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Serum free amino acid levels in rheumatoid arthritis according to therapy and physical disability

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A B S T R A C T

Background: In presented study the amino acid analysis was performed in serum derived from rheumatoid arthritis patients (RA) according to undertaken therapy and classification of physical disability. The results were compared with previously published data.

Methods: The levels of 31 free amino acids were determined in 50 serum samples derived from RA subjects and 51 controls. The RA patients were divided into two groups according to the therapy (methotrexate/leflunomide, infliximab/adalimumab/etanercept/tocilizumab, prednisolone/NSAID) and classification of physical disability of the patients. Levels of amino acids were measured by LC-MS/MS. The obtained results were subjected to multivariate statistical tests.

Results: According to the therapy that was being used, threonine differentiated RA patients treated with methotrexate/leflunomide - infliximab/adalimumab/etanercept/tocilizumab ($p = 0.00954$) and infliximab/adalimumab/etanercept/tocilizumab - prednisolone/NSAID ($p = 0.03109$), while tryptophan differentiated RA patients treated with methotrexate/leflunomide - infliximab/adalimumab/etanercept/tocilizumab ($p = 0.01723$). In the functional classification, arginine differentiated RA samples between class III and IV ($p = 0.02332$), while glycine differentiated them between class I + II and III of the Steinbrocker functional classification ($p = 0.03366$).

Conclusions: An analysis of the metabolome profile requires the use of validated bioanalytical methods that are strictly dedicated for this purpose. The obtained results are not accidental (p value less than 0.05), and all of the selected amino acids play an important role in inflammation and immune response. It is suggested that studied amino acids can be considered as a markers for diagnosis of RA and monitoring pharmacotherapy of the disease.

1. Introduction

It is generally known that amino acids play a crucial role in human body homeostasis, including a regulatory role in gene expression, protein turnover, cellular signaling, DNA and RNA synthesis, and acid-base balance regulation [1]. It is well known, that the overall amino acid profiles of the human body are dynamic and vary depending on different physiological or pathological conditions, what makes a great opportunity to looking for amino acid serum levels as new potential biomarkers in disease diagnosis or therapy monitoring. Disease-associated alterations of amino acid profiles were described for various diseases, e.g. cancer [2], insulin resistance [3–5], inflammatory diseases [6], sepsis [7], and in rheumatoid arthritis [8]. It has been suggested that the metabolic fingerprint reflects inflammatory disease activity, demonstrating that underlying inflammatory processes cause significant changes in the metabolism, which can be measured in the peripheral blood. The identification of metabolic alterations may provide insight into disease mechanisms operating in patients with

Abbreviations: AA, Amino acids; ANOVA, Analysis of variance; Bio-DMARDs, biological disease-modifying anti-rheumatic drugs; DMARDs, Synthetic disease-modifying anti-rheumatic drugs; DAS28, Disease activity score for rheumatoid arthritis; ESI, Electrospray ionization; GC, Gas chromatography; HPLC, High performance liquid chromatography; IL, interleukin; MS, Mass spectrometry; NAISD, Nonsteroidal anti-inflammatory drugs; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NMR, Nuclear magnetic resonance; NYHA, New York Heart Association; RA, Rheumatoid arthritis; sMRM, Scheduled multiple reaction monitoring; TLR, Toll like receptor; TNF-alpha, Tumor necrosis factor-alpha; TOF, Time of flight; VAS, Visual analog scale

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inflammatory arthritis [9].

The study presents an analysis of serum free amino acids in rheumatoid arthritis (RA) patients. The aim was to compare the metabolomics profile of free serum amino acids between RA patients and controls, thus first the concentration levels were established for both. This allowed to estimate the relative amino acid levels in the group of both patients and controls. The aim and novelty of the study presented here was to assess the feasibility of employing a serum free amino acid profile as a potential instrument for therapy monitoring of rheumatoid arthritis in patients treated with biological disease-modifying anti-rheumatic drugs. An amino acid metabolomics profile was also used as a tool to estimate the extent of physical disability in RA patients.

The authors also conducted an analysis of recent publications describing the utilization of metabolomics studies, particularly amino acids analysis, as a potential tool for predicting disease progression and in therapy monitoring in patients suffering from RA. The authors pointed out that the proper selection of a validated analytical methodology plays an important and crucial role in proper inference of experimentally obtained data.

2. Materials and methods

2.1. Patients

The project was focused on a targeted analysis of up to 42 proteinogenic and non-proteinogenic amino acids in blood serum samples derived from RA subjects and a matched control group. The investigations presented in this article were divided into several steps. First, the serum free amino acid level in both RA and control samples was determined. Second, amino acid levels were measured in RA patients, who were divided for this purpose into two separate subgroups according to the therapy used (DMARDs, Bio-DMARDs and other) and their physical disability as denoted by the Steinbrocker functional scale. Furthermore, statistical analysis was applied according to the selection of amino acids that differentiated the group of RA patients from the controls as well as the RA subgroups. Finally, based on the experimental data that were obtained the authors conducted an analysis of previously obtained data that were available in the literature.

