Summary of the Ph.D. Thesis

Targeted metabolomics of tryptophan and its metabolites in neurological diseases

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INTRODUCTION

Metabolomics is the large-scale analysis of small (typically <1000 Da) molecules in living organism, which are generally known as metabolites. In practice, metabolomics presents a significant analytical challenge because it aims to measure molecules that have disparate physical properties (e.g., ranging in polarity from highly water-soluble organic acids to very nonpolar lipids). There are two distinct strategies of metabolomics: untargeted (global) and targeted. A targeted metabolomics analysis is the quantitation of defined groups of chemically characterized and biochemically annotated metabolites. The main advantage of the targeted approach is its sensitivity. Through the use of internal standards, analysis can be undertaken in a quantitative or semi-quantitative manner. This approach enables us to comprehensively understand a vast array of metabolic enzymes, their kinetics, end products, and the known biochemical pathways to which they contribute.

In general, metabolomics is used in various fields such as drug discovery, metabolic pathways confirmation, disease, and biomarker research. The metabolome is the closest point in the omics cascade to the phenotype. By the analysis of metabolome valuable information can be collected on effective biomarkers of diseases (diagnostic, prognostic, and predictive), metabolites can be relevant indicators of physiological or pathological states.

Tryptophan (TRP) is a non-polar aromatic α-amino acid that is used in the biosynthesis of proteins and plays a critical role in numerous metabolic functions. There are two main metabolic pathways of TRP. The major degradation pathway is the kynurenine (KYN) pathway (KP) which includes both neuroprotective and neurotoxic compounds. Concentration changes of neuroprotectants e.g., kynurenic acid (KYNA) and picolinic acid (PICA), the free radical generator 3-hydroxykynurenine (3-HK), and the excitotoxic quinolinic acid (QUIN) have been observed in different neurological disorders, including multiple sclerosis.

The other pathway of TRP metabolism is the serotonin (SERO) pathway (SP) which comprises the synthesis of 5-hydroxyindoleacetic acid (5-HIAA) and melatonin (MELA) in addition to SERO (Figure 1). SERO has a role in aggression, anxiety, and stress. Alterations in the serotonergic system are significantly responsible in the pathogenesis of neurological diseases and neuropsychiatric disorders. SERO and its metabolites appear to be useful as diagnostic and prognostic markers for neurological diseases or as targets for more effective therapies in neurology in the future.

