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Clinical significance of plasma tryptophan, kynurenine, and kynurenine/tryptophan ratio in rheumatoid arthritis patients

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ABSTRACT

Background: The catabolism of tryptophan (Trp) in the kynurenine (Kyn) pathway is thought to have a critical immunosuppressive effect.
Aim of the work: To evaluate plasma Trp and metabolite levels to identify their diagnostic potential in rheumatoid arthritis (RA).
Patients and methods: 50 RA patients and 41 control were included in this study. The Trp and Kyn were analyzed and the Kyn\Trp ratio was calculated to estimate Indolamine 2,3 dioxygenase (IDO) enzyme activity involved in Trp degradation.
Results: The 50 patients had a mean age of 58·5 ± 10·6 years; 37 females and 13 males, F:M 2.8:1 and median disease duration was 10·1 (7–16) years. Rheumatoid factor was positive in 64 %. The median Trp value was significantly lower in RA (11120·7; 3259–16352 ng/ml) compared to control (12372·3; 7217–31,936 ng/ml) ($p = 0·001$). Kyn/Trp was significantly higher in RA (4·04; 2·5-12·3) compared to control (3.2; 2·1-4·7) ($p < 0·0001$). The median Kyn value was higher in RA (451·02; 264–1292 ng/ml) than in control (391·4; 236–1494 ng/ml) ($p = 0·04$). Trp significantly inversely correlated with the morning stiffness ($r = −0·32$, $p = 0·025$), Kyn significantly correlated with the C-reactive protein (CRP) ($r = 0·31$, $p = 0·028$) and erythrocyte sedimentation rate (ESR) ($r = 0·41$, $p = 0·003$) and the Kyn/Trp ratio correlated with the morning stiffness, CRP and ESR ($r = 0·26$, $p = 0·045$; $r = 0·32$, $p = 0·025$ and $r = 0.32$, $p = 0·024$ respectively). Kyn/Trp, Kyn and Trp significantly predicted RA at a cut-off value of 4.72, 589.1 ng/ml and 9921.1 ng/ml ($p < 0.0001$, $p = 0.04$ and $p = 0.001$ respectively).
Conclusion: Our study showed that there is a significant relationship between RA and Kyn and Trp levels and IDO enzyme activity.

1. Introduction

Rheumatoid Arthritis (RA) is a systemic, autoimmune, inflammatory disease in which excessive proinflammatory cytokine release causes inflammation and tissue destruction. Although the exact etiology of RA is unknown, much information has been obtained recently about its molecular and cellular mechanisms [1]. It is known that immune system activation and excessive cytokine release have essential roles in pathophysiology [2]. Proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8 are involved in expressing cell adhesion molecules, migration, and adhesion of leukocytes in inflamed tissue. They also have a co-stimulatory effect on leukocyte activation and lymphocyte proliferation [3]. T lymphocytes have been shown to play an essential role in the pathophysiological processes in the initiation and maintenance of RA. It has been found that the number of active T lymphocytes in the synovial fluid and peripheral blood of patients with RA is increased [4].

Tryptophan (Trp), an essential amino acid, is a precursor for protein synthesis and the production of various molecules involved in fundamental biological processes [5]. The majority (99 %) of Trp

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is metabolized via the Kynurenine (Kyn) pathway, and the remaining 1 % is converted to serotonin and melatonin via the serotonin pathway [6,7]. It is thought that an important immunosuppressive effect occurs with the catabolization of Trp in the Kyn metabolic pathway [8]. Decreased Trp concentrations in peripheral blood have been found in RA patients [9,10], various neoplasms [11], and autoimmune diseases such as antineutrophil cytoplasmic antibody-associated vasculitis [12]. Indolamine 2,3 dioxygenase (IDO) is an enzyme responsible for Trp catabolism in the Kyn metabolic pathway [13]. Local or systemic up-regulation of IDO expression in tissues due to immune activation and inflammation increases Trp catabolism in the inflammatory cell microenvironment and lowers its level [14,15]. Because T cells are sensitive to low Trp concentrations, it has been reported that potentially harmful autoimmune response is down-regulated, peripheral intolerance is prevented, and persistent immune hyperactivation is reduced due to IDO activity [16,17]. IDO activity causes stress kinase general control non-derepressible-2 (GCN2) activation due to Trp depletion and accumulation of uncharged tRNAs. Thus, a state of anergy is induced in effector T cells leading to cell cycle arrest and proinflammatory responses, and impaired cell proliferation. In addition, the accumulation of Kyn causes immunosuppressive effects by cell differentiation to T-regulatory lymphocytes and concomitant weakening of effector T-cell responses [18].

