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Persons With Multiple Sclerosis Reveal Distinct Kynurenine Pathway Metabolite Patterns

A Multinational Cross-Sectional Study

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Abstract

Background and Objectives

Kynurenine pathway (KP) metabolites modulate inflammatory activity and neuronal viability. The consequences of KP imbalance partly resemble the molecular mechanisms of multiple sclerosis (MS). An improved understanding of KP imbalance and its relevance in MS requires holistic approaches beyond single-metabolite investigations. Thus, we aimed to explore the presence of KP metabolite patterns in MS and to evaluate their relevance in relation to participant characteristics and clinical measures.

Methods

In this multinational cross-sectional analysis, we determined serum concentrations of KP metabolites in persons with MS and healthy individuals using targeted metabolomics (LC-MS/MS). Analyses were conducted between March 24, 2022, and August 9, 2024. The source studies were conducted in Denmark, Germany, and Switzerland. All participants were aged 18 years or older and free of acute or chronic diseases besides MS. Persons with MS had mild to moderate disease severity (Expanded Disability Status Scale [EDSS] score ≤6.5). Following the investigation of individual metabolites, we explored KP metabolite patterns using exploratory factor analysis. Associations between KP metabolite patterns and participant characteristics, MS symptoms, and MRI metrics were investigated using correlation analyses, proportional odds regression, and multiple linear regression.

Results

The MS cohort included 353 participants (67.1% female) with a mean (SD) age of 46.1 (12.4) years. The mean (SD) EDSS score was 3.1 (1.8). The healthy control (HC) cohort included 111 participants (53.2% female) with a mean (SD) age of 45.7 (16.6) years. Persons with MS showed 2 distinct KP metabolite patterns: an inflammation-driven neurotoxic pattern (*NeuroTox*) and a neuroprotective pattern (*NeuroPro*). Greater *NeuroTox* was associated with a higher EDSS score, older age, and higher body fat percentage. Greater *NeuroPro* was associated with a lower EDSS score and higher cardiorespiratory fitness.

Discussion

Using a data-driven approach, we demonstrate the presence of 2 KP metabolite patterns, *NeuroTox* and *NeuroPro*, in MS. Greater *NeuroTox* and lower *NeuroPro* were both associated

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Supplementary Material

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Glossary

3-HAA = 3-hydroxyanthranilic acid; **3-HK** = 3-hydroxykynurenine; **6MWT** = 6-Minute Walk Test; **AA** = anthranilic acid; **BIA** = bioelectrical impedance analysis; **BMI** = body mass index; **DMT** = disease-modifying treatment; **DXA** = dual-energy x-ray absorptiometry; **EDSS** = Expanded Disability Status Scale; **EFA** = exploratory factor analysis; **FDR** = false discovery rate; **HC** = healthy control; **IDO1** = indoleamine 2,3-dioxygenase 1; **KA** = kynurenic acid; **KP** = kynurenine pathway; **KTR** = kynurenine-to-tryptophan ratio; **Kyn** = kynurenine; **MFIS** = Modified Fatigue Impact Scale; **MLR** = multiple linear regression model; **MS** = multiple sclerosis; **NAD^+** = nicotinamide adenine dinucleotide; **Neopt** = neopterin; **(s)NfL** = (serum) neurofilament light chain; **NMDAR** = N-methyl-D-aspartate receptor; **Pic** = picolinic acid; **QA** = quinolinic acid; **Qld** = quinaldic acid; **SDMT** = Symbol Digit Modalities Test; **SRT** = Selective Reminding Test; **Trp** = tryptophan; **XA** = xanthurenic acid.

with greater disease severity. Future studies need to investigate the KP metabolite patterns across the MS disability spectrum and may use comparable approaches to investigate whether KP imbalance follows similar or disease-specific patterns in diseases other than MS.

Trial Registration Information

NCT03322761, NCT02661555, NCT04762342, NCT04356248, DRKS00017091, DRKS00031445, DRKS00028792, DRKS00029105.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the CNS characterized by focal inflammatory lesions and diffuse, smoldering disease activity. The resulting neuroaxonal damage is believed to emerge from an interplay of multiple neurotoxic pathomechanisms combined with a reduced neuroprotective capacity of neurons and glia to resist neurotoxic damage. Understanding the complex interactions between molecular mechanisms and the various neuroimmunologic pathways that contribute to the neurotoxic CNS microenvironment in MS has recently been highlighted as key to increasing neuronal resilience and slowing disease progression.[1]

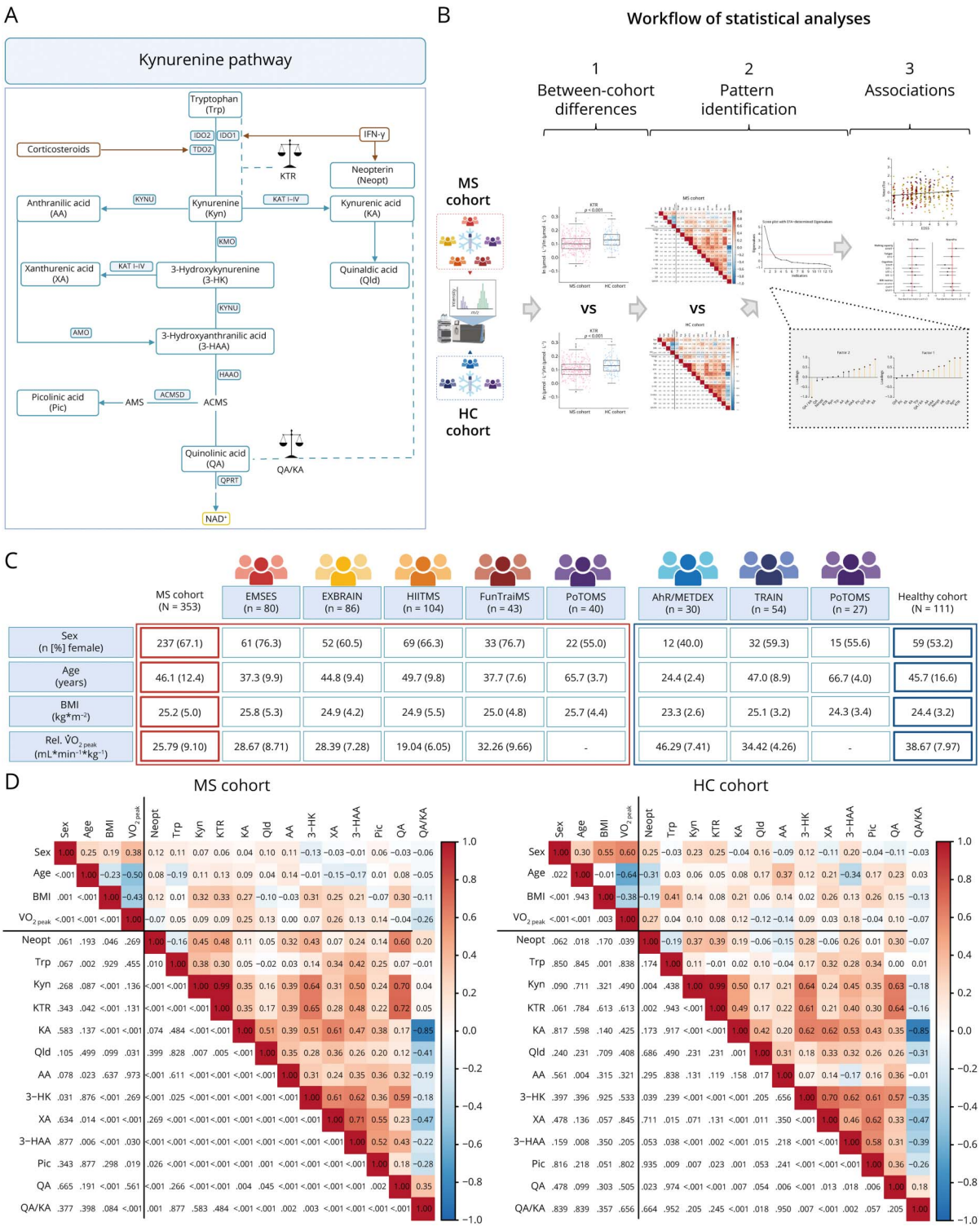
The kynurenine pathway (KP) of tryptophan (Trp) metabolism (Figure 1A) may represent 1 of the neuroimmunologic pathways involved in the neurotoxic CNS microenvironment associated with the pathophysiology of MS.[2] Trp metabolites, collectively termed kynurenines, not only serve as precursors in nicotinamide adenine dinucleotide (NAD^+) de novo synthesis but also modulate inflammatory activity, neuronal and glial homeostasis, and neurotransmission. Moreover, the consequences of KP imbalance partly resemble the molecular mechanisms currently discussed as central to MS pathophysiology (e.g., glutamate excitotoxicity).[3]