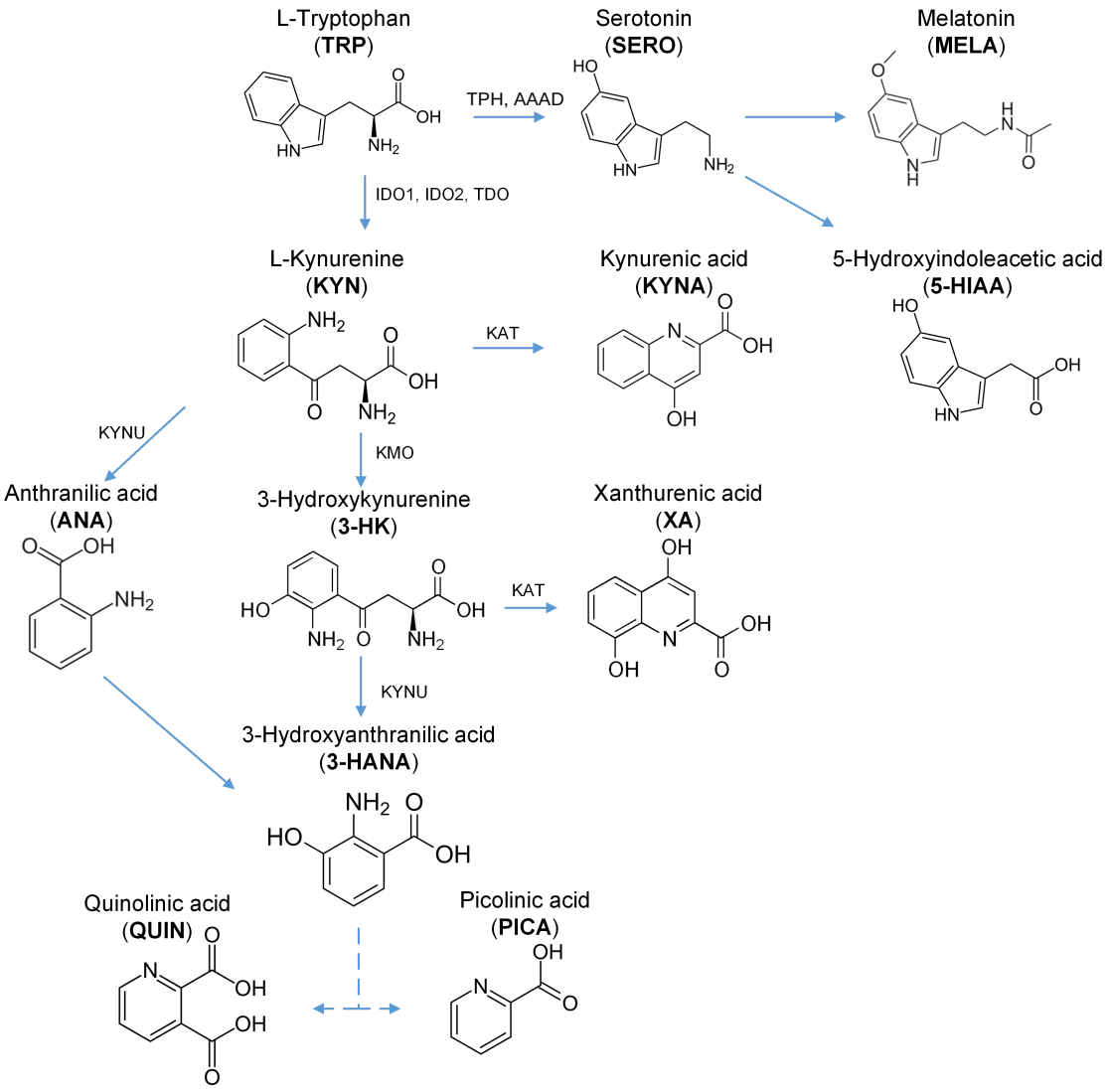

Figure 1. The simplified pathways of tryptophan metabolism, indicating the principal enzymes tryptophan-5-hydroxylase (TPH), aromatic L-amino acid decarboxylase (AAAD), indoleamine-2,3-dioxygenase (IDO), tryptophan-2,3-dioxygenase (TDO), kynurenine aminotransferase (KAT), kynureninase (KYNU), and kynurenine-3-monooxygenase (KMO)

AIMS

In medicine, it is essential to be able to determine precisely a given compound from different samples with the highest possible accuracy, as false results are unacceptable when diagnosing a patient or monitoring the course of the disease. Even though more and more methods are available, there is still a great need for liquid chromatography-mass spectrometry (LC-MS) methods that can quantify as many analytes as possible simultaneously and accurately. Many metabolites are present in the matrices in very low concentrations; hence, the proper coordination and optimization of the sample preparation, the chromatography, and mass spectrometry are essential and challenging.

Our aims were:

I. The development of a new, robust ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method by which TRP and its 11 most important metabolites (including the rarely measured analytes PICA and QUIN) can be quantified in three different human matrices:

- **a,** cerebrospinal fluid (CSF)
- **b,** serum
- **c,** plasma.

II. The determination of the most appropriate, optimized conditions for rapid, sensitive, precise, and reproducible chromatographic separation of components in complex samples; minimizing time and resources spent developing methods using DryLab®4 software.

III. The validation of the developed method including selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and recovery according to the protocols of the International Conference on Harmonisation (ICH) and the United States Food and Drug Administration (FDA).

IV. The application of the developed method for the diagnosis of neurological diseases.

- **a,** The discovery of putative biomarkers of multiple sclerosis.
- **b,** The exploration of TRP metabolite profile changes in migraine.

MATERIALS AND METHODS

The female subjects included in the multiple sclerosis study underwent both lumbar puncture and blood sample collection, were enrolled at the Department of Neurology, University of Szeged. Approval for the human study was granted by the local Ethical Committee of the University of Szeged (46/2014 and 143/2015), and the study protocol adhered to the tenets of the most recent revision of the Declaration of Helsinki for experiments involving human subjects. All subjects enrolled in this study provided voluntary written informed consent. Multiple sclerosis group consisted of 20 individuals and the control group consisted of 14.

All patients enrolled in the migraine study were treated as outpatients at the Department of Neurology. Investigations were conducted after the approval of the local Ethical Committee of the University of Szeged (87/2009) and the Department of Health Administration of National Public Health Centre (29022-5/2019/EÜIG, 28324-5/2019/EÜIG) adhering to the most recent revision of the Declaration of Helsinki. Fifty episodic migraine patients and 34 healthy control subjects were recruited. From the 50 migraineurs, 47 samples were acquired during the interictal and 12 samples during the ictal phases.

Sample preparation

To determine TRP and its metabolites in CSF, serum, and plasma samples calibration standards were prepared at 12 concentration levels, and the quality control (QC) samples were prepared at three levels (low-level QC [LQC], middle-level QC [MQC], and high-level QC [HQC]) in artificial CSF or in charcoal-stripped human serum ("blank" serum) or plasma ("blank" plasma), respectively.

The calibration and QC samples were prepared by adding standard solution mix of the twelve analytes (SERO, KYN, 3-hydroxyanthranilic acid (3-HANA), TRP, 5-HIAA, anthranilic acid (ANA), KYNA, 3-HK, xanthurenic acid (XA), MELA, PICA, and QUIN) to aCSF or "blank" serum/plasma. Samples were deproteinized by mixing with acetonitrile (ACN) –for CSF– or acetone-methanol (MeOH) (1:1, v/v) –for serum or plasma– (containing the stable isotope labeled internal standards (SIL-IS): d4-SERO, d4-KYN, d3-3-HANA, d5-TRP, d5-5-HIAA, d5-KYNA, d3-3-HK, d4-XA, d4-MELA, d4-PICA, and d3-QUIN). Carboxyl group containing TRP metabolites were derivatized by esterification with different aliphatic alcohols to compensate their unfavorable chromatographic behavior (Figure 2).