50 patients (Caucasian strain) were selected to participate in this study and were recruited by specialists in rheumatology from the Clinic of Rheumatology and Rehabilitation, Poznan University of Medical Sciences.

All of selected patients were examined due the fulfilling of the following inclusion criteria: (1) age of participant over 18 years; (2) proved diagnosis of RA according to the American-European Consensus Group classification [10]; (3) RA duration longer than 3 months; 4) all included RA patients were seropositive. Moreover, the group of RA patients was also described by the Steinbrocker functional classification (Class I to Class IV) according to the observed changes on radiographs; as well as in all cases the following laboratory tests were carried out and taken into the consideration: hematocrit (HCT), red blood cells (RBC), hemoglobin (HGB), white blood cells (WBC), platelets (PLT), lymphocytes, neutrophils, C reactive protein (CRP), rheumatoid factor (RF), anti-cyclic citrullinated peptide antibody (anti-CCP antibody), erythrocyte sedimentation rate (ESR), creatinine (with calculated glomerular filtration rate-GFR), aspartate and alanine transaminase ratio (AST, ALT).

The exclusion criteria were set as follows: (1) occurrence of symptoms of opportunistic infections in the last two months before blood sample collection; (2) occurrence of symptoms of acute infections in a last quarter before blood sample collection; (3) lung, kidney or liver insufficiency; (4) coexistence of heart failure (NYHA class II above); (5) HIV infection; (6) cancer disease and anti-cancer therapy during last 5 years; (7) other autoimmune disorders including demyelinating diseases and (8) pregnancy.

All patients were informed about the course of the study and gave written, informed consent for participation in the study.

2.2. Therapy involved

Conventional synthetic disease-modifying anti-rheumatic drugs (DMARDS) (methotrexate or leflunomide) or biological anti-TNF DMARDS, including etanercept, tocilizumab, adalimumab or infliximab were administered to the RA-patients. Any of medication changes were introduced during last 3 months. Some of the RA patients required additional supplementation of the treatment with non-steroidal anti-inflammatory drugs or administration of 5 mg per day (or less) of prednisolone.

To evaluate disease activity the DAS-28 sore was used [11]. Despite the treatment, none of the patients had complete remission (mean DAS-28 was 5.24 ± ; ranging from 3.11 to 8.08).

2.3. Control group

The control group included 64 healthy blood donors (with no inflammatory disease). The serum samples of subjects selected for the control group were purchased from the Regional Blood Center in Poznan and underwent the same laboratory tests as the RA patients (2.1. inclusion criteria), and were in the range of the reference values. Moreover, control subjects samples were also tested for the HIV and anti-HIV antigen, anti-HCV and HBs antigen.

2.4. Preparation of the physiological samples

All patients had to fast overnight before their blood samples were collected. The collected blood samples were immediately centrifuged, frozen and stored at −80 °C until further analysis. All RA and control samples used in the study were analyzed within one analytical (frozen-thawing) cycle.

The samples were mixed directly after thawing, and 40 μL of serum was transferred to an Eppendorf tube. Then 10 μL of sulfosalicylic acid containing norleucine (for recovery and labeling efficiency) was added. The tube was vortexed promptly and centrifuged at 10 000 rpm for 2 min to ensure full protein precipitation and separation from the supernatant. A total of 10 μL of supernatant was transferred to a clean Eppendorf tube and diluted with 40 μL of labeling buffer to ensure an appropriate pH value for further reactions. The labeling buffer contained norvaline to ensure secondary labeling efficiency. After mixing and centrifugation, 10 μL of the mixture was transferred again to a clean Eppendorf tube. In the next step, AA labeling was conducted. To label amino acid residues in a physiological sample, 5 μL of freshly prepared aTRAQ Reagent Δ8 solution was added to 10 μL of the mixture sample/labeling buffer. After vortexing and centrifugation, the vial was incubated for 30 min at room temperature. The labeling reaction was stopped by adding 5 μL of hydroxylamine. After mixing and centrifugation, 15 min of incubation at room temperature was required, according to the protocol. In the last step, 32 μL of internal standards solution was mixed with the labeled sample. The centrifuged labeled sample with internal standard was evaporated for 15 min at 50 °C in a vacuum concentrator (miVac Duo Concentrator, Genevac, Stone Ridge, NY, USA). Prior to analysis, the sample was diluted with 20 μL of water and transferred to an autosampler vial.

2.5. HPLC-MS/MS analysis

The applied procedure allowed simultaneous qualitative and quantitative analysis of 42 amino acids in human serum and provided superior specificity via use of the sMRM mode. It can be stated that the aTRAQ™ methodology is one of the most reliable and comprehensive methods of measurement of free amino acids in biological fluids [12,13].