According to the traditional view, KP imbalance is believed to result from chronic inflammatory stimulation of the KP, as indicated by an elevated kynurenine-to-tryptophan ratio (KTR), together with a shift of KP activity toward a neurotoxic branch, primarily characterized by the accumulation of the neurotoxic KP metabolite quinolinic acid (QA). QA exerts its neurotoxic effects through several molecular mechanisms, such as N-methyl-D-aspartate receptor (NMDAR)–mediated glutamate excitotoxicity or the promotion of oxidative stress.[4] Concurrently, KP activity toward a neuroprotective branch is reduced, which is characterized by kynurenic acid (KA), an NMDAR antagonist and antioxidant.[2,5] The neurotoxic imbalance of kynurenines, expressed as an elevated QA/KA ratio, was demonstrated in the CSF and serum of persons with MS compared with healthy individuals.[6,7] A higher serum QA/KA ratio in persons with MS is related to higher disease severity,[7] lower cognitive performance,[8] and higher plasma neurofilament light chain (NfL) concentration.[8]

These findings suggest that systemic kynurenines may reflect aspects of CNS pathophysiology in MS.[3] A narrow focus on QA and KA, or their (im)balance, however, does not capture the inherent complexity of the KP. An improved understanding of KP imbalance in MS requires holistic approaches beyond single-metabolite investigations. This is especially relevant as so far scarcely investigated kynurenines may also modulate the neurotoxic CNS microenvironment in MS, given their broad involvement in inflammatory processes, redox regulation, and glutamatergic neurotransmission.[3,5]

In this multinational cross-sectional study, we applied a state-of-the-art *targeted metabolomics* approach to determine serum concentrations of kynurenines in 353 persons with MS and 111 healthy individuals. Our objectives were to (1) validate MS-specific differences in the serum concentrations of kynurenines compared with healthy individuals, (2) explore KP metabolite patterns, and (3) assess their clinical relevance concerning disease severity, MS symptoms, MRI metrics, and relevant demographic, lifestyle-related, and disease-specific factors.

Figure 1 Study Overview and Results of Correlation Analysis



(A) Schematic illustration of the KP. Neopt and the KTR indicate inflammatory stimulation of the KP, as IFN-γ induces both the activation of the enzyme IDO1 and the formation of Neopt by activated macrophages and dendritic cells. (B) Workflow of statistical analyses. (C) Characteristics of the pooled MS and HC cohorts with individual subcohorts. Categorical data are given as total numbers and percentages (%). Continuous data are given as mean (SD). Cardiorespiratory fitness (relative $\dot{V}O_{2\ peak}$) was not assessed in the PoTOMS study. $\dot{V}O_{2\ peak}$ was missing for individual participants of the FunTraiMS (n = 4), EXBRAIN (n = 2), TRAIN (n = 2), and AhR/METDEX (n = 1) studies. (D) Heatmaps showing correlations between participant characteristics and KP metabolites/ratios, presented separately for the pooled MS cohort (left) and HC cohort (right). Pearson correlation analysis was partialized for sex, age, BMI, and relative $\dot{V}O_{2\ peak}$. The lower triangles of the heatmaps show false discovery rate–adjusted *p* values. The upper triangles of the heatmaps show Pearson's r correlation coefficients. AA = anthranilic acid; BMI = body mass index; HC = healthy control; IDO1 = indoleamine 2,3-dioxygenase; IFN-γ = interferon-gamma; KA = kynurenic acid; KP = kynurenine pathway; KTR = kynurenine-to-tryptophan ratio; Kyn = kynurenine; MS = multiple sclerosis; Neopt = neopterin; Pic = picolinic acid; Trp = tryptophan; QA = quinolinic acid; QA/KA = quinolinic acid-to-kynurenic acid ratio; Qld = quinaldic acid; $\dot{V}O_{2\ peak}$ = peak oxygen consumption during cardiopulmonary exercise testing divided by body weight; XA = xanthurenic acid; 3-HAA = 3-hydroxyanthranilic acid; 3-HK = 3-hydroxykynurenine.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The source studies included 5 MS cohorts (pooled MS cohort) and 4 cohorts of healthy controls (pooled HC cohort). Individual MS cohorts comprised participants of EMSES (Denmark, NCT03322761), EXBRAIN (Denmark, NCT02661555), PoTOMS (Denmark, NCT04762342), HIITMS (Switzerland, NCT04356248), and FunTraiMS (Germany, DRKS00017091). Individual HC cohorts comprised matched healthy participants of PoTOMS and participants of TRAIN (Germany, DRKS00031445), AhR (Germany, DRKS00028792), and METDEX (Germany, DRKS00029105). Ethical approval for each study was obtained from the regional or institutional review boards. For this retrospective analysis, no additional approval was required. All studies were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study Participants

Eligibility criteria for all studies were adult age (≥18 years) and the absence of acute or chronic diseases besides MS. All persons with MS were diagnosed according to the McDonald criteria,[9,10] had mild to moderate disease severity (Expanded Disability Status Scale [EDSS] score ≤6.5), and were stable on disease-modifying treatment (DMT), if any. Participants did not receive corticosteroid treatment at the time of blood sampling. Persons with relapsing-remitting MS were in the remission phase. HIITMS was conducted in an inpatient rehabilitation setting. All other studies were conducted in outpatient settings.

KP Metabolite Profiling

Blood samples were obtained from the antecubital vein after at least 5 minutes of rest. After clotting, samples were centrifuged at 1,200–4,300 g for 10 minutes. The supernatant was aliquoted and stored at −80°C at the study sites until analysis. Serum concentrations of kynurenines were analyzed in the same laboratory (Bevital AS, Bergen, Norway) between March 24, 2022, and August 9, 2024. Targeted metabolomics was performed using high-throughput liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), as previously described.[11] Serum concentrations of the following metabolites were determined: anthranilic acid (AA), KA, kynurenine (Kyn), neopterin (Neopt), picolinic acid (Pic), QA, quinaldic acid (Qld), Trp, xanthurenic acid (XA), 3-hydroxyanthranilic acid (3-HAA), and 3-hydroxykynurenine (3-HK). The assay's lower limit of detection ranged from 0.01 to 8.00 nmol*L^{-1}. Within-day coefficients of variation ranged from 3.78% to 9.32%.