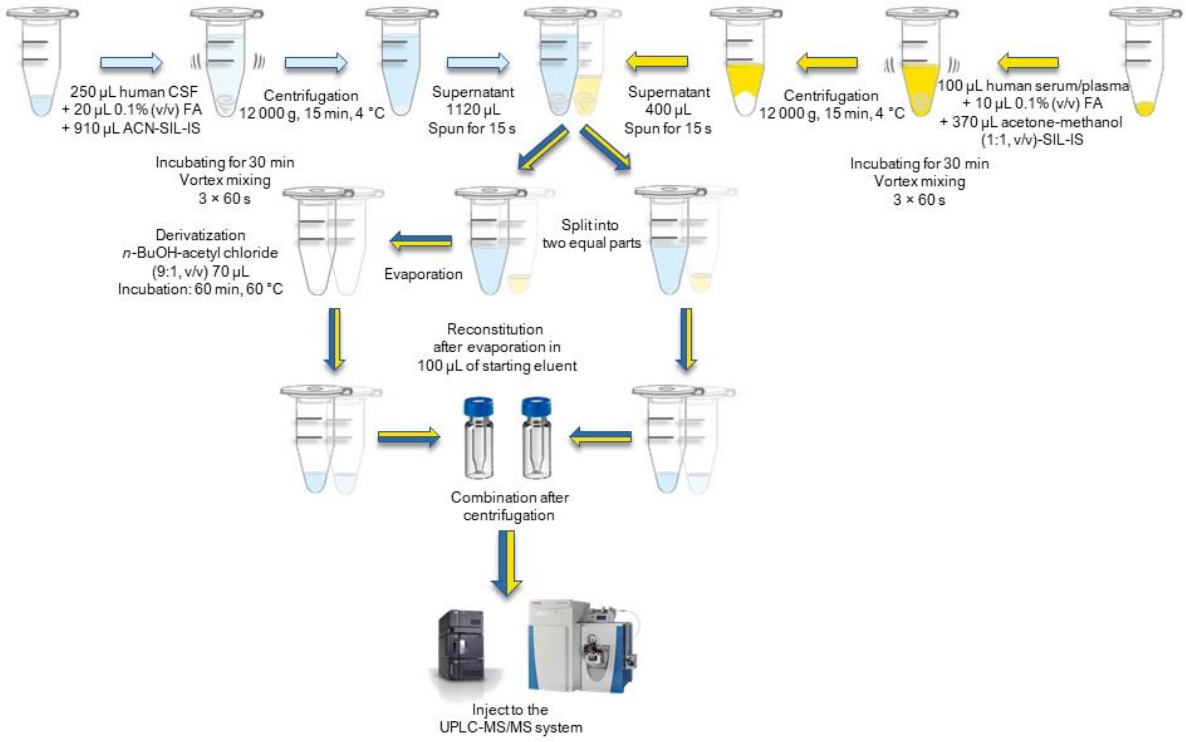

Figure 2. The flow chart of the sample preparation process of human CSF, serum, and plasma samples.

Chromatography

Chromatographic separation of TRP and its 11 metabolites was carried out on a ACQUITY I-Class UPLC™ (ultra-performance liquid chromatography) liquid chromatography system (Waters, Manchester, UK). Separation parameters were optimized using DryLab®4 software (Molnár-Institute, Berlin, Germany). Clinical samples were analyzed on a pentafluorophenyl (PFP) column (Phenomenex, 100 Å, 100 mm × 2.1 mm, particle size 2.6 µm) using 0.1% (v/v) aqueous FA as solvent A and MeOH containing 0.1% (v/v) FA as solvent B at 25°C. The final gradient was set as follows: 0.0 min, 10% B; 1.0 min, 30% B; 3.0 min, 50% B; 3.5 min, 90% B; 5.0 min, 90% B; 5.1 min, 10% B; and 7 min 10% B. For each measurement, the flow rate was set at 300 µL/min. Finally, 20 µL of the sample were injected into the UPLC–MS/MS system.

Mass spectrometry

The quantitative measurements were conducted using a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) connected online to the ACQUITY I-Class UPLC™ system. The mass spectrometer was operated in the positive-ion mode using the equipped HESI-II source with the following parameters: capillary T, 256°C; spray voltage, 3.5 kV; aux gas heater T, 406°C; sheath gas flow, 48; aux gas flow, 11; sweep gas flow, 2; and S-lens RF level, 50.0 (source auto-defaults). The full scan was conducted with a mass range of 50–300 m/z and a resolution of 17,500. The automatic gain control (AGC) setting was defined as 3×10^6 charges, and the maximum injection time was set to 60 ms.

For the analysis of TRP and its metabolites via MS/MS, the PRM data acquisition mode was chosen. To reach the best precursor/product transition for quantitation and maximize sensitivity, the optimal collision energies of each analyte were identified.