Separation and measurement of the amino acids was conducted

using a QTRAP 4000 mass spectrometer (Sciex, Framingham, MA, USA) coupled with an Agilent Infinity 1260 LC system (Agilent Technologies, Santa Clara, CA, USA). A Sciex C18 (5 µm, 4.6 mm × 150 mm) column for amino acid separation, maintained at 50 °C, was used. A binary gradient of 0.1% formic acid (FA) and 0.01% heptafluorobutyric acid in water (mobile phase A) and 0.1% FA and 0.01% heptafluorobutyric acid in methanol (mobile phase B) was applied at a flow rate of 0.8 mL/min. The following gradient was used: 0–6 min 2–40% B, 6–10 min 40% B, 10–11 min 40–90% B, 11–12 min 90% B, 12–13 min 90–2% B and 13–18 min 2% B.

The detection was performed using a QTRAP 4000 tandem mass spectrometer equipped with an electrospray ionization source (ESI). A positive ionization mode and a scheduled multiple reaction monitoring (sMRM) mode were applied. According to the aTRAQ protocol, the ion spray parameters were as follows: ion spray voltage 4500 V, entrance potential (EP) 10 V, declustering potential (DP) 30 V, and collision cell potential (CXP) 50 V. All MRM transitions and collision energy parameters were provided in the supplementary materials (Supplementary Table 1). Validation results using serum samples were described in our previous paper [12]. Data acquisition and determination of the AA concentrations were conducted using Analyst 1.5.2. software (Sciex).

2.6. Data analysis

Statistical analysis was conducted using STATISTICA 12.5 software to mark the amino acids that differentiated the study and control group as well as the various subgroups of RA patients. First, the Shapiro-Wilk test of normality was performed to check that the obtained concentration levels were normally distributed. For normally distributed data, Levene's test was carried out to assess the equality of variances between the study and control group. Then the student's *t*-test and Welch test were applied for the population with equal variances and different variances, respectively. The data that were non-normally distributed were analyzed with the Mann–Whitney *U* test. For all tests, the *p* value for statistical significance was defined as $p \leq 0.05$. In the second step of statistical analysis the authors tried to find correlations between the amino acid concentration and treatment, clinical disease activity (as the DAS28 parameter), and the Steinbrocker functional classification of the RA disease. After verifying the assumption of *normality of data* and *confirming* the equality of variances, one-way analysis of variance (ANOVA) was applied. If the data distribution was non-normal or there was no equality of variances, the Kruskal–Wallis test by ranks was used for comparison of the subgroups.

3. Results

3.1. Alteration in serum free amino acid profiles between RA patients and the control group

The project involved the application of a targeted metabolomics investigation focused on profiles of free amino acids and their derivatives in the serum of RA patients and a matched control group. The assays were performed using an advanced analytical method of liquid chromatography-tandem mass spectrometry which is characterized by high sensitivity and accuracy. The method allowed to obtain a very broad profile of free amino acids, including both proteinogenic and non-proteinogenic amino acids and some of their derivatives, and thereby provided data on a wide variety of metabolic changes occurring in RA patients.

The applied bioanalytical method allowed for the detection and quantification of 31 out of 42 amino acids in all of the serum samples, and their levels were then subjected to statistical analysis while the remaining 11 amino acids were found in a part of the analyzed samples or their concentrations were below the level of quantification. A total of 31 serum free amino acid concentrations were established for 50 samples derived from RA subjects and 51 control samples (Table 2).

Table 1
The structure of rheumatoid arthritis (RA) patients and control group. The RA patients group was divided into three subgroups according to: DAS28, applied pharmacotherapy and Steinbrocker functional classification.

Comparison of RA patients and control group			Rheumatoid Arthritis (RA) Patients – structure of subgroups										
			DAS28			Applied pharmacotherapy				Steinbrocker classification;			
	RA	Control	DAS28 ≤ 3.2	DAS28 3.2 – 5.2	DAS28 ≥ 5.2	Synthetic DMARDs	BiologicalDMARDs	Additional NSAIDs or Steroids		I	II	III	IV
Group size (n or %)	50	51	19%	44%	37%	74%	26%	28%		4%	20%	60%	16%
Number of females in a group	45	41	17%	40%	17%	51%	14%	24%		2%	20%	50%	18%
Number of males in a group	5	10	2%	4%	4%	2%	4%	4%		2%	–	8%	–
Range – age (years)	19–73	19–58	19–64	32–73	22–70	28–70	32–64	19–73		26	19–62	32–73	37–70
Average age (years)	51	31.78	48.75	55.2	47.37	50.17	48.33	52.30		41	41.87	54.17	57.75
Median – age (years)	54	30	56	57	50.5	54	53	59		41	42.5	55	60

Table 2
The determined concentration levels of free amino acids in the analyzed serum samples with the results of univariate statistical analyses.

