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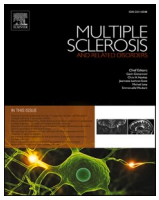

Association between microglial activation and serum kynurenine pathway metabolites in multiple sclerosis patients

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ABSTRACT

Background: Microglial activation associates with MS progression but it is unclear what drives their persistent pro-inflammatory state. Metabolites of the kynurenine pathway (KP), the main metabolism route of tryptophan, can influence the function of brain innate immune cells.
Objective: To investigate whether tryptophan metabolites in blood associate with TSPO-PET measurable microglial activation in MS brain.
Methods: Microglial activation was detected using PET imaging and the TSPO-binding radioligand [^{11}C]PK11195. Distribution volume ratios (DVR) for specific [^{11}C]PK11195-binding in the normal appearing white matter (NAWM), lesions, and thalamus were calculated. Ultrahigh performance liquid chromatography-tandem mass spectrometry was used to measure serum levels of tryptophan and kynurenine pathway metabolites.
Results: The study cohort consisted of 48 MS patients. Increased DVR in the NAWM and thalamus correlated with decreased serum 3-hydroxykynurenine level ($R = -0.31$, $p = 0.031$ and $R = -0.32$, $p = 0.028$). Increased EDSS correlated with decreased 3-hydroxykynurenine and xanthurenic acid ($R = -0.36$, $p = 0.012$ and $R = -0.31$, $p = 0.034$) and increased DVR in the NAWM and thalamus ($R = 0.33$, $p = 0.023$ and $R = 0.34$, $p = 0.020$, respectively).
Conclusions: This clinical study demonstrates an association between low serum 3-hydroxykynurenine and high microglial activation in MS. Further investigations are warranted for elucidation of the biological mechanisms behind this association.

1. Introduction

Relapsing remitting multiple sclerosis (RRMS) is characterized by blood-brain barrier (BBB) breakdown and emergence of focal inflammatory lesions in the central nervous system (CNS), where organ-specific autoreactive T lymphocytes are driving the process with involvement of a network of other cell types, e.g. B lymphocytes and the innate immune system players macrophages and microglia (Dobson and Giovannoni, 2019). Innate immune system activation can be detected *in vivo* using positron emission tomography (PET) and 18-kDa translocator protein (TSPO) binding radioligands. PET is a powerful and sensitive tool that offers valuable information of the disease pathology in various

Abbreviations: Ahr, Aryl-hydrocarbon receptor; BBB, Blood-brain barrier; CNS, Central nervous system; DVR, Distribution volume ratio; EDSS, Expanded disability status scale; FLAIR, Fluid attenuated inversion recovery; IQR, Interquartile range; KYN, Kynurenine; KYNA, Kynurenic acid; MS, Multiple sclerosis; NAWM, Normal appearing white matter; PET, Positron emission tomography; QUIN, Quinolinic acid; RRMS, Relapsing-remitting multiple sclerosis; SD, Standard deviation; TRP, Tryptophan; TSPO, Translocator protein; 3HK, 3-hydroxykynurenine.

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areas of the brain (Bodini et al., 2021), and numerous PET studies have confirmed that the CNS innate immune cell activation is a phenomenon particularly associated with multiple sclerosis (MS) disease progression (Sucksdorff et al., 2020; Giannetti et al., 2015; Politis et al., 2012; Rissanen et al., 2018; Singhal et al., 2019).

The essential amino acid tryptophan (TRP) is a precursor for neuroactive compounds that can influence the function of microglia or astrocytes, and have either neurotoxic or neuroprotective effects (Schwarcz et al., 2012). More than 95% of the TRP is metabolized via the kynurenine pathway (Fig. 1), while the rest is directly converted to serotonin. Alterations in TRP metabolism have been associated with various human pathologies including psychiatric disorders, autoimmunity and neurodegeneration (Platten et al., 2019).

The kynurenine pathway metabolites are involved in inflammation, immune response and excitatory neurotransmission and they serve as important mediators of inter-organ communication (Savitz, 2020; Cervenka et al., 2017). Regulation of the kynurenine pathway is complex, and involves factors such as TRP availability, impacted both by TRP intake and by the gut microbiome (consumption), and the activity of the individual kynurenine pathway-associated enzymes in various compartments of the body. Of the kynurenine metabolites, TRP, kynurenine (KYN) and 3-hydroxykynurenine (3HK) penetrate avidly through the BBB, whereas kynurenic acid (KYNA) and quinolinic acid (QUIN) cross the BBB less efficiently, but can be produced from KYN and 3HK by astrocytes and microglia, respectively (Schwarcz et al., 2012; Fukui et al., 1991). The 3HK-QUIN arm of the kynurenine pathway is enhanced under inflammatory condition (Connor et al., 2008; Molteni et al., 2013) with resulting adverse outcomes in the CNS, as 3HK is a known free radical generator while QUIN is a known neurotoxin and gliotoxin.