RESULTS AND DISCUSSION

1. Optimization process of sample preparation

To improve sensitivity, the protein content of the samples had to be eliminated. In our preliminary experiments 3- and 5-times volume of MeOH, ACN, acetone–MeOH (3:7, v/v), and acetone–MeOH (1:1, v/v) were used to precipitate human serum, and we applied MeOH and ACN to eliminate proteins from CSF samples. The recovery of each TRP metabolite was monitored. 3-HANA could not be detected properly using MeOH neither in serum nor CSF samples (data not shown). The best result was achieved using a 3-times volume of ACN, which gave the highest recovery of TRP metabolites from CSF samples. A 3-times volume of acetone–MeOH (1:1, v/v) resulted in the same results for serum samples.

Esterification of TRP and its metabolites was optimized by assessing the effect of reaction solvent (MeOH, ethanol, n-propanol, and n-butanol) and for n-butanol the reaction time (0, 20, 30, 40, 50, and 60 min; n = 3). Because of the longest hydrophobic aliphatic chains, the butylated products exhibited the highest retention on reversed-phase columns, and there was no coelution between the esterified and non-derivatized other components (data not shown). Although esterification was not complete after 60 min for all components (74–95%), the method could be reliably used because of the presence of the SIL-IS, which has nearly identical chemical and physical properties as the target analyte. Although the absolute response may be affected, the analyte to IS peak area ratio should be unaffected, and the method should be accurate, precise, and rugged.

Esterification with n-butanol changed the polarity of the molecules so that 3-HK, PICA and QUIN appear in the chromatogram as sufficiently retained, well-shaped peaks.

2. Design, development, and optimization of the UHPLC method

The first stage of chromatographic method development is identifying the most promising column chemistry, organic modifier, and pH of the mobile phase that will be used to separate the analytes. To scout the most appropriate column and organic solvent, we performed two initial experiments using 13 columns eluting with an MS-compatible generic gradient. The "best" column and organic modifiers were selected via visual comparison of the resultant chromatograms, considering the overall peak shapes, retention of highly polar compounds, and baseline separation of analytes in the shortest time. DryLab®4 software was then used to optimize the separation on the selected column.

To optimize the separation of TRP and its 11 metabolites on a Kinetex PFP column using MeOH as an organic modifier, we performed four initial linear gradient chromatographic runs to test the effects of the gradient steepness/t_G (t_{G1} = 5 min and t_{G2} = 15 min) and column temperature (T_1 = 25°C and T_2 = 50°C) on retention times and resolution. Based on these chromatograms, DryLab®4 software simulated chromatograms and created a 2D color-coded resolution map plotting critical resolution as a function of t_G and T (Figure 3).

The best separation was achieved using a gradient with four linear segments: 0–1 min, 10–30% B; 1–3 min, 30–50% B; 3–3.5 min, 50–90% B; and 3.5–5 min, 90% B. The retention time prediction was experimentally verified using the selected parameters (Figure 4).

According to our best knowledge, this is the first research to use DryLab®4 software to optimize the LC/MS analysis of endogenous multianalyte-containing biological samples.

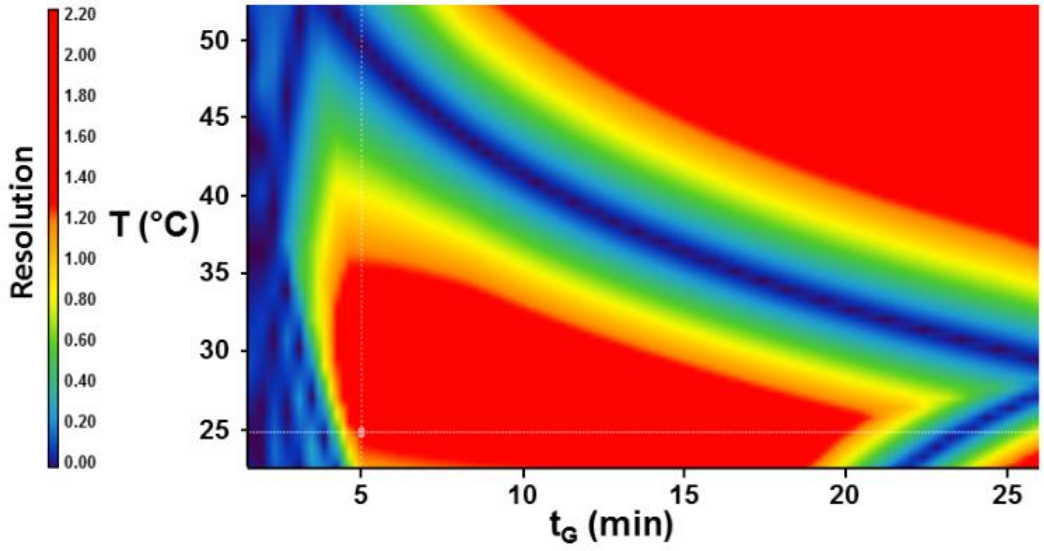

Figure 3. DryLab resolution map with working point (t_G = 5 min, T = 25°C).