Assessment of Clinical Measures

The EDSS score was rated by neurologists in all MS studies. MRI metrics and data on walking capacity, fatigue, and cognitive performance were similarly collected in EMSES, EXBRAIN, and PoTOMS. MRI scans were performed on the same 3-T MRI scanner (MAGNETOM Skyra, Siemens Medical Systems, Erlangen, Germany). Structural T1-weighted (T1w) MP2RAGE images and T2-weighted fluid-attenuated inversion recovery (T2FLAIR) images were acquired. For this analysis, gray matter parenchymal fraction, white matter parenchymal fraction, total lesion volume, and volumes of the following regions of interest were considered: hippocampus, thalamus, caudate, putamen, and globus pallidus. Serum NfL (sNfL) concentrations were determined in EXBRAIN using an ultra-sensitive single-molecule array (Simoa).[12] Walking capacity was assessed using the 6-Minute Walk Test (6MWT).[13] Fatigue impact was queried using the 21-item Modified Fatigue Impact Scale (MFIS).[14,15] Cognitive performance was tested using the Selective Reminding Test ([SRT], verbal learning and memory) and the Symbol Digit Modalities Test ([SDMT], information processing speed).[16] Higher scores indicate greater fatigue and better cognitive performance, respectively. Body composition metrics included body mass index ([BMI], all studies) and total body fat percentage (EMSES, EXBRAIN, and PoTOMS). Body fat percentage was determined by bioelectrical impedance analysis ([BIA]; EMSES and EXBRAIN) or dual-energy X-ray absorptiometry ([DXA]; PoTOMS). Cardiorespiratory fitness was assessed as peak oxygen consumption during cardiopulmonary exercise testing divided by body weight (relative $\dot{V}O_{2\ peak}$) in all studies except PoTOMS.

Statistical Analysis

The workflow of statistical analyses is visualized in Figure 1B. The distribution of KP metabolite data (i.e., serum concentrations of Neopt, Trp, and kynurenines) was assessed visually using histograms. Owing to relevant skewness, serum concentrations of all metabolites were log transformed to approximate normal distribution. The KTR and QA/KA ratio were calculated subsequently.

In the first step, we assessed differences in serum concentrations of KP metabolites between the pooled MS and HC cohorts. We performed independent *t* tests and adjusted the resulting *p* values for false discovery rate (FDR).[17] Subsequently, 1-way ANCOVAs were computed to evaluate the influence of the covariates sex, age, BMI, and sample storage time on between-group differences in KP metabolite concentrations and ratios.

In the second step, we assessed correlations among KP metabolites and ratios separately for the MS and HC cohorts using Pearson correlation analysis (*R* package "ppcor" [version 1.1]).[18] The results were refined using partial correlation analysis to account for potential confounding effects of sex, age, BMI, and relative $\dot{V}O_{2\ peak}$. Correlations were FDR-adjusted. Data suitability for exploratory factor analysis (EFA) was checked by comparing correlations and partial correlations and using the Kaiser-Meyer-Olkin criterion. EFA was conducted using maximum likelihood estimation with promax rotation (*R* package "EFAtools" [version 0.4.4]).[19] The

number of factors to retain was determined using the scree test and the Kaiser-Guttman criterion. The EFA-derived factors were designated according to current evidence on the neuroactive properties ascribed to the KP metabolites and ratios with relevant loadings ($|\lambda| \geq 0.3$) to characterize KP metabolite patterns. Trp, the precursor metabolite, and metabolites with relevant loadings on more than 1 factor (i.e., 3-HAA) were neglected in pattern designation. The consistency of KP metabolite patterns across individual MS cohorts was checked using EFAs with a predefined 2-factor solution. Using the same approach, we additionally explored the presence of similar KP metabolite patterns in the HC cohorts.

In the third step, we investigated associations of the KP metabolite patterns with clinical outcomes. Correlation analyses were repeated to assess associations between the KP metabolite patterns and demographic (i.e., age), anthropometric (i.e., BMI and body fat percentage), and disease-related characteristics (i.e., EDSS score, sNfL concentration, and relative $\dot{V}O_{2\ peak}$). Associations of the KP metabolite patterns with the EDSS score were computed using logistic proportional odds regression models (*R* package "VGAM" [version 1.1–11]).[20] Associations of the KP metabolite patterns with MRI metrics and MS symptoms were assessed using multiple linear regression models (MLRs). MLRs included the KP metabolite patterns, sex, age, BMI, EDSS score, DMT (yes/no), total lesion volume, and study (EMSES, EXBRAIN, and PoTOMS) as predictors, and 6MWT distance, MFIS total score, SRT and SDMT scores, and MRI metrics as predicted variables. In all analyses, the significance level was set at $p = 0.05$. Statistical analyses were conducted using *R* (version 4.4.2).[21] Figures were created with *R* and biorender.com.

Details on study cohorts, outcome assessment, and statistical analyses are provided in the eMethods.

Data Availability

Anonymous raw data can be accessed on reasonable request to the corresponding author, subject to approval from the study sites regarding institutional data protection regulations.

Results

Cohort Characteristics

Characteristics of the pooled MS and HC cohorts, along with individual subcohorts, are presented in Figure 1C. Disease-specific characteristics of the MS cohorts, both pooled and individual, are given in Table 1. In brief, the pooled MS cohort included 353 participants (67.1% female) with a mean (SD) age of 46.1 (12.4) years. Most participants had the relapsing-remitting MS phenotype (74.5%) and were receiving DMT (73.7%). The mean (SD) EDSS score was 3.1 (1.8). The pooled HC cohort included 111 participants (53.2% female), with a mean (SD) age of 45.7 (16.6) years.

Serum Concentrations of KP Metabolites and Ratios Differ Between Persons With MS and Healthy Individuals

To validate the presence of MS-specific differences in the KP metabolome, we first compared serum concentrations of KP metabolites between the MS and HC cohorts. Our results showed that persons with MS have higher concentrations of the inflammation marker Neopt and a higher QA/KA ratio, but lower concentrations of most KP metabolites, including Trp, Kyn, KA, AA, XA, Pic, and QA. In addition, the KTR was lower in persons with MS. The only kynurenine revealing higher concentrations in persons with MS was 3-HAA. Serum concentrations of Qld and 3-HK did not differ between cohorts. Detailed results and nontransformed concentrations of metabolites are provided in eFigure 1 and eTables 1 and 2.

Correlation Matrices Differ Between Persons With MS and Healthy Individuals

Visual inspection of the correlation matrices revealed marked differences in the strength and direction of partialized correlations among KP metabolites and ratios between the pooled MS and HC cohorts (Figure 1D). For example, a higher Neopt concentration was correlated with a higher QA/KA ratio only in the MS cohort. In addition, Neopt showed stronger positive correlations with KTR, 3-HK concentration, and QA concentration in the MS cohort compared with the HC cohort.

We also identified some MS-specific differences in correlations between KP metabolites and ratios and lifestyle-related factors. For example, a higher BMI was correlated with a higher KTR and higher concentrations of most KP metabolites, including Neopt, Kyn, KA, 3-HK, XA, 3-HAA, and QA, in the pooled MS cohort but not in the HC cohort.

Persons With MS Reveal 2 Distinct KP Metabolite Patterns: *NeuroTox* and *NeuroPro*

Both differences in serum concentrations of kynurenines and the heterogeneity of correlation matrices between the pooled MS and HC cohort indicated the presence of an MS-specific shift in the KP metabolome. Therefore, we performed EFA for the MS cohort only. Correlations among KP metabolites, which were not simply explained by bivariate correlations, and a Kaiser-Meyer-Olkin criterion of 0.715 (middling) in the pooled MS cohort indicated data suitability for EFA. Application of the Kaiser-Guttman criterion and the scree test resulted in a 2-factor solution (Figure 2A). The cumulative proportional variance explained by the 2 factors was 54.2%.