There are a limited number of studies evaluating Trp and its metabolism in RA [9,10,19–21]. In these studies, it has been reported that Trp, Kyn, and IDO may be closely related to the pathophysiology of RA and represent a possible checkpoint that can be targeted for the diagnosis and treatment of RA. This study has been conducted to support the potential role of IDO and to clarify the usefulness of determining Trp degradation and Kyn levels in RA.

2. Patients and methods

A total of 50 RA patients diagnosed according to the American College of Rheumatology/European League Against Rheumatism [22] and followed-up in the rheumatology department of Ankara Yıldırım Beyazıt University Ankara City Hospital, were included. A total of 41 matched healthy volunteers were considered as a control group. All RA patients were receiving at least one disease-modifying antirheumatic drug (DMARD) or biologic therapy. To minimize the change in serum levels of Trp and its metabolites due to acute phase reactants and to more accurately predict the possible effect of Trp metabolites on RA pathophysiology, patients with morning stiffness <20 minutes and with a low acute-phase response and with no active synovitis on physical examination were selected and considered to have low disease activity. The ethics committee approved the study of Ankara Yıldırım Beyazıt University Faculty of Medicine. All patients included in the study gave informed consent.

Collection of samples and calculation of IDO enzyme activity: Blood samples were taken from patients into tubes containing EDTA after 12 h of fasting and centrifuged at 4000 rpm for 10 min and the plasma samples were obtained and stored in a freezer at −80 °C until analysis. Trp and Kyn analyses were performed in plasma samples. Erythrocyte sedimentation rates (ESR) and C-reactive protein (CRP) levels from patients and controls were measured within 3 hours of sample collection. Rheumatoid factor (RF) and anti-cyclic citrulline peptide (anti-CCP) were assessed in the patients.

Trp and kynurenine were measured by the in-house method. L-TRP (CAS No.: 73-22-3), L-KYN (CAS No. 2922-83-0), L-TRP-(indol-d5) (Catalog No: 615862), high-performance liquid chromatography-grade water (CAS No.: 7732-18-5), and acetonitrile (CAS No: 75-05-8) were obtained from Sigma Aldrich (St. Louis, MO, USA). L-KYN-d4 trifluoroacetic acid salt (Catalog No: K661007) was obtained from Toronto Research Chemicals (North York, ON, Canada). Methanol (Catalog No: M1060182500), formic acid (Catalog No: M100253.1000), and trifluoroacetic acid (TFA, %99, Catalog No: M110255.0025) were obtained from Merck (Darmstadt, Germany).

Analysis of Trp and Kyn was performed to determine IDO enzyme activity. IDO, the rate-limiting enzyme of the kynurenine pathway, converts Trp to Kyn. Kyn/Trp ratio gives us the IDO enzyme activity, which means the kynurenine pathway's speed, which constitutes 99 % of Trp metabolism in the participants. Trp and Kyn were analyzed using the modified in-house method by Huang et al. [23] in the LC-MS/MS device (Thermo Scientific™ TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer, San Jose, USA). Calibrator and control samples for Trp and Kyn were produced using Trp and Kyn stocks in combination. Internal standard (IS) stock solutions of Trp-d5 and Kyn-d4 were prepared at 1 mg/ml in 50 % acetonitrile. IS working solution containing 3.5 µg/ml Trp-d5 and 1.1 µg/ml Kyn-d4 was prepared using distilled water and stored at 4 °C. Briefly while preparing the samples, 100 µl of a plasma/calibrator/control samples, 300 µl of internal standard (containing Trp-d5, kyn-d4, and acetonitrile), and 50 µl of TFA (trifluoroacetic acid) were taken into Eppendorf tubes and mixed for 30 s. Then, the mixture was centrifuged at 12000 rpm for 15 min. Supernatants were transferred into clean autosampler vials for analysis. The synergy polar RP (reverse phase) column (75 × 4.6 mm, Phenomenex, CA, USA) was used for chromatographic separation of analytes by an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase contains 2 % acetonitrile, 5.2 % methanol, and 0.1 % formic acid in HPLC-grade water. The flow rate is 0.8 ml/min, and the analysis time of each sample is 8 min. The injection volume is 2 µl. The Autosampler temperature was set at 4 °C. Trp and Kyn were detected by TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA USA) in positive electrospray ionization SRM mode. Precursor/product transitions are *m/z* 204.9 > 187.9 for Trp, *m/z* 209.0 > 192.1 for Kyn, *m/z* 210.0 > 193.0 for Trp-d5 and *m/z* 213.0 > 196.0 for Kyn-d4.