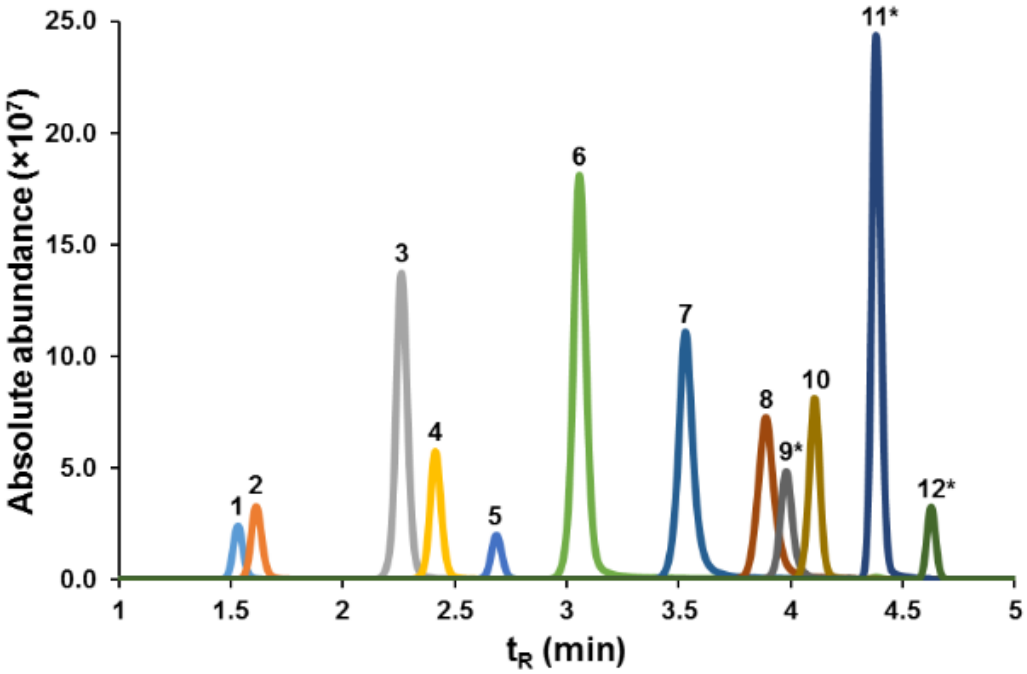

Figure 4. Extracted ion chromatogram of the measured analytes: SERO (1), KYN (2), 3-HANA (3), TRP (4), 5-HIAA (5), ANA (6), KYNA (7), XA (8), 3-HK (9*), MELA (10), PICA (11*), QUIN (12*). *, indicating the derivatized analytes.

3. Validation of the developed method

For all analytes, r^2 exceeded 0.99. The LODs were less than 2.7 nM, and the LOQs were lower than 8.3 nM for all metabolites excluding SERO and QUIN (9.8 and 6.8 nM for LOD and 29.6 and 20.6 nM, for LOQ, respectively).

The intra- and inter-day precision (RSD) of the method for the three matrices were obtained by analyzing five replicates of the three QC levels on 3 consecutive days. According to the results, the developed method has reliable precision.

The accuracy ranged from 86.7 to 112.0% of intra- and inter-day measurements in aCSF, from 89.1 to 107.7% in “blank” serum, and from 85.1 to 114.8% in “blank” plasma, which agree with the guideline data.

To determine the concentrations of the analytes, a simple protein precipitation method was chosen. The recoveries were determined at three different concentration levels to prove that the recovery of each analyte was concentration-independent, reproducible, and consistent. The recoveries of the analytes ranged between 93.8 and 105.3% for aCSF, between 84.7 and 109.4% for “blank” serum, and between 90.3 and 101.8% for “blank” plasma. Our values are in the range recommended by the official guidelines.

4. TRP metabolite profile changes in multiple sclerosis

Our findings conform to the literature data, and QUIN-induced excitotoxic effects can be counterbalanced by KYNA. In the context of the disease, the amount of QUIN was increased dramatically in both CSF and serum, whereas KYNA levels were slightly decreased, resulting in a significantly higher QUIN/KYNA ratio in patients with multiple sclerosis compared with those in controls (Figure 5 and 6). The QUIN/KYNA ratio reflects the excitotoxic potential, as excitotoxicity is increasingly favored as the ratio increases. The hypothesis that excitotoxic TRP metabolites can cause neurodegeneration in multiple sclerosis is supported by these data.