Factor 1 was characterized by positive loadings of the inflammation marker Neopt and the KTR, indicating inflammatory stimulation of KP activity. Factor 1 was also characterized by positive loadings of the predominantly neurotoxic kynurenines 3-HK and QA, alongside AA and the neurotoxicity index, the QA/KA ratio. Thus, factor 1 was considered to reflect an inflammation-driven neurotoxic KP metabolite pattern, and hereafter referred to as *NeuroTox*.

Table 1 Disease-Related Characteristics of the Pooled MS Cohort Along With Subcohorts

	Overall n = 353	EMSES n = 80	EXBRAIN n = 86	HIITMS n = 104	FunTraiMS n = 43	PoTOMS n = 40
MS phenotype						
RRMS	263 (74.5)	80 (100)	75 (87.2)	52 (50.0)	43 (100)	13 (32.5)
SPMS	63 (17.8)	—	5 (5.8)	37 (35.6)	—	21 (52.5)
PPMS	27 (7.6)	—	6 (7.0)	15 (14.4)	—	6 (15.0)
EDSS score	3.1 (1.8)	1.6 (1.0)	2.7 (1.5)	4.6 (1.3)	1.6 (1.1)	3.9 (1.4)
TSD (y)	8.93 (9.09)	0.89 (0.56)	9.77 (7.10)	13.14 (9.08)	6.26 (6.34)	16.11 (10.67)
DMT						
None	92 (26.3)	7 (8.8)	16 (18.6)	38 (36.5)	6 (14.0)	25 (62.5)
Alemtuzumab	6 (1.7)	—	5 (5.8)	1 (1.0)	—	—
Cladribine	2 (0.6)	—	—	—	2 (4.7)	—
Dimethyl fumarate	30 (8.5)	6 (7.5)	11 (12.8)	6 (5.8)	4 (9.3)	1 (2.5)
Diroximel fumarate	3 (0.8)	—	—	—	3 (7.0)	—
Fingolimod	27 (7.6)	4 (5.0)	11 (12.8)	10 (9.6)	2 (4.7)	—
Glatiramer acetate	9 (2.5)	3 (3.8)	1 (1.2)	—	4 (9.3)	—
Interferon β	13 (3.7)	2 (2.5)	4 (4.7)	2 (1.9)	2 (4.7)	1 (2.5)
Peginterferon β-1a	1 (0.3)	—	2 (2.3)	—	—	1 (2.5)
Natalizumab	49 (13.9)	5 (6.3)	26 (30.2)	10 (9.6)	7 (16.3)	3 (7.5)
Ocrelizumab	39 (11.0)	2 (2.5)	—	27 (26.0)	7 (16.3)	3 (7.5)
Ofatumumab	7 (2.0)	—	—	2 (1.9)	4 (9.3)	1 (2.5)
Ozanimod	1 (0.3)	—	—	—	1 (2.3)	—
Rituximab	5 (1.4)	—	—	5 (4.8)	—	—
Siponimod	2 (0.6)	—	—	2 (1.9)	—	—
Teriflunomide	65 (18.4)	48 (60.0)	10 (11.6)	1 (1.0)	1 (2.3)	5 (12.5)

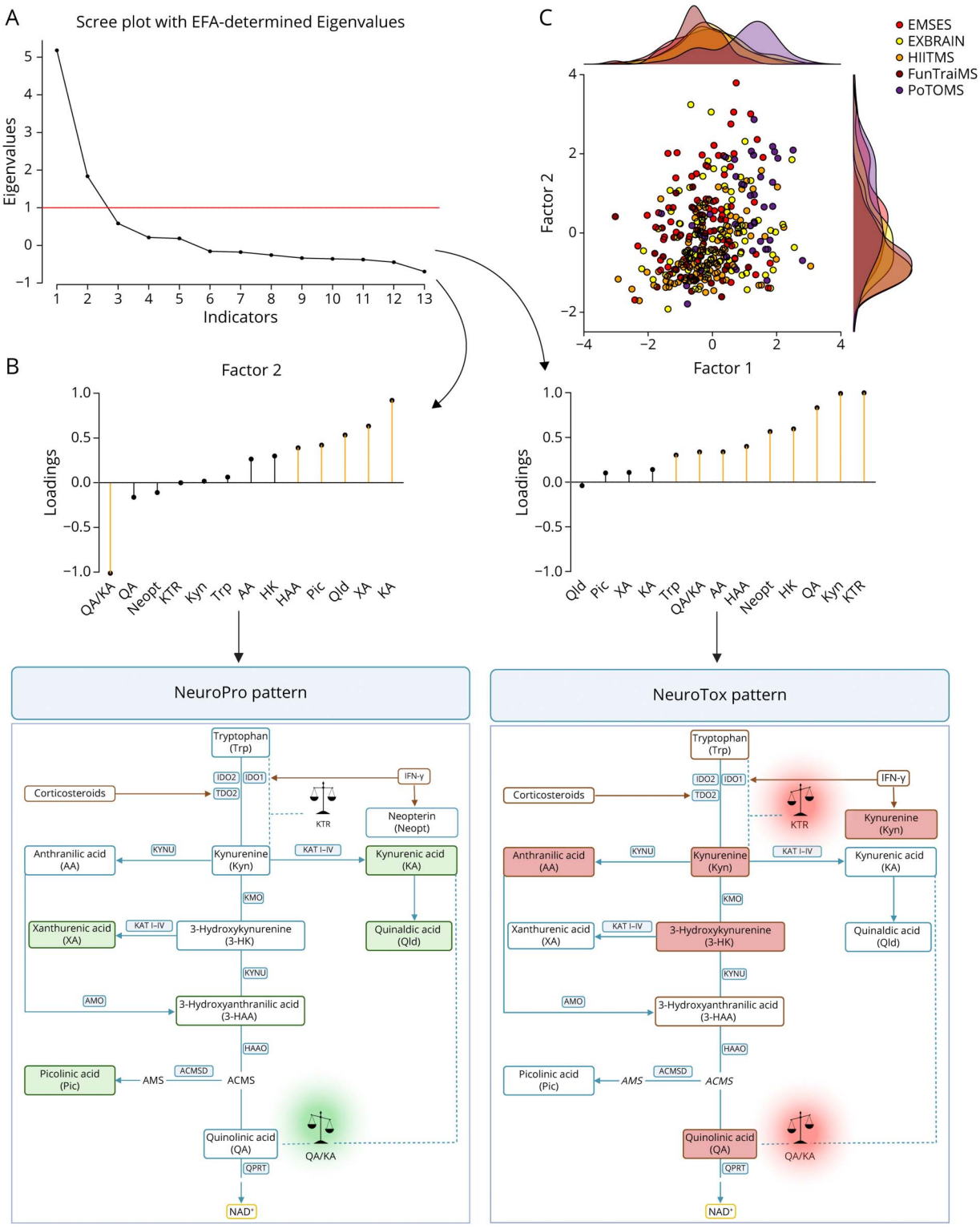
Abbreviations: DMT = disease-modifying treatment; EDSS = Expanded Disability Status Scale; MS = multiple sclerosis; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; TSD = time since diagnosis.
Categorical data are reported as total numbers and percentages (%). Continuous data are expressed as mean (SD). Missing data include information on DMT (n = 3) and EDSS scores (n = 9) for individual participants of the EMSES study. TSD was missing for 1 participant of the FunTraiMS study.