Statistical analysis: It was performed using the Statistical Package for the Social Sciences (SPSS) version 22·0. Data were presented as mean and standard deviation (mean ± SD), median and IQR (Inter-quartile range), or numbers and percentages (%).Sample t-tests, Kruskal Wallis test, Mann-Whitney U, and Chi-square test were considered for comparison. Receiver operating characteristics (ROC) analysis was used to test the diagnostic accuracy measures of the indices with the AUC 95 % confidence interval and the optimum cut-off value was determined using the Youden index. $p < 0{\cdot}05$ level was considered significant.

3. Results

The 50 patients had a mean age of 58·5 ± 10·6 years; 37 females and 13 males, F:M 2.8:1, and 41 matched control (54·3 ± 14 years; 31 females and 10 males; p = 0.09 and p = 0.33 respectively). Characteristics of the patients and control are presented in Table 1.

In patients, there was no significant relationship between Trp, Kyn, and Kyn/Trp with the presence of hand erosions (p = 0·65, p = 0·99, p = 0·75, respectively), lung parenchyma involvement (p = 0·71, p = 0·09, p = 0·2, respectively), RF (p = 0·31, p = 0·84, p = 0·28, respectively), and anti-CCP positivity (p = 0·7, p = 0·24, p = 0·61, respectively). There was no significant relation to medical treatment type (p = 0·09, p = 0·25, p = 0·35, respectively).

Table 2 shows the correlation between patient age and other disease parameters in the RA group and Trp, Kyn, and Kyn/Trp. Table 3 shows the ROC analysis of Trp, Kyn, and Kyn/Trp with significance at certain cut-off values (Fig. 1).

Table 1
Characteristics of rheumatoid arthritis patients and control.

Parameters n(%) or mean ± SD or median (IQR)	RA (n = 50)	Control (n = 41)	p
Female:male	37:13 (2.8:1)	31:10 (3.1:1)	0·33
Age (years)	58·5 ± 10·6	54·3 ± 14	0·09
Disease duration (years)	10·1 (7–16)	–	–
Body mass index	27·24 ± 4·05	28·4 ± 4·89	0·14
Smoking	10 (20)	8 (19·5)	0·46
Lung involvement	5 (10)	–	–
Comorbidities	16 (32)	14 (34·1)	0·17
Morning stiffness (min)	12·4 (5–20)	–	–
Hemoglobin (x10^9/L)	11·9 ± 4·3	12·6 ± 4·3	0·19
Platelets (x10^9/L)	308 ± 105	285 ± 76	0·46
WBC (x10^9/L)	7030 ± 2640	6130 ± 2040	0·11
Lymphocyte (x10^9/L)	2646 ± 559	2045 ± 454	0·18
Neutrophil (x10^9/L)	4220 ± 958	3860 ± 742	0·13
ESR (mm/1st h)	20·2 ± 7·5	17·5 ± 5·8	0·09
CRP (g/L)	8·2 ± 2·8	5 ± 1·7	0·07
Creatinine (mg/dl)	0·94 ± 0·44	0·82 ± 0·38	0·25
ALT (U/L)	24·8 ± 7·4	18·6 ± 6·9	0·21
AST (U/L)	33 ± 10·8	26 ± 8·9	0·13
LDH (U/L)	215 ± 54	191 ± 43	0·14
RF positivity	32 (64)	–	**–**
Anti-CCP positivity	24 (48)	–	**–**
Tryptophan (ng/ml)	11120·7 (3259–16352)	12372·3 (7217–31936)	**0·001**
Kynurenine (ng/ml)	451·02 (264–1292)	391·4 (236–1494)	**0·04**
Kyn/Trp	4·04 (2·5-12·3)	3·2 (2·1-4·7)	**<0·0001**
Erosions (hand X-ray)	13 (26)	–	**–**
Corticosteroids	42 (84)	–	**–**
csDMARD	34 (68)	–	**–**
anti-TNF	3 (6)	–	**–**
tsDMARD	6 (12)	–	**–**
IL-6 blocker	3 (6)	–	**–**
Rituximab	3 (6)	–	**–**