	RA group			Control group			p Value	Fold change
	Median	Mean	SD	Median	Mean	SD		
Taurine (Tau)	59.27	65.64	27.84	149.89	147.36	44.66	< **0.00001**[*]	0.45
Asparagine (Asn)	46.99	48.07	10.19	53.80	57.88	16.45	**0.00096**[*]	0.83
Serine (Ser)	102.63	110.03	30.85	146.82	150.27	39.22	< **0.00001**[**]	0.73
Glycine (Gly)	230.27	253.42	89.60	291.95	314.72	91.51	**0.00040**[*]	0.81
Hydroxy-L-proline (Hyp)	9.93	11.70	7.24	8.96	11.40	6.94	0.96478[*]	1.03
Ethanolamine (EtN)	7.15	7.47	1.74	10.09	10.19	2.04	< **0.00001**[*]	0.73
Glutamine (Gln)	619.62	614.89	115.26	547.53	567.98	121.73	**0.04954**[**]	1.08
Aspartic acid (Asp)	3.50	4.25	2.03	18.88	21.14	11.36	< **0.00001**[*]	0.20
Citrulline (Cit)	27.61	28.01	7.78	28.32	28.39	6.53	0.79269[**]	0.99
Threonine (Thr)	97.46	101.98	22.93	119.93	126.06	38.86	**0.00086**[*]	0.81
Sarcosine (Sar)	0.99	1.07	0.42	1.25	1.33	0.49	**0.00338**[*]	0.81
β-Alanine (bAla)	15.12	16.02	7.16	19.19	18.70	8.73	0.11903[*]	0.86
Alanine (Ala)	340.19	359.90	104.02	491.80	478.12	129.84	< **0.00001**[**]	0.75
Glutamic acid (Glu)	60.31	63.13	21.36	68.44	74.26	35.19	0.14701[*]	0.85
Histidine (His)	67.65	66.41	14.13	80.46	78.68	18.79	**0.00035**[***]	0.84
1-Methyl-L-Histidine (1MHis)	3.78	7.23	7.71	6.10	8.02	7.49	0.16379[*]	0.90
3-Methyl-L-Histidine (3MHis)	3.46	3.86	1.73	3.61	3.74	1.16	0.68361[*]	1.03
α-Aminoadipic acid (Aad)	0.66	0.75	0.30	0.84	0.90	0.46	0.10093[*]	0.83
β-Aminoisobutyric acid (bAib)	1.44	1.62	0.96	1.38	1.68	0.96	0.79894[*]	0.97
α-Amino-n-butyric acid (Abu)	22.63	23.41	7.67	23.98	24.38	7.42	0.41308[*]	0.96
Arginine (Arg)	92.04	90.38	21.72	99.77	102.70	30.72	0.03991[*]	0.88
Proline (Pro)	149.02	148.42	42.25	182.65	195.48	78.26	**0.00005**[*]	0.76
Ornithine (Orn)	74.54	76.30	21.21	99.20	100.23	31.06	**0.00002**[***]	0.76
Lysine (Lys)	216.31	216.53	45.42	204.95	215.05	63.32	0.77283[*]	1.01
Methionine (Met)	17.37	17.46	4.70	21.27	23.22	7.05	< **0.00001**[*]	0.75
Valine (Val)	196.92	201.99	37.43	235.56	238.40	61.22	**0.00052**[***]	0.85
Tyrosine (Tyr)	57.18	58.74	13.70	53.90	58.28	19.78	0.47783[*]	1.01
Isoleucine (Ile)	59.64	60.83	13.73	62.33	66.52	20.99	0.11120[*]	0.91
Leucine (Leu)	99.21	100.18	23.87	116.36	121.12	36.90	**0.00047**[*]	0.83
Phenylalanine (Phe)	55.38	57.33	10.72	66.14	65.94	16.24	**0.00136**[*]	0.87
Tryptophan (Trp)	44.563	45.45	10.93	49.16	50.3	10.80	**0.02701**[**]	0.90

[*] Mann-Whitney *U* test.
[**] *t*-Test.
[***] Welch test.

According to the Human Metabolome Database (www.hmdb.ca), the observed concentrations of the analyzed amino acids, in both the RA as well as control group, did not exceed the reference limit concentration in blood as established for healthy subjects (adults > 18 years). This is taken as evidence that both the RA and control group subjects did not suffer from any disease that could interfere with the obtained results. Data shown in Table 2 indicate that the serum concentrations of 18 out of the total 31 analyzed amino acids were significantly different ($p < 0.05$). The observed fold-change levels (Table 2) of the serum free amino acids between the group of patients and controls varied from 0.2 (aspartic acid, Asp) to 1.1 (glutamine, Gln).