Factor 2 was characterized by positive loadings of the neuroprotective KP metabolite KA, its degradation product Qld, XA, and Pic, and an inverse loading of the QA/KA ratio. Factor 2 was thus designated as a neuroprotective KP metabolite pattern and referred to as *NeuroPro* (Figure 2B).

NeuroTox and *NeuroPro* were differentially distributed among participants as a function of the source cohort. *NeuroTox* was particularly pronounced in the PoTOMS cohort, while it was less pronounced and more homogeneously distributed in the EMSES cohort. *NeuroPro* was less pronounced and more homogeneously distributed in the EMSES and PoTOMS cohorts (Figure 2C).

Translating the KP metabolite patterns to the MS subcohorts revealed consistent loadings of KP metabolites and ratios on *NeuroTox* and *NeuroPro*. In all MS subcohorts, *NeuroTox* was characterized by positive loadings of the KTR, Kyn, 3-HK, and QA, whereas *NeuroPro* was characterized by positive loadings of KA and XA and an inverse loading of the QA/KA ratio. Taking into account limited translatability, we exploratively assessed the presence of similar KP metabolite patterns in the pooled HC cohort. The pooled HC cohort showed some similarities in the loadings of KP metabolites and ratios on both predefined factors, such as positive loadings of Neopt, KTR, Kyn, 3-HK, and QA on *NeuroTox*, and positive loadings of KA, Qld, XA, and Pic together with an inverse loading of the QA/KA ratio on *NeuroPro*. However, in comparison with the pooled MS cohort, *NeuroTox* revealed reduced loading of QA and no relevant (positive) loadings of AA and the QA/KA ratio (Figure 3).

Figure 2 Pooled MS Cohort Reveals 2 Distinct KP Metabolite Patterns



(A) Scree plot illustrating the number of factors to be retained for EFA. (B) Lollipop plots showing the loadings of KP metabolites and ratios on the EFA-derived factors. Relevant loadings ($|\lambda| \geq 0.3$) are highlighted in yellow. Below, the EFA-derived factors have been illustrated as distinct serum KP metabolite patterns according to Figure 1A. Factor 1 represents an inflammation-driven neurotoxic KP metabolite pattern (*NeuroTox*). Factor 2 represents a neuroprotective KP metabolite pattern (*NeuroPro*). As a precursor metabolite, Trp was not considered in factor designation of *NeuroTox*. 3-HAA showed relevant loadings on both factors and was therefore neglected in factor designation. (C) Combined score and density plot showing the EFA-derived factors of each participant, colored by the source cohort (score plot), and the distribution of both factors at the cohort level (density plot). AA = anthranilic acid; EFA = exploratory factor analysis; KA = kynurenic acid; KP = kynurenine pathway; KTR = kynurenine-to-tryptophan ratio; Kyn = kynurenine; MS = multiple sclerosis; Neopt = neopterin; Pic = picolinic acid; Trp = tryptophan; QA = quinolinic acid; QA/KA ratio = quinolinic acid-to-kynurenic acid ratio; Qld = quinaldic acid; XA = xanthurenic acid; 3-HAA = 3-hydroxyanthranilic acid; 3-HK = 3-hydroxykynurenine.

KP Metabolite Patterns Are Associated With Clinical Measures

According to previous evidence on the neurotoxic effects of QA, the neuroprotective effects of KA, and the clinical relevance of the neurotoxicity index QA/KA, our results show that greater *NeuroTox* (Figure 4A) and lower *NeuroPro* (Figure 4B) were correlated with higher disease severity (EDSS score). Greater *NeuroTox* was also correlated with older age, as well as with higher BMI and higher body fat percentage, as determined by BIA and DXA (Figure 5A). By contrast, greater *NeuroPro* was associated with lower body fat percentage and higher cardiorespiratory fitness (relative $\dot{V}O_{2\ peak}$) (Figure 5B). No correlations were observed between KP metabolite patterns and sNfL concentration (EXBRAIN cohort) (eFigure 2).

In line with the results of the correlation analyses, the standardized estimates obtained from our proportional odds regression models indicated a trend toward associations between greater *NeuroTox* and lower *NeuroPro* and higher EDSS score. When considering individual cohorts, we identified a significant association between greater *NeuroTox* and higher EDSS score in the EMSES cohort (Figure 4C). *NeuroTox* and *NeuroPro* were not associated with walking capacity (6MWT), fatigue (MFIS), cognitive performance (SDMT, SRT), or MRI metrics (Figure 4D, eFigure 3). Details on clinical measures and results of regression models are provided in eTables 3–18.

Discussion

In this multinational cross-sectional analysis, we determined serum concentrations of kynurenines in a large cohort of 353 persons with MS and 111 healthy individuals. First, we investigated MS-specific differences in serum concentrations of KP metabolites and ratios compared with healthy individuals.

Figure 3 Loadings of KP Metabolites and Ratios on the EFA-Derived Factors in the MS and the HC Cohorts (Pooled and Individual)

A

Kynurenine pathway metabolite pattern NeuroTox

	MS cohort						HC cohort			
	POOLED	EMSES	EXBRAIN	PoTOMS	HIITMS	FunTraiMS	POOLED	PoTOMS	TRAIN	AhR/ METDEX
Neopt	**0.565**	**0.618**	**0.548**	**0.629**	**0.536**	0.270	**0.442**	**0.100**	**0.650**	0.157
Trp	**0.302**	0.201	**0.405**	0.232	0.202	**0.394**	0.226	**−0.100**	**−0.314**	**0.456**
Kyn	**0.991**	**0.959**	**0.993**	**0.943**	**0.956**	**0.993**	**1.023**	**0.141**	**0.957**	**1.037**
KTR	**0.998**	**0.961**	**0.997**	**0.991**	**0.960**	**0.994**	**1.027**	**0.171**	**1.002**	**1.043**
KA	0.141	0.160	0.130	0.110	0.262	0.032	0.177	**0.728**	0.216	0.124
Qld	−0.040	−0.023	−0.132	−0.125	0.166	−0.002	0.056	**0.100**	0.057	0.066
AA	**0.338**	0.200	**0.314**	0.169	**0.410**	0.198	0.092	**0.033**	0.267	0.263
3-HK	**0.595**	**0.664**	**0.553**	**0.460**	**0.669**	**0.408**	**0.524**	**0.461**	**0.362**	0.286
XA	0.108	0.224	0.071	−0.013	**0.313**	−0.024	0.003	**0.608**	**−0.306**	0.067
3-HAA	**0.399**	**0.344**	**0.447**	**0.343**	**0.578**	0.071	**0.401**	**0.150**	0.138	0.197
Pic	0.102	−0.005	0.012	−0.055	0.258	−0.010	0.190	**0.593**	0.013	−0.190
QA	**0.832**	**0.816**	**0.801**	**0.927**	**0.850**	**0.562**	**0.681**	**−0.319**	**0.617**	**0.404**
QA/KA	**0.337**	0.272	0.285	**0.418**	**0.328**	0.216	0.217	**−1.164**	0.087	0.099

