquat:** Validation, Software, Resources. **Hamdi Nsairat:** Methodology, Investigation, Formal analysis. **Tahia H. Saleem:** Investigation, Data curation, Conceptualization. **Samar H. Goma:** Writing – review & editing, Validation, Supervision. **Ahmed H. Fangary:** Validation, Supervision, Software. **Mahmoud S. Abu-Samak:** Writing – original draft, Visualization, Software, Resources, Project administration. **Marwa A. Gaber:** Project administration, Methodology, Investigation, Formal analysis.

Informed consent statement

Informed consent was obtained from all subjects involved in the study. Written informed consent was obtained from the patient(s) to publish this paper.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki. Initial ethics approval was obtained on February 24, 2020 for study registration and protocol development. Due to COVID-19 pandemic restrictions, all participant recruitment and sample collection (starting August 2021) were conducted under the authority of the original **February 24, 2020** approval.

Data availability statement

Data included in the article/supplementary material is referenced in the article. Additional data will be made available on request. For requesting data, please write to the corresponding author.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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