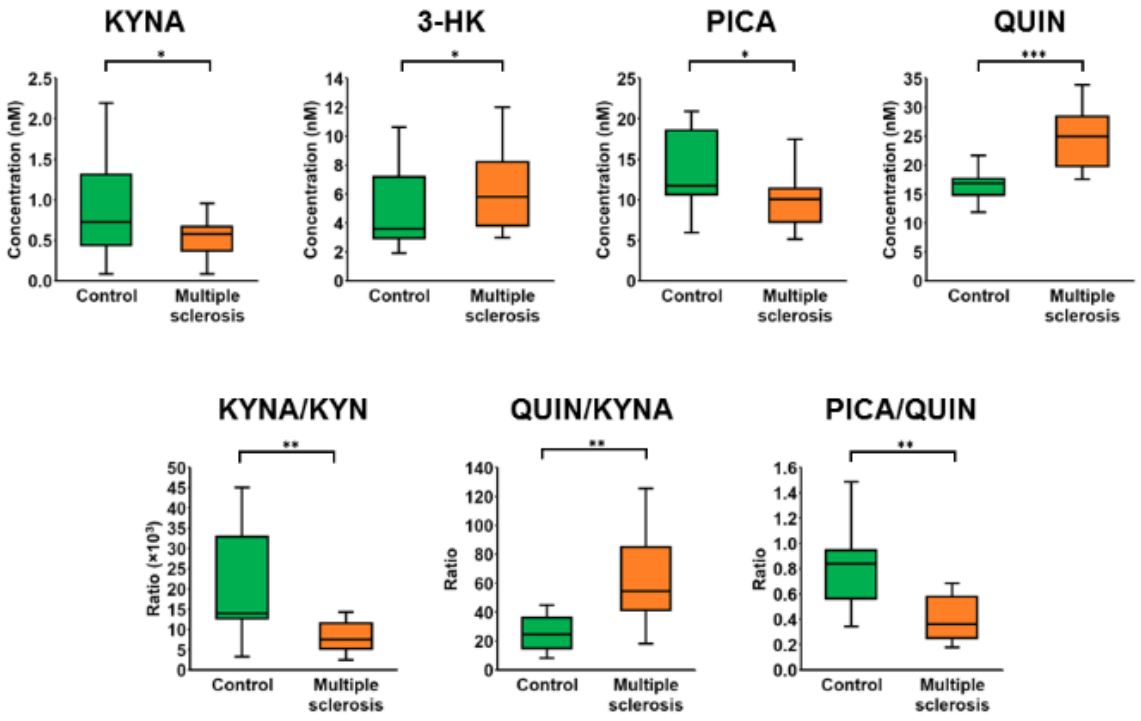

Figure 5. Box plots of significant changes between the control and multiple sclerosis groups in cerebrospinal fluid, in the concentrations of KYNA, 3-HK, PICA, QUIN, KYNA/KYN, QUIN/KYNA, and PICA/QUIN. Significance was evaluated using an independent samples *t*-test or a two-sample Wilcoxon test after the F-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

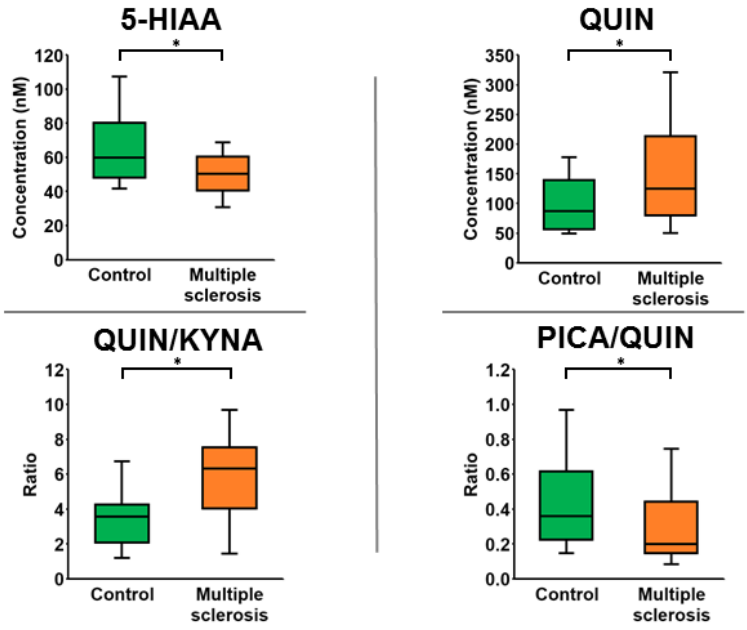

Figure 6. Box plots of significant changes between the control and multiple sclerosis groups in serum, in the concentrations of 5-HIAA, QUIN, QUIN/KYNA, and PICA/QUIN. Significance was evaluated using an independent samples *t*-test or a two-sample Wilcoxon test after the F-test: * $p < 0.05$.

The KYNA/KYN ratio, a potential surrogate marker of KAT activity, is decreased in patients with multiple sclerosis compared with that in controls, as a consequence of decreased metabolism of the neuroprotective branch of the KP, a phenomenon described in other disorders. In addition, the PICA/QUIN ratio, which is increased in patients with multiple sclerosis, may be the result of the ability of neuroprotective PICA to antagonize the neurotoxicity of QUIN or representative of the inflammatory processes of the disease.

In CSF samples, the measured concentration of 3-HK was significantly increased, which is not surprising, as the neurotoxic metabolite is known to potentiate QUIN-induced excitotoxicity. Moreover, QUIN is involved in the phosphorylation of neurofilaments, the structural components of axons. The increased level of neurofilaments in the CSF and sera of patients with multiple sclerosis reflects the extent of neuroaxonal damage, as described in previous studies.

5. TRP metabolite profile changes in migraine

The importance of TRP, SERO, and MELA has already been described in migraine, however, the KP has received limited attention in human studies. The KP is the main branch (95%) of TRP catabolism, and its several metabolites can affect different pain-related mechanisms, including glutamate-mediated neurotransmission, immunological or antioxidant processes. Simultaneous investigation of the different routes of TRP metabolism can get us closer to the metabolomic alterations characteristic of migraine.