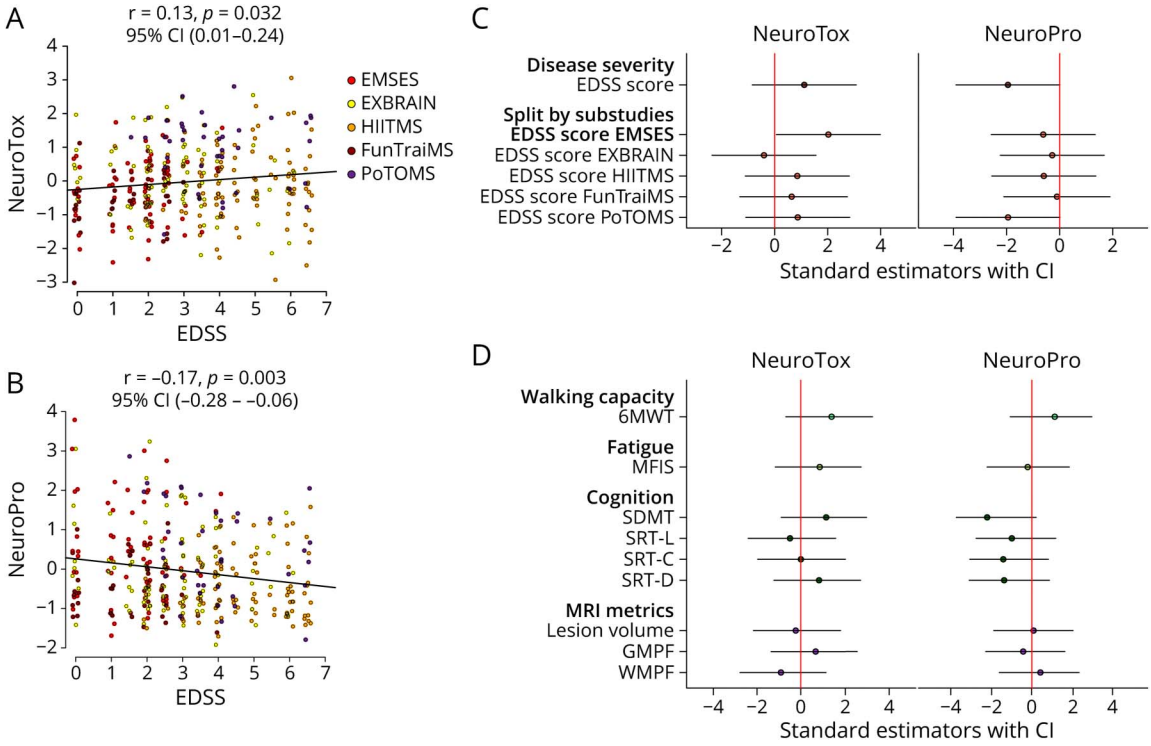
B

Kynurenine pathway metabolite pattern NeuroPro

	MS cohort						HC cohort			
	POOLED	EMSES	EXBRAIN	PoTOMS	HIITMS	FunTraiMS	POOLED	PoTOMS	TRAIN	AhR/ METDEX
Neopt	−0.110	−0.250	−0.192	−0.083	−0.047	−0.161	0.043	**0.488**	−0.251	0.282
Trp	0.062	0.158	−0.007	**0.529**	0.097	−0.265	0.003	**0.543**	**0.626**	0.039
Kyn	0.017	0.200	0.014	0.100	0.114	0.036	−0.048	**0.850**	0.082	−0.066
KTR	−0.001	0.189	0.003	0.015	0.105	0.032	−0.062	**0.823**	−0.011	−0.085
KA	**0.920**	**0.928**	**0.953**	**0.923**	**0.798**	**0.993**	**0.887**	**0.395**	**0.581**	**0.786**
Qld	**0.533**	**0.531**	**0.576**	**0.747**	0.198	0.158	**0.390**	**0.390**	**0.305**	**0.433**
AA	0.264	**0.358**	**0.305**	**0.407**	0.116	0.128	0.130	**0.680**	−0.077	0.129
3-HK	0.298	0.204	**0.346**	**0.557**	0.113	**0.439**	**0.367**	**0.509**	**0.649**	**0.625**
XA	**0.632**	**0.468**	**0.649**	**0.927**	**0.427**	**0.437**	**0.638**	0.294	**0.926**	**0.945**
3-HAA	**0.389**	0.289	**0.330**	**0.681**	0.161	**0.313**	0.267	**0.596**	**0.692**	**0.766**
Pic	**0.420**	0.158	**0.446**	**0.655**	**0.308**	**0.381**	**0.425**	0.213	**0.776**	**0.871**
QA	−0.163	**−0.380**	−0.194	−0.213	−0.231	0.019	−0.017	**1.133**	0.069	**0.312**
QA/KA	**−1.012**	**−0.985**	**−0.983**	**−1.076**	**−1.049**	**−0.895**	**−0.988**	**0.439**	**−0.535**	**−0.782**

Heatmaps illustrating the similarities and differences in KP metabolite patterns across MS subcohorts and between the MS and HC (sub)cohorts. (A) Loadings on Factor 1 (*NeuroTox*). (B) Loadings on Factor 2 (*NeuroPro*). Darker color indicates stronger positive or inverse loading of the KP metabolite or ratio on the respective factor. KP metabolites and ratios with relevant loadings ($|\lambda| \geq 0.3$) in the main analysis are printed in bold. AA = anthranilic acid; HC = healthy control; KA = kynurenic acid; KP = kynurenine pathway; KTR = kynurenine-to-tryptophan ratio; Kyn = kynurenine; MS = multiple sclerosis; Neopt = neopterin; Pic = picolinic acid; Trp = tryptophan; QA = quinolinic acid; QA/KA = quinolinic acid-to-kynurenic acid ratio; Qld = quinaldic acid; XA = xanthurenic acid; 3-HAA = 3-hydroxyanthranilic acid; 3-HK = 3-hydroxykynurenine.

Figure 4 Associations Between the KP Metabolite Patterns and Disease Severity, MS Symptoms, and MRI Metrics in the Pooled MS Cohort and Subcohorts