In general it can be observed, that serum free amino acids levels were decreased in the RA patients samples, especially significant in the case of alanine, arginine, aspartic acid, glycine, proline and ornithine (Table 2). However, in the case of glutamine (Gln), valine (Val), and lysine (Lys) concentration levels were increased in the RA patients group when compared with controls; and levels of hydroxyl-L-proline (Hyp), citrulline (Cit), and tyrosine (Tyr) were nearly equal in both groups, respectively.

3.2. Serum amino acid concentrations and their correlation to the RA therapy used

All of the 50 analyzed blood serum samples derived from the RA patients were divided into three subgroups according to the therapy that was being used, namely DMARDs, Bio-DMARDs, and Other (Table 1). The DMARDs subgroup comprised RA patients treated with synthetic disease-modifying anti-rheumatic drugs, including methotrexate or leflunomide, and accounted for nearly 74% of the total RA subjects (Table 1). The Bio-DMARDs subgroup (accounting for nearly 26% of total RA patients) comprised RA subjects treated only with biological anti-TNF-alpha disease-modifying anti-rheumatic drugs (Bio-DMARDs), including infliximab, adalimumab, etanercept or tocilizumab, respectively. The OTHER subgroup (28% of the total RA patients) comprised only those RA subjects who, despite DMARDs or Bio-DMARDs treatment, were supported with stable doses of prednisolone or non-steroidal anti-inflammatory drugs. The statistical analysis found a correlation between two amino acids and the analyzed subgroups of RA patients according to the treatment that was being used. The selected amino acids were (Fig. 1) threonine (Thr) and tryptophan (Trp). The different levels of threonine allowed for statistical discrimination of the RA samples into DMARDs, Bio-DMARDs, or OTHER subgroups of patients. The following statistically significant differences were observed: DMARDs versus Bio-DMARDs ($p = 0.00954$) and Bio-DMARDs versus OTHER ($p = 0.03109$). In other words, the concentration levels of threonine in the RA serum blood samples varied according to the therapy procedure that was being used. Significantly highest concentrations of threonine were observed for patients treated with biological therapy only (Bio-DMARDs, 122.71 μM), while the lowest concentration levels of threonine were observed for subjects treated with DMARDs (96.55 μM) only (Fig. 1). In the case of tryptophan (Fig. 2), statistically significant differences were observed only for DMARDs versus Bio-DMARDs ($p = 0.01723$), respectively. However, samples representing the Bio-DMARDs subgroup of RA patients exhibited a higher concentration of Trp (52.17 μM) than in the DMARDs subgroup (42.06 μM).

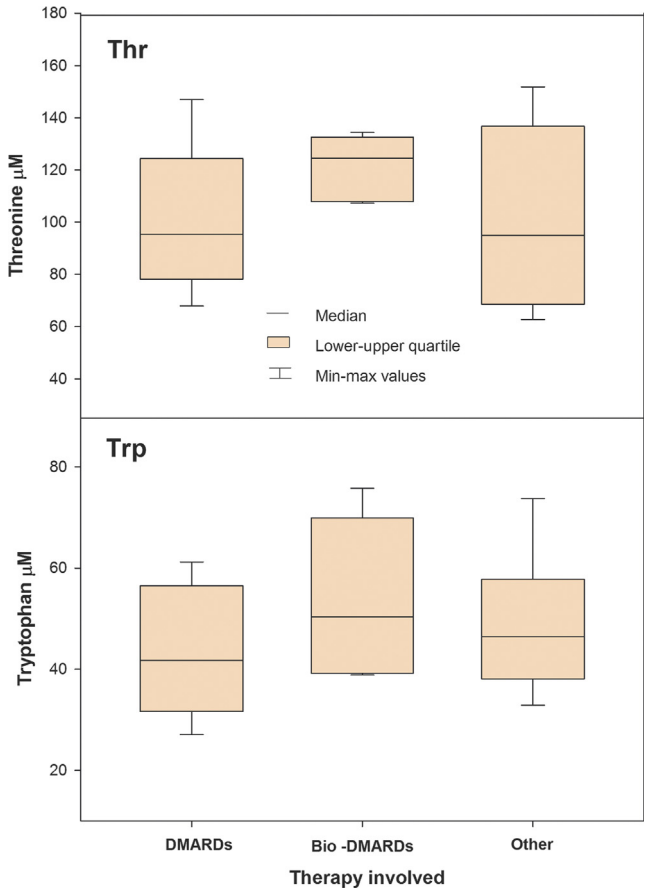

Fig. 1. Serum concentration of threonine (Thr) and typtophan (Trp) in rheumatoid arthritis patients divided according the therapy involved. DMARDs - patients treated with methotrexate or leflunomide, Bio-DMARDs - patients treated with biological disease-modifying anti-rheumatic drugs (infliximab, adalimumab, etanercept or tocilizumab), OTHER – patients with non-steroidal anti-inflammatory drugs or stable doses of prednisone of 5 mg per day or less. The statistically significant differences observed: **Thr:** DMARDs versus Bio-DMARDs, $p = 0.00954$ and Bio-DMARDs versus OTHER, $p = 0.03109$ **Trp:** DMARDs versus Bio-DMARDs, $p = 0.01723$.