Our results show that the entire metabolic route (except SERO) is depressed during the interictal (attack free) period in migraineurs, but a tendency of elevated metabolite levels was found during the ictal (attack) period. We detected significantly lower plasma concentrations of TRP metabolites (TRP, KYN, ANA, XA, and PICA) in the interictal phase of migraineurs (n = 38) compared to the healthy control group (n = 34) (Figure 7). MELA showed a similar tendency but did not reach statistical significance ($n_{control}$ = 30 vs. $n_{interictal}$ = 37 vs. n_{ictal} = 10) and the concentrations were below LOQ. A tendency of elevated TRP metabolite levels was revealed in the ictal phase of migraineurs (n = 12) compared to the attack-free period but only PICA levels differed significantly (34.9 ± 13.7 vs. 46.0 ± 24.4; $p < 0.049$). The level of SERO showed opposite alterations between controls (n = 17) and patients ($n_{interictal}$ = 25, n_{ictal} = 8) compared to other metabolites, but the difference was not significant in any of the cases. The interictal data of 10 migraineurs, whose plasma samples were collected from both periods, were excluded from this analysis to avoid statistical problems.

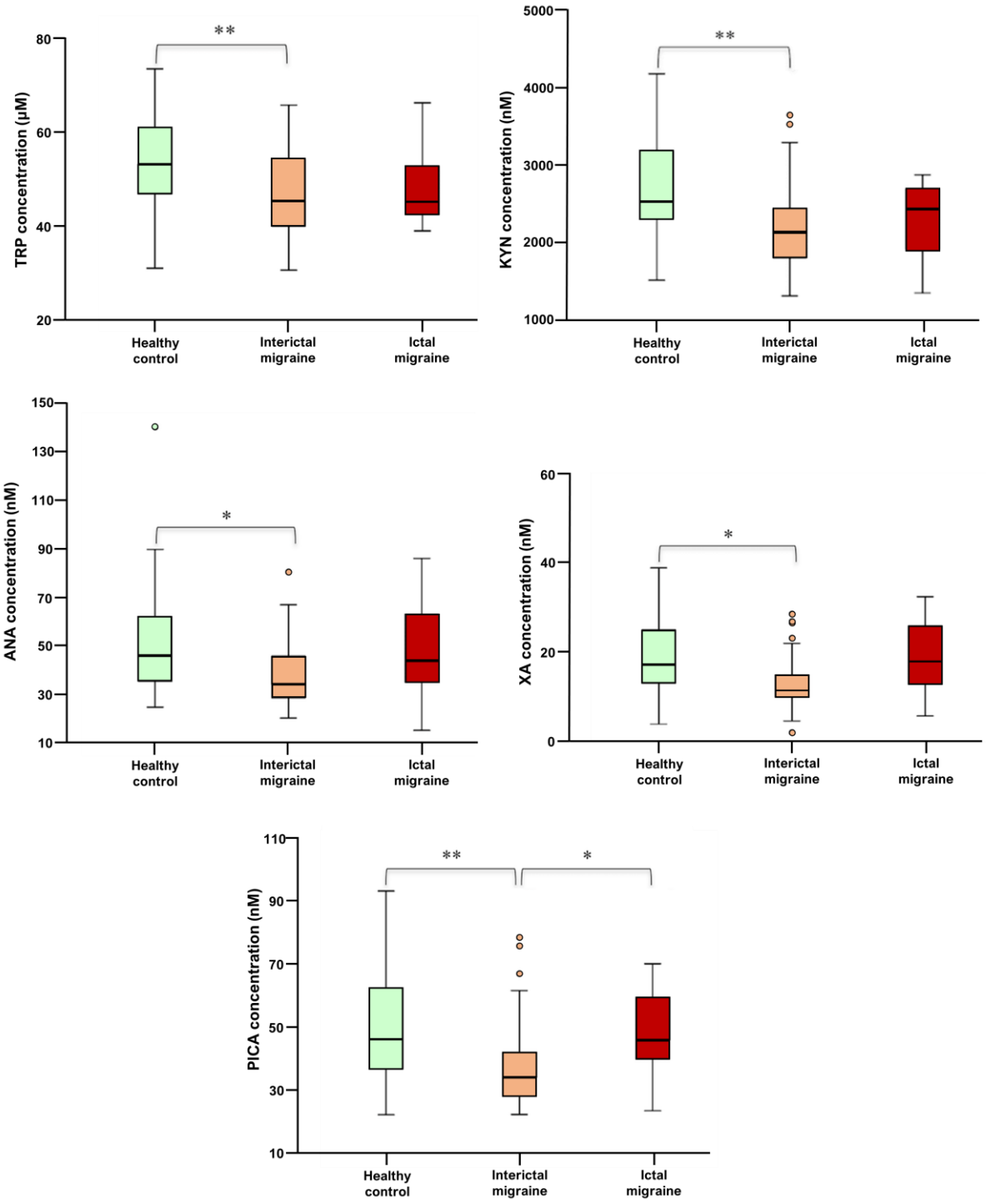

Figure 7. Plasma concentrations of TRP, KYN, ANA, XA, and PICA in controls and migraineurs in both interictal/ictal periods (median, interquartile range, minimum, maximum values, and outliers were presented in this figure). Sample sizes in groups: healthy control (n = 34), interictal phase of migraineurs (n = 38), ictal phase of migraineurs (n = 12). Significance levels: * $p < 0.05$, ** $p < 0.01$.