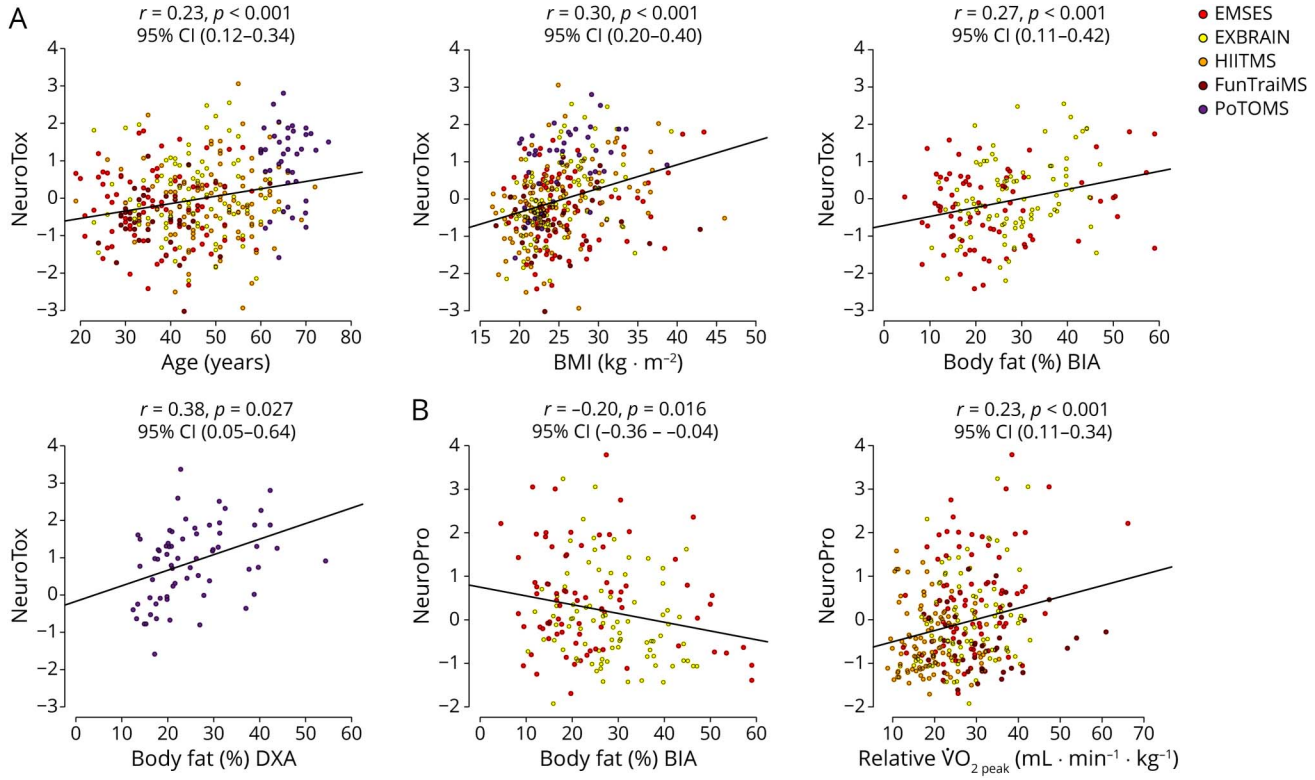
(A and B) Scatter plots showing correlations of *NeuroTox* and *NeuroPro* with EDSS scores across MS cohorts. *p* Values were FDR-adjusted. (C) Estimator plot showing the standardized estimators of the proportional odds regression models with *NeuroTox* and *NeuroPro* as predictor variables and EDSS score as predicted variable. Regression analyses were performed based on data from the pooled MS cohort (EMSES, EXBRAIN, HIITMS, FunTraiMS, and PoTOMS) and subcohorts. Greater *NeuroTox* was associated with higher EDSS score in the EMSES cohort (bold). (D) Estimator plot showing the standardized estimators of the multiple linear regression models with *NeuroTox* and *NeuroPro* as predictor variables and walking capacity (6MWT), fatigue (MFIS), cognitive performance (SDMT, SRT), or MRI metrics (Lesion volume, GMPF, WMPF) as predicted variables. Regression analyses were performed based on data from the pooled EMSES, EXBRAIN, and PoTOMS cohorts. EDSS = Expanded Disability Status Scale; FDR = false discovery rate; GMPF = gray matter parenchymal fraction; MFIS = Modified Fatigue Impact Scale (total score); MS = multiple sclerosis; SDMT = Symbol Digit Modalities Test; SRT-L = Selective Reminding Test-Long-term Retrieval Test; SRT-C = Selective Reminding Test-Consistent Long-term Retrieval Test; SRT-D = Selective Reminding Test-Delayed Recall Test; WMPF = white matter parenchymal fraction; 6MWT = 6-Minute Walk Test.

Second, we explored KP metabolite patterns in persons with MS, which were then evaluated in terms of consistency across individual MS cohorts and compared with KP metabolite patterns in healthy individuals. Third, we investigated associations between the emergent KP metabolite patterns with participant characteristics and clinical measures to study the role of the KP as one of the neuroimmunologic pathways involved in MS pathophysiology.

We show that persons with MS had a lower KTR and lower concentrations of most kynurenines compared with healthy individuals. These kynurenines include both metabolites ascribed with neurotoxic properties and those ascribed with neuroprotective properties. The serum concentrations of Neopt and 3-HAA and the QA/KA ratio were higher in persons with MS (eFigure 1, eTables 1 and 2). Neopt is believed to indicate Th1-mediated inflammatory stimulation of the KP.[22] Therefore, higher systemic concentrations of kynurenines in healthy individuals may result from inflammation-independent mechanisms. It may be hypothesized that persons with MS, presenting with a lower physical activity level[23] and reduced cardiorespiratory fitness[24] compared with healthy individuals, have a lower bioenergetic turnover to generate NAD^+, considering that both the hepatic and skeletal muscle KP substantially contribute to systemic concentrations of kynurenines.[25,26] In line with that, we recently discussed exercise-induced KP modulation in persons with MS as a potential endogenous mechanism to cover increased NAD^+ demands.[27] In addition, it has also been shown that persons with MS exhibit reduced expression of the KP enzyme indoleamine 2,3-dioxygenase 1 (IDO1) in peripheral blood mononuclear cells.[28] As IDO1 induces the first step of the KP,[26] reduced IDO1 expression in immune cells may contribute to lower concentrations of kynurenines in the systemic circulation.

Earlier studies yielded discrepant results concerning differences in systemic concentrations of kynurenines between persons with MS and healthy individuals, challenging comparisons with our findings. Nevertheless, we replicate the most consistent finding of human MS trials, that is a higher QA/KA ratio in persons with MS.[29,30]

Figure 5 Associations Between the KP Metabolite Patterns and Participant Characteristics in the Pooled MS Cohort or Subcohorts



(A) Scatter plots showing correlations of *NeuroTox* with age, BMI, and body fat percentage, derived from BIA (EMSES and EXBRAIN) or DXA (PoTOMS). (B) Scatter plots showing correlations of *NeuroPro* with body fat percentage, derived from BIA (EMSES and EXBRAIN), and cardiorespiratory fitness (relative $\dot{V}O_{2\ peak}$; all MS cohorts except PoTOMS). *p* Values were FDR-adjusted. BIA = bioelectrical impedance analysis; BMI = body mass index; DXA = dual-energy X-ray absorptiometry; FDR = false discovery rate; MS = multiple sclerosis; relative $\dot{V}O_{2\ peak}$ = peak oxygen consumption during cardiopulmonary exercise testing divided by body weight.

Using a data-driven approach, we identified 2 distinct KP metabolite patterns (Figure 2) that were consistent across individual MS studies (Figure 3). These 2 patterns separated neurotoxic and neuroprotective kynurenines, together with the respective precursors and degradation products, according to the traditional view of a neurotoxic and neuroprotective KP branch.[2] We further show that most kynurenines, with as yet unclear roles in MS, also clearly allocated to either *NeuroTox* or *NeuroPro*. Of note, we did not assess the molecular effects of kynurenines in our study and relied on previous evidence for factor designation. Our results nevertheless suggest that persons with pronounced *NeuroTox* present with elevated systemic inflammation, increased KP activity, and neurotoxic KP imbalance toward the formation of the neurotoxin QA. *NeuroTox* also included 3-HK, another neurotoxin, pro-oxidant, and QA agonist.[3] By comparison, persons with pronounced *NeuroPro* may have an increased neuroprotective capacity, considering that *NeuroPro* was characterized by the neuroprotective and antioxidative metabolite KA[2,5] and its precursor Qld, in line with a shift of the QA/KA ratio toward KA. *NeuroPro* also included XA and Pic. XA and Pic both have been shown to attenuate aspects of QA-related neurotoxicity and may thus synergize the neuroprotective effects of KA.[3] 3-HAA was the only kynurenine with higher concentrations in persons with MS (eFigure 1, eTables 1 and 2). Preclinical studies have shown that 3-HAA accumulates locally in response to neuronal damage in a rodent stroke model,[31] and induces immunosuppressive and proapoptotic effects on activated T cells,[32,33] suggesting a counterregulatory response to neurodegenerative or inflammatory processes. Other studies found ambiguity in the pro-oxidant or antioxidant effects of 3-HAA, which likely depend on the redox environment.[34-36] These findings correspond to the indistinct loading of 3-HAA on either *NeuroTox* or *NeuroPro* (Figure 2B).