RA: Rheumatoid Arthritis**,** WBC: white blood cells, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, ALT: alanine aminotransferase, AST: aspartate aminotransferase, LDH: lactate dehydrogenase, Kyn: kynurenine, Trp: tryptophan, RF: rheumatoid factor, anti-CCP: anti-cyclic citrullinated peptides, csDMARD: conventional synthetic disease-modifying antirheumatic drugs, anti-TNF: anti-tumor necrosis factor, tsDMARD: targeted synthetic disease-modifying antirheumatic drugs, IL-6: interleukin-6. Bold values are significant at p < 0.05.

4. Discussion

Rheumatoid arthritis (RA) is a multifactorial disease. Genetic predisposition and environmental triggers including infections are the major players in autoimmunity [24]. Furthermore, pro-inflammatory cytokines play an important role in the initiation and perpetuation of inflammation in RA [25]. Oxidative stress is profound in RA [26] and the tryptophan metabolites via the kynurenine pathway are proposed as new and sensitive markers of oxidative stress status [27]. Activation of the kynurenine pathway leads to the accumulation of its pro-oxidative metabolites and an increase in the oxidative stress level, which increases mitochondrial damage, and disrupts the cellular energy metabolism [28]. Despite the many treatment options available for RA, there is still a need for new therapeutic options with fewer side-effect profiles and can ideally cure the disease.

This work focused on the known roles of Trp and its metabolic products, which are thought to be a possible checkpoint mechanism in the pathogenesis of RA. The present results confirmed a limited number of studies [9,10,19–21] showing that RA is associated with low Trp levels, high Kyn/Trp ratio, and high Kyn levels.

An animal study of RA induced by collagen-induced arthritis (CIA) showed that pharmacological inhibition of IDO resulted in increased incidence and severity of arthritis [29]. Similarly, another study showed that increased Trp catabolism following administration of iDO-containing exosomes ameliorated CIA [30]. *Desvignes and Ernst* showed that an equimolar Kyn mixture could inhibit IL-17 production in a dose-dependent manner. In addition, they reported that Kyns were able to suppress the capacity of IL-23 to stimulate Th17 [31]. In addition, it has been reported that Kyns have an immunosuppressive effect by simultaneously inducing Treg cells and attenuating effector T cell responses [32]. In studies on IDO and Trp metabolism in RA patients, although Trp levels in the serum of patients were found to be significantly decreased, no significant increase was observed in Kyn concentrations [8,9,21]. Trp metabolism is active in the synovial tissue of patients with arthritis [20,33]. Similar to these studies, in the present work, Trp levels were found to be significantly lower and Kyn levels significantly higher in RA patients compared to controls.

Decreased dietary intake of Trp reduces plasma Trp levels along with Kyn concentrations but does not change the Kyn/Trp ratio. Therefore, it has been stated that the IDO enzyme activity estimated by the Kyn/Trp ratio for Trp degradation is a better indicator than the absolute Trp concentration in serum [34,35]. It has been reported that RA patients show a Trp metabolite distribution characterized by a high Kyn/Trp ratio in their serum [10,19,21]. Similarly, in this study, IDO enzyme activity was significantly higher in RA than in controls.

No significant difference was found between RA patients receiving different treatments in terms of Trp, Kyn concentrations, and Kyn/Trp ratio. Similarly, in other studies, no significant relationship was observed in Trp, Kyn concentrations, and Kyn/Trp ratio between patients receiving treatment from different groups used in RA [9,10,36].