SUMMARY AND CONCLUSIONS

I. We designed a UHPLC–MS/MS method to simultaneously quantify TRP and its 11 major metabolites of its two metabolic pathways (KP and SP), in human CSF, serum, and plasma. Although three metabolites (3-HK, PICA, and QUIN) were analyzed in derivatized forms, they were assessed together with nine underivatized metabolites in one single run.

II. Chromatographic separation was *in silico*-optimized with the DryLab®4 software using data from four test runs. Instead of a long, manual optimization of the composition, shape, and time of the gradient, and the column temperature, we did all this fast with the software. The 12 analytes were separated in a 3.5 min chromatographic time window and the retention times of the components were in excellent match with the values "predicted" by the software.

III. The values of the parameters examined during the validation of the new method (selectivity, linearity, LOD and LOQ, precision, accuracy, and recovery) were within the acceptable ranges provided by the ICH and the FDA.

IV. Our results indicate that TRP and its metabolites could serve as diagnostic markers for multiple sclerosis because the ratios of KYNA/KYN, PICA/QUIN, and QUIN/KYNA are significantly different in patients with multiple sclerosis compared with those in controls. The concentration levels of TRP and its catabolic products in patients with migraine shows that the entire metabolic route (except SERO) is depressed during the interictal period in migraineurs, but in the ictal phase they reach the values measured in control patients. Since migraine is a multifactorial disease, it may be beneficial to pay more attention to complex metabolic pathways to improve therapeutic strategies.

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LIST OF PUBLICATIONS

Original publications directly related to the Ph.D. thesis:

I. **Ferenc Tömösi,** Gábor Kecskeméti, Edina Katalin Cseh, Elza Szabó, Cecília Rajda, Róbert Kormány, Zoltán Szabó, László Vécsei, Tamás Janáky
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IF_{2020}: 3.935

II. Bernadett Tuka, Aliz Nyári, Edina Katalin Cseh, Tamás Körtési, Dániel Veréb, **Ferenc Tömösi**, Gábor Kecskeméti, Tamás Janáky, János Tajti, László Vécsei
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The Journal of Headache and Pain, 22(1), 60 (2021)
IF_{2020}: 7.277

Total impact factor of the directly related publications[1]: 11.212

[1] Based on JCR Clarivate – Web of Science

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1. Ádám Annus, **Ferenc Tömösi**, Ferenc Rárosi, Evelin Fehér, Tamás Janáky, Gábor Kecskeméti, József Toldi, Péter Klivényi, László Sztriha, László Vécsei
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IF$_{2020}$: 1.727

2. Dezső P. Virok, **Ferenc Tömösi**, Anikó Keller-Pintér, Kitti Szabó, Anita Bogdanov, Szilárd Poliska, Zsolt Rázga, Bella Bruszel, Zsuzsanna Cseh, Dávid Kókai, Dóra Paróczai, Valéria Endrész, Tamás Janáky, Katalin Burián
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IF_{2020}: 6.321

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IF_{2019}: 3.216

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9. Róbert Berkecz, **Ferenc Tömösi,** Tímea Körmöczi, Viktor Szegedi, János Horváth, Tamás Janáky

Comprehensive phospholipid and sphingomyelin profiling of different brain regions in mouse model of anxiety disorder using online two-dimensional (HILIC/RP)-LC/MS method

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IF_{2018}: 2.983

10. Róbert Berkecz , Tímea Körmöczi, **Ferenc Tömösi**, Viktor Szegedi, János Horváth, Nóra Kovács, Tamás Janáky

Plasma phospholipid profiling of a mouse model of anxiety disorder by hydrophilic interaction liquid chromatography coupled to high-resolution mass spectrometry

Biomedical Chromatography, 32, 6, e4202, 9 p. (2018)

IF_{2018}: 1.748

Total impact factor of the not closely related publications[2]: 41.953

Total impact factor[2]: 53.165

[2] Based on JCR Clarivate – Web of Science

1.sz. melléklet

Társszerzői lemondó nyilatkozat

Co-author certification

Alulírott Prof. Dr. Vécsei László (felelős szerző) kijelentem, hogy Tömösi Ferenc (pályázó) PhD értekezésének tézispontjaiban bemutatott - közösen publikált - tudományos eredmények elérésében a pályázónak meghatározó szerepe volt, ezért ezeket a téziseket más a PhD fokozat megszerzését célzó minősítési eljárásban nem használta fel, illetve nem kívánja felhasználni.

2021. július 15.
Dátum

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Felelős szerző

A pályázó tézispontjaiban érintett, közösen publikált közlemények:

Bernadett Tuka, Aliz Nyári, Edina Katalin Cseh, Tamás Körtési, Dániel Veréb, **Ferenc Tömösi**, Gábor Kecskeméti, Tamás Janáky, János Tajti, László Vécsei

Clinical relevance of depressed kynurenine pathway in episodic migraine patients: potential prognostic markers in the peripheral plasma during the interictal period

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