Both an elevated QA/KA ratio in persons with MS and a shift in KP metabolite patterns may mirror KP imbalance at the CNS level and its potential contribution to the neurotoxic CNS microenvironment inherent to MS, given the concordance of CSF and systemic concentrations of kynurenines in MS.[7] Persons with pronounced *NeuroTox* and/or reduced *NeuroPro* would thus be particularly vulnerable to neuroaxonal damage, albeit this needs to be proven in future studies.

We do not show associations between KP metabolite patterns and MRI metrics or MS symptoms, which might be explained

by the investigation of a rather well-functioning study cohort (mean [SD] EDSS score of 3.1 [1.8]) that likely does not show extensive structural brain damage, walking impairment, fatigue, or cognitive impairment (eTable 3).[37,38] Nevertheless, we demonstrate that a greater *NeuroTox* and its components, Neopt, KTR, AA, QA/KA ratio, and QA, correlated with higher disease severity (Figure 4A, eFigure 2). Concordantly, a greater *NeuroPro* and its components, KA and XA, correlated with lower disease severity (Figure 4B, eFigure 2). Although the same trend was reproduced in the logistic regression models, the associations remained nonsignificant in the adjusted analyses, potentially due to the significant association between age and EDSS score. In support of this notion, both greater *NeuroTox* and lower *NeuroPro* were significantly associated with higher EDSS scores when excluding the covariates age and study cohort from the models.

Aging and MS are believed to induce a cumulative effect on neuroaxonal damage in older persons with MS through the interaction of age-specific and disease-specific mechanisms that are detrimental to CNS health.[39] These mechanisms likely involve the KP, given that immunologic changes during the aging process, referred to as inflammaging, are themselves associated with KP imbalance.[40] In that sense, inflammaging contributes to systemic KP imbalance due to the perpetuation of chronic systemic low-grade inflammation and may fuel CNS KP imbalance due to an increased activation of microglia and CNS recruitment of proinflammatory macrophages,[39] both of which are the main sources of QA in the CNS.[2] In line with that, we show that older age was associated with greater *NeuroTox* (Figure 5A) and that participants of the oldest cohort PoTOMS (mean [SD] age: 65.7 [3.7] years) revealed particularly pronounced *NeuroTox*, especially when compared with the youngest cohort EMSES (mean [SD] age: 37.3 [9.9] years) (Figures 1C and 2C).

Our findings additionally suggest that both *NeuroTox* and *NeuroPro* are modulated by lifestyle-related factors, such as body composition and regular physical exercise, both of which shape the proinflammatory microenvironment in MS and influence MS-related disability.

In this regard, it has been shown that hypertrophic and dysfunctional adipose tissue contributes to chronic systemic low-grade inflammation through the release of proinflammatory cytokines, such as interferon-gamma, thereby contributing to increased inflammatory stimulation of the KP.[41] Accordingly, we show that a higher BMI and a higher amount of body fat were associated with greater *NeuroTox* across measurement methods (Figure 5A). These findings confirm our previous results showing that overweight and obese persons with MS had a higher KTR and higher serum concentrations of most kynurenines compared with normal-weight and underweight persons with MS.[42] Expanding our previous results, we now demonstrate that a higher body fat percentage is associated with a predominant formation of neurotoxic metabolites and an unfavorable shift in KP metabolite ratios. These findings are of particular relevance for the potential involvement of the KP in MS pathophysiology, considering that obesity in childhood and adolescence significantly increases MS risk,[43] and that obese persons with MS exhibit higher disease severity and accelerated disability accumulation.[44]

By contrast, regular physical exercise, which is indicated by increased cardiorespiratory fitness, has a beneficial effect on the proinflammatory microenvironment in MS and KP imbalance. We previously showed that exercise reduced systemic markers of inflammation[45] and downregulated the CD49d expression of $CD8^+$ T cells, which is a migration marker on immune cells targeted by natalizumab.[46] Moreover, we previously reported that an acute intense exercise bout shifted the QA/KA ratio toward an increased formation of KA.[8] The results of this study show that higher cardiorespiratory fitness was associated with greater *NeuroPro* (Figure 5B) and its components, including KA, Qld, XA, Pic, and QA/KA ratio (inversely) (eFigure 2). Kynurenic acid, XA, and Pic may induce a concerted neuroprotective and/or anti-inflammatory action, given that, for example, both KA and XA are ligands to the aryl hydrocarbon receptor,[47] which promotes the differentiation of anti-inflammatory regulatory T cells.[48] In this regard, laquinimod, another aryl hydrocarbon receptor ligand with structural similarity to KA, has been shown to induce potent disease-modifying effects in 2 phase III MS trials.[49]

Our study comes with strengths and limitations. To our knowledge, this study is the largest investigation of the KP in MS. The participants included are well characterized in relation to demographic, anthropometric, and disease-specific characteristics, and cover a wide age (19–76 years) and disability (EDSS ≤6.5) range. By applying a holistic data-driven approach that, beyond single-metabolite investigations, enabled the identification of distinct KP metabolite patterns, we accounted for the complexity of the KP. Using the KP metabolite patterns *NeuroTox* and *NeuroPro*, we comprehensively investigated associations between the systemic KP and markers of neuroaxonal damage (e.g., MRI metrics), disease severity, MS symptoms, and a broad collection of MS-relevant participant characteristics. Thereby, the results of this analysis improve the current understanding of the KP and its potential clinical relevance in MS. The genuine neurotoxic or neuroprotective effects of *NeuroTox* and *NeuroPro*, as designated based on previous evidence, need to be validated in follow-up studies, including molecular markers of excitotoxicity or oxidative stress. We refrained from sex-matching of cohorts to retain the sample size of the smaller HC cohort. The differences in both the proportion of sexes and cohort sizes complicate a fair comparison of correlation matrices. The additional control for diet or time since the last relapse would have been optimal. The inclusion of such factors may have increased the cumulative proportional variance explained by the EFA-derived factors. We did not assess CSF samples, which are the preferred choice when studying the role of kynurenines in CNS integrity and function. Nevertheless, a recent study showed that systemic kynurenines

outperformed CSF-derived kynurenines in the prediction of Parkinson disease diagnosis,[50] highlighting the value of systemic kynurenines as informative and easily accessible markers in neurologic diseases. Inherent to the cross-sectional nature of this analysis, our results do not allow statements on the cellular or tissue-specific origin of kynurenines, intercompartmental exchange across the blood-brain barrier, or causality of associations. In this regard, future studies are warranted that include participants across all disability levels and perform repeated blood sampling.

Author Contributions

M. Kupjetz: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data. M. Langeskov-Christensen: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. M. Riemenschneider: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. S. Inerle: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. U. Ligges: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. T. Gaemelke: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. N. Patt: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. J. Bansi: drafting/revision of the manuscript for content, including medical writing for content. R.R. Gonzenbach: drafting/revision of the manuscript for content, including medical writing for content. M. Reuter: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. F. Rosenberger: drafting/revision of the manuscript for content, including medical writing for content. T. Meyer: drafting/revision of the manuscript for content, including medical writing for content. A. McCann: drafting/revision of the manuscript for content, including medical writing for content. P.M. Ueland: drafting/revision of the manuscript for content, including medical writing for content. S.F. Eskildsen: drafting/revision of the manuscript for content, including medical writing for content. M.K.E. Nygaard: drafting/revision of the manuscript for content, including medical writing for content. N. Joisten: drafting/revision of the manuscript for content, including medical writing for content; study concept or design. L. Hvid: drafting/revision of the manuscript for content, including medical writing for content. U. Dalgas: drafting/revision of the manuscript for content, including medical writing for content. P. Zimmer: drafting/revision of the manuscript for content, including medical writing for content; study concept or design.

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