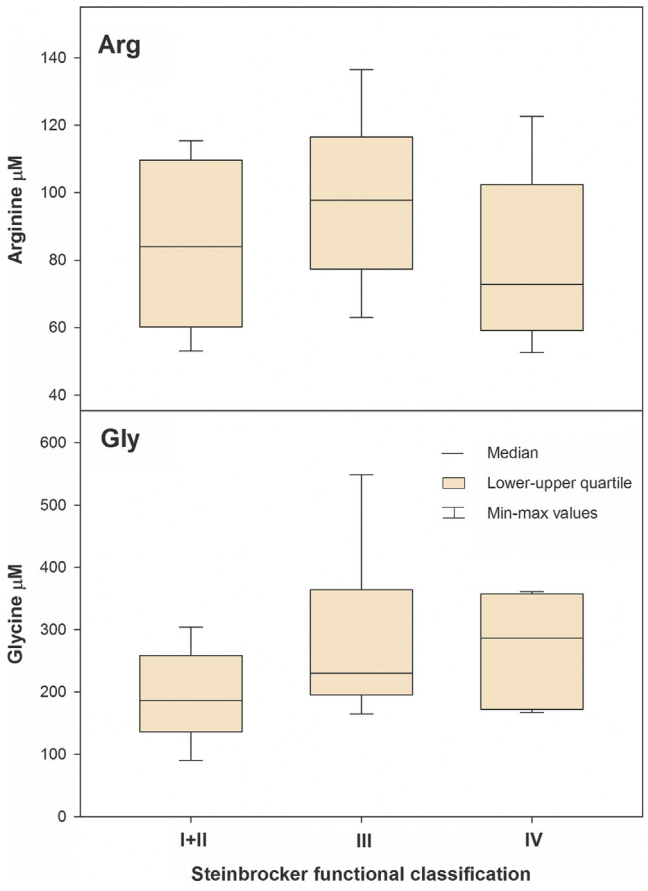

Fig. 2. Serum concnetration of arginine (Arg) and glycine (Gly) in rheumatoid arthritis patients divided according the Steinbrocker functional classification. I + II - patients classified to the I and II class of the Steinbrocker functional classification, III - patients classified to the III class of the Steinbrocker functional classification, IV - patients classified to the IV class of the Steinbrocker functional classification. The statistically significant differences observed. **Arg:** III versus IV, $p = 0.02332$, **Gly:** I + II versus III, $p = 0.03366$.

3.3. Serum amino acid concentrations and their correlation to the Steinbrocker functional classification of RA patients

According to Table 1, all of the analyzed RA patients were classified (from Class I to Class IV) according to the Steinbrocker functional classification of RA. The Steinbrocker functional classification is used to rate the extent of physical disability on a four-level scale, ranging from the patient's complete functional capacity to being largely or wholly incapacitated, bedridden, or confined to a wheelchair [14]. In this study the authors combined Classes I and II because only two RA patients fulfilled the clinical conditions of Class I of the Steinbrocker scale (4% of total RA patients), while 10 RA subjects (20% of total RA patients) fell into Class II of the Steinbrocker scale. The combination of Classes I and II was further consequently denoted as I + II. Moreover, Classes III and IV of the Steinbrocker classification reflected 60% and 16% of all the RA patients, respectively.

The obtained results show that two amino acids can differentiate RA subjects depending on the Steinbrocker classification. The selected amino acids are arginine (Arg) and glycine (Gly), respectively (Fig. 2). The estimated arginine levels allowed for differentiation of the RA samples into subgroups III and IV of the patients ($p = 0.02332$) according to the functional Steinbrocker classification. Significantly highest levels of arginine were found in the RA patients, subgroup III (97.05 µM), while the lowest were stated in group IV (77.32 µM), respectively.

In contrast, the different levels of glycine allowed to classify the RA patients into classess I + II and III of the Steinbrocker functional classification ($p = 0.03366$); the highest average concentration of glycine was observed in patients from class III (267.23 µM) than IV (263.79 µM) and, finally, class I + II (192.11 µM), respectively.

4. Discussion

Determining the amino acid profile of the human body allows to find new potential amino acid biomarkers that will be useful during the RA diagnosis process as well as therapy monitoring. The applied analytical procedure allowed for a simultaneous, qualitative, and quantitative analysis with superior specificity of up to 42 amino acids in human serum. Thus, this is the first study of such a wide profile of serum free amino acids in RA patients that utilized the most modern mass spectrometry-based methodology designed directly for this purpose.