In the current literature, there are not enough studies evaluating the relationship between disease activation parameters and Trp and its metabolites in RA. Contradictory results were obtained in two previous studies with a small number of patients. Özkan et al. found a significant correlation between CRP and Kyn in RA patients [36]. On the contrary, Kang et al. found a negative correlation between Kyn and ESR and CRP in RA [20]. This study found a significant correlation between Kyn/Trp ratio and CRP, ESR, and morning stiffness, between Kyn and CRP and ESR, and inversely between Trp and morning stiffness.

The limitations of this study include that it was cross-sectional, on a small sample of patients with low acute phase reactants and short morning stiffness.

This study focused on the known roles of Trp, Kyn, and IDO, which are thought to represent critical immune checkpoint mechanisms in the pathogenesis of RA. Although patients with RA have increased tryptophan metabolism, the significance of this finding is still unclear. No association was found with RF or anti-CCP positivity, radiological damage, or lung involvement. Although a correla-

Table 2
Correlation between tryptophan metabolites and rheumatoid arthritis patient's age and disease activity parameters.

Parameter r(p)	Trp	Kyn	Kyn/Trp
Age	−0·06 (0·69)	−0·04 (0·79)	−0·06 (0·97)
Duration of disease	0·07(0·65)	−0·03 (0·85)	−0·002(0·99)
Morning stiffness	**−0·32(0·03)**	0·14(0·34)	**0·29(0·045)**
CRP	−0·16(0·27)	**0·31(0·03)**	**0·32(0.03)**
ESR	−0·003(0·98)	**0·41(0·003)**	**0·32(0·02)**

CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, Kyn: kynurenine, Trp: tryptophan Bold values are significant at p < 0.05.

Table 3
The cut-off levels, specificity, and sensitivity of indexes in rheumatoid arthritis patients.

Parameters	Cut-off	AUC (%95Cl)	Sensitivity (%)	Specificity (%)	LR	p
Trp (ng/ml)	9921·1	0·7 (0·59-0·81)	26	95·1	5.3	**0·001**
Kyn (ng/ml)	589·1	0·63 (0·51-0·74)	18	97·6	7·5	**0·04**
Kyn/Trp	4·72	0·73 (0·63-0·83)	30	97·6	12·5	**<0·0001**

AUC: area under the curve, LR: likelihood ratio, Kyn: kynurenine, Trp: tryptophan. Bold values are significant at p < 0.05.

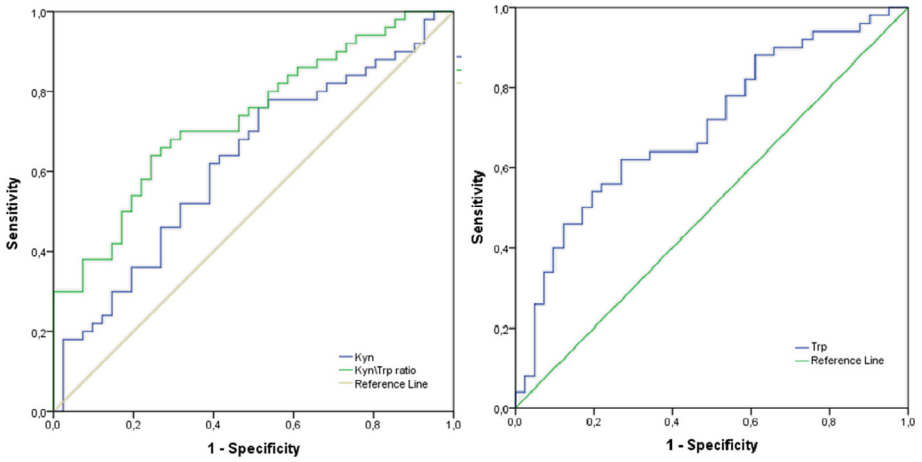

Fig. 1. Receiver operating characteristic (ROC) curve of Kynurenine (Kyn) level and Kyn/Trp ratio (left) and Tryptophan (Trp) level (right) in rheumatoid arthritis patients.

tion was found between Kyn/Trp ratio and CRP, ESR, and morning stiffness, the relationship between tryptophan metabolism and disease activity should be investigated. More extensive studies are needed to support the potential role of IDO in understanding disease progression in RA and to clarify the usefulness of determining Trp degradation and Kyn levels.

In conclusion, this study showed that the ratio of Kyn/Trp showing IDO enzyme activity, Trp levels, and Kyn levels confirmed a significant association with RA.

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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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