The associations between metabolic biomarkers and CNS immunopathology in MS disease are largely unexplored (Vécsei et al., 2013). The primary objective of the present study was to search potential associations between peripheral kynurenine metabolite levels and TSPO-PET measurable microglial activation in MS to obtain further understanding of the relations between the CNS innate immune system and TRP metabolism products.

2. Patients and methods

MS patients were recruited from the outpatient clinic of the Division of Clinical Neurosciences at the University Hospital Turku, Turku, Finland during 2015–2019. The study protocol included [^{11}C]PK11195 PET imaging to detect microglial activation, MRI to provide anatomical reference and to evaluate pathology related to MS, blood sampling to measure the concentration of serum kynurenine metabolites, and clinical assessment by an experienced clinician to evaluate the Expanded Disability Status Scale (EDSS) score using standardized examination form (neurostatus.net). Exclusion criteria included inability to tolerate PET or MRI, active autoimmune or neurological disease other than MS, another comorbidity considered significant, and current or desired pregnancy.

The Ethical Committee of the Hospital District of Southwest Finland approved the study and written informed consent was obtained from all participants according to the Declaration of Helsinki.

2.1. MRI acquisition

Brain MRI scans were performed in Turku PET center with a 3 T Ingenuity TF PET/MR scanner (Philips Healthcare, Cleveland, OH) as previously described (Bezukladova et al., 2020). Axial T2, 3D fluid attenuated inversion recovery (FLAIR), 3D T1, and gadolinium enhanced 3D T1 sequences were acquired. An 8-channel SENSE head coil was used.

2.2. PET acquisition

The radioligand [^{11}C]PK11195 was synthesized according to previously described methodology (Rissanen et al., 2018). Dynamic 60-minute PET imaging was performed in Turku PET center with the ECAT High Resolution Research Tomograph (HRRT) Scanner (CTI, Siemens Medical Solutions, Knoxville, TN, USA). The intrinsic spatial resolution of the HRRT scanner is approximately 2.5 mm (de Jong et al., 2007). A thermoplastic mask was used to minimize head movements during the scan. Before the administration of [^{11}C]PK11195-radioligand, a transmission scan (6 min) with ^{137}Cs point source was performed to obtain attenuation correction. The radioligand was administered as a smooth intravenous bolus injection. The mean (SD) injected dose was 488 (14.3) MBq.

2.3. MRI and PET data preprocessing and analysis

Preprocessing of MRI data included following steps: 1) creation of T2 lesion masks by identifying T2 hyperintense lesions from FLAIR images using Lesion Segmentation Toolbox 2) manual correction of T2 lesion masks 3) manual creation and correction of T1 lesion masks 4) segmentation of white matter, gray matter and thalamus with Freesurfer