In this paper the authors showed that the blood serum levels of 18 out of 31 determined amino acids differed in the group of RA patients in comparison with the control group. Based on data presented in Table 2, it can be stated in general, that amino acid concentration levels are decreased in RA subjects. A comparison of the obtained experimental data with those available from the scientific literature showed some discrepancies among previously reported studies as well as between them and our investigation [8,15–17]. There are a few possible reasons

for this lack of consistency between the obtained and previously published data, e.g. different methodologies, including sample collection and preparation, and a different bio-analytical methodology, thus the lack of complete consistency among the studies was not surprising. Madsen et al. utilized validated liquid-chromatography-mass spectrometry and gas-chromatography-mass spectrometry methods that required an additional derivatization process. It should be emphasized that 83 different metabolites were assigned by Madsen et al., while 52 of them were positively identified in both the study and the validation study; however, it was clearly stated that the obtained results were semi-quantitative [8]. Smolenska et al. used a methodology that was originally designed for analysis of coenzyme NAD metabolites (nicotinamide adenine dinucleotide) than amino acids in their study [15,18]. The results reported in the Priori review are combined and based on experiments conducted according to two different bioanalytical methodologies, namely mass spectrometry and nuclear magnetic resonance (NMR), respectively [16], while data reported by Kim et al. were obtained by utilization of the GC/TOF MS system which requires an additional derivatization process [17]. Thus it is extremely difficult to compare the literature data among these studies and with those presented in this paper.

The authors thus tried to compare the obtained experimental data with those available in the literature in order to find and equate the levels of serum free amino acids in the group of RA patients versus the control group, although different bioanalytical approaches were used. In an interesting and valuable paper presented by Madsen et al. [8], utilization of the untargeted metabolomics approach was described, 52 different metabolites were analyzed while 24 of them covered the amino acids analyzed in the current study; 11 of them exhibited different serum levels. Furthermore, Smolenska [15] established the blood concentration of 24 serum metabolites, in which 20 of them covered the amino acids used in our study. Only the concentrations of 8 of them revealed a tendency compatible with our findings. The serum amino acid levels reported by two other and independent groups [16,17] also varied when compared to our data or to data presented by Madsen and Smolenka. In the Priori review [16] only 4 out of 11 and only 4 out of 7 analyzed amino acids in Kim et al. [17] exhibited the same tendency as that presented in our results, respectively. Based on our data, we are strongly convinced that the analytical methodology used in our study allowed to obtain a metabolome profile that was possibly closest to real levels of analyzed amino acids occurring in RA patients as well as in the controls.

Despite the apparent differences in the serum free amino acid levels between the RA-derived samples and controls, it was also shown that some of the analyzed amino acid concentrations varied within the group of RA subjects according to the therapy that was being used and the Steinbrocker functional classification.

It was found that monitoring of the threonine and tryptophan serum levels allowed to distinguish RA patients depending on the therapy that was being used, namely DMARDs, Bio-DMARDs, or that supported with NSAIDs or steroids. The authors are convinced that these findings have some practical value in therapy progress and monitoring; however, the results presented in this paper do not strictly correspond with data that were recently published by Smolenska et al., in which arginine, asparagine, glutamic acid, and proline exhibited significant statistical differences in RA patients treated with DMARDs only, steroids only, a combination of DMARDs and steroids, and patients without any treatment, respectively.[15] Most of the amino acids selected by Smolenska et al. do not overlap with the results presented here. Furthermore, the data and results obtained in this study were compared with data reported by Madsen et al., who used metabolite profiling to assess its usefulness during the diagnostic process of early stage RA subjects and psoriatic arthritis according to an evaluation of a specific RA metabolome profile. They evaluated the relationships between metabolite levels and their fluctuations in both early and advanced stages of RA as well as in patients treated with different DMARDs regimens [8]. Ignoring the different bioanalytical methodologies proposed by either Smolenska et al. or Madsen et al., the lack of consistency between obtained and previously published data probably results from the structure of the RA subjects classified for the metabolomics studies. Kapoor et al. demonstrated that biological DMARDs (infliximab and etanercept) strongly altered the metabolite profile in urine, e.g. increased levels of hippuric acid, citrate, and lactic acid were associated with infliximab treatment, while increased choline, phenylacetic acid, urea, creatine, and methylamine levels were associated with etanercept treatment, and the presence of choline suggested that etanercept may alter the lipid metabolism [19]. Therefore, it can be concluded that Bio-DMARDs antirheumatic therapy causes meaningful changes in the metabolic profile when compared with other therapies.

As was stated above, the threonine and tryptophan serum levels allowed to distinguish RA patients depending on the therapy that was being used, namely DMARDs, Bio-DMARDs, or that supported with NSAIDs or steroids. An analysis of the scientific literature describing the threonine and tryptophan metabolic pathways shows that the selection of these two amino acids as potential indicators of the implemented anti-rheumatic therapy is not accidental. Both threonine and tryptophan are involved in the metabolic pathways that regulate cell danger response, stimulation of IL6 synthesis and T cell and TLR (Toll-like receptor) functions, and are involved in protein phosphorylation, O-linked glycosylation, nitric oxide and reactive oxygen species pathways that directly influence the interleukin, tumor necrosis and lipopolysaccharides metabolism [20–28]. Priori et al. indicated that some endogenous metabolites may discriminate RA patients with different disease activity or even predict the response to a particular treatment [16]. RA patients treated with methotrexate, after 24 weeks of introduced monotherapy exhibited elevated levels of taurine, methionine, and glycine. On the contrary, aspartate and tryptophan serum levels were significantly decreased after methotrexate therapy [16], which is in agreement with the findings presented in this paper. The presented threonine pathway is also connected with the tryptophan-dependent Toll-Like receptor (TLR) activation as mentioned above [21,29]. Threonine is reported to regulate epithelial cell migration and proliferation, cell differentiation, restoration of epithelial barrier functions, and modulation of cell apoptosis [30]. Moreover, threonine plasma level changes were observed in rabbits with an anterior cruciate ligament reconstruction injury [30].

The unique proposal of this study is to use an amino acid profile to estimate the extent of physical disability in RA patients. Some publications describe the correlation between DAS28 (denoting disease activity) and/or VAS (visual analog scale) and the metabolic profile. Nonetheless, both the DAS28 and VAS estimations are encumbered with some error due to an individual patient's estimation of his/her levels of pain. No publications were found that connected the Steinbrocker functional classification of RA patients with the metabolomics profile. The Steinbrocker functional classification ranges from class I (complete functional capacity) to class IV (incapacitated, largely or wholly bedridden or confined to a wheelchair with little or no self-care) [14]. It was found that significant changes between the arginine and glycine serum levels allowed for differentiation of RA patients depending on the Steinbrocker functional classes. The selection of arginine and glycine as potential indicators of RA patients according to the Steinbrocker functional classification is also unfortunate. Both arginine and glycine are known to be important amino acids in metabolism and nutrition, nitric oxide synthase regulation, or neurotransmission of the central nervous system. It was proven that both influence the immune responses by enhancing NO production (arginine) or stimulation of polarization of the cell membrane (glycine), thus resulting in the stimulation of TNF alpha or interleukin production. Therefore, concentrations of arginine in plasma are markedly reduced in response to inflammation or infection [31–34]. In the study presented here, the established arginine levels were lower in the RA group of patients in general when compared with the controls, and the lowest concentration of arginine was

observed in RA patients who belonged to class IV of the Steincbrocker classification.

Glycine is a nonessential amino acid. It is well known as an inhibitory neurotransmitter, mainly in the central nervous system. Glycine also stimulates the glycine-gated chloride channels, leading to increased chloride influx that hyperpolarizes the neuronal membranes and inhibits excitatory signal transduction [35]. However, several studies have described the immunosuppressive properties of the glycine amino acid [35,36]. Glycine inhibits superoxide production and blunts both the nuclear translocation of NF-kB and TNF-a production [37]. It is thus an amino acid that determines the structure of collagen [38].

5. Conclusions

The current article extends earlier reports on the search for new rheumatoid arthritis biomarkers in the group of free amino acids. The authors also conducted an analysis of previous reports to point out that an analysis of the metabolomics profile, which is extremely dynamic and variable by its nature, needs to utilize validated bioanalytical methods that are strictly dedicated for this purpose.

It was also proven (p value less than 0.05) that the selection of threonine, tryptophan, arginine, and glycine as potential indicators of RA patients treated with DMARDs and Bio-DMARDs as well as when used to estimate the extent of physical disability was not accidental. The metabolic pathways of each of these amino acids are involved in the inflammatory or immune response of the human body. Some of them, e.g. glycine, directly modulate the organism's immune response and are considered to be immune modulators. Since the metabolic profile exhibits a highly dynamic range when compared with genomics or proteomics, it would be worth studying how selected metabolites correlate with response to a given treatment and disease progression. Ultimately, a given patient's metabolite profile could be used as a tool to predict the disease course and thereby facilitate optimal treatment.

Acknowledgments

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The investigation has been approved by the Bioethical Commission of Poznan University of Medical Sciences: Decisions No 135/15 and 536/16. A protocol of the conducted research conforms to the principles of the World Medical Association's Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

Contributions

Bartosz Urbaniak (BU) processed samples, acquired data, performed data analysis, performed statistical analysis and interpretation of data and mainly drafted the manuscript. Agnieszka Klupczynska (AK) and Szymon Plewa (SzP) processed samples, acquired data, and participated in data analysis, performed statistical analysis of experimental data, participated in data interpretation. Dorota Sikorska (DS) and Włodzimierz Samborski (WS) participated in study design and coordination, and coordinated patients sample acquisition. Zenon J. Kokot (ZJK) conceived of the study, participated in study design and coordination, and helped draft the manuscript. All authors read and contributed to manuscript drafts, and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.10.002.

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