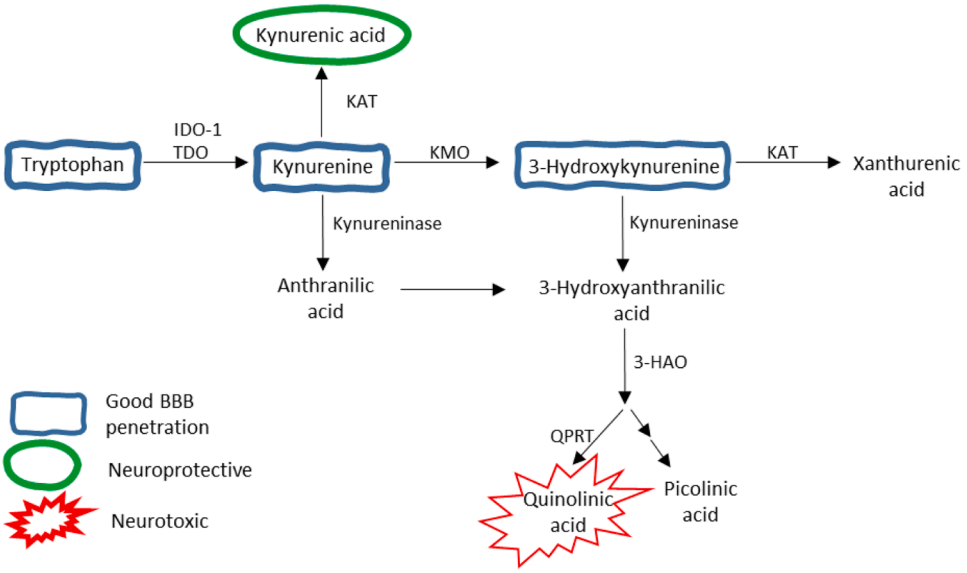

Fig. 1. Scheme of the kynurenine pathway. Essential amino acid tryptophan is mainly catabolized via the kynurenine pathway. 3-HAO = 3-hydroxyanthranilate-3,4-dioxygenase, IDO-1 = Indoleamine-2,3-dioxigenase-1, IDO-2 = indoleamine-2,3-dioxigenase-2, KAT = kynurenine aminotransferase, KMO = kynurenine 3-monooxigenase, QPRT = quinolinate phosphoribosyltransferase, TDO = tryptophan-2,3-dioxigenase.

software. The details of the preprocessing steps have been described earlier (Rissanen et al., 2018). Normal appearing white matter (NAWM) region of interest was created by excluding T2 lesions from the white matter. The volumes of NAWM, T1 and T2 lesions, cortical gray matter and thalamus were defined with Freesurfer software according to our previously reported methodology (Rissanen et al., 2018).

PET images were reconstructed using 17 time frames, smoothed and preprocessed as previously described (Rissanen et al., 2018; Rissanen et al., 2014). Distribution volume ratio (DVR) was used to evaluate specific [^{11}C]PK11195-binding in NAWM, T1 and T2 lesions, and thalamus. The time-activity curve corresponding to a reference region, which is devoid of specific [^{11}C]PK11195 binding, was acquired using a supervised clustering algorithm (SuperPK software) as previously described (Rissanen et al., 2014). The reference tissue-input Logan method with a time interval of 20–60 min was applied to the regional time-activity curves using the supervised clustering algorithm gray reference input.

2.4. Measurement of serum kynurenine-pathway metabolites

Blood was collected in 10 ml Vacuette® serum clot-activator tubes (Greiner Bio-one, product number 455,092) before 12 AM. Blood was allowed to clot for 30 min at room temperature and serum was stored in aliquots at -80 °C within 2 h of sampling. Serum concentrations of TRP, KYN, 3HK, 3-hydroxyanthranilic acid, QUIN, KYNA, anthranilic acid, xanthurenic acid and picolinic acid were assessed using ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) according to previously published methodology (Galla et al., 2021).

2.5. Statistical analysis

Statistical analyses were performed using R software (version 4.1.1). Wilcoxon rank-sum test was used to assess differences between females and males. To test our main hypothesis, the associations between the concentrations of serum kynurenine pathway metabolites and [^{11}C]PK11195 DVR in NAWM, thalamus, and T1 and T2 lesions were assessed using Spearman correlation analysis and multiple linear regression model in all study patients and separately in patients under and over 50 years old. The model was adjusted with age, disease duration, sex and disease modifying treatment [no disease modifying treatment, 1st line treatment (dimethyl fumarate, fingolimod, glatiramer acetate, interferon β-1a and teriflunomide) or 2nd line treatment (natalizumab)]. Due to the exploratory nature of the study correction for multiple testing was not performed. In addition, Spearman correlation coefficients between metabolites and clinical (age, EDSS, disease duration) and MRI parameters were calculated. A p-value < 0.05 was considered statistically significant in all analyses.

3. Results

The demographics, clinical characteristics and volumetric MRI data of the 48 MS patients taking part in the study are shown in Table 1. Most of the patients had relapsing-remitting disease type (92 %) and were using a disease modifying therapy at the onset of the study (73 %). Brain TSPO-radioligand uptake and serum concentrations of kynurenine pathway metabolites were evaluated in all study patients (Table 2).

In the Spearman correlation analysis, low serum 3HK correlated with high [^{11}C]PK11195 DVR both in the NAWM and in thalamus (Fig. 2). The negative association between 3HK and NAWM DVR was sustained in multiple regression analysis of the data [estimate (*10^{-4}): -6.13 (95 % CL -11.5 - -0.79), p = 0.026]. No other statistically significant correlations between kynurenine pathway metabolites and [^{11}C]PK11195 DVR were observed among the study cohort (data not shown). When subgroups with patients younger (n = 31) and older (n = 17) than 50 years were evaluated separately, we observed among younger patients a stronger correlation between serum 3HK and NAWM and thalamus DVR (Fig. 2). These associations were similarly sustained in multiple regression analysis of the data [estimate (*10^{-4}): -10.3 (95 % CL -16.2 - -4.4), p = 0.0015 for NAWM and -11.1 (95 % CL -19.9 - -2.4), p = 0.014 for thalamus]. In older patients, there were no correlations between 3HK and microglial activation in the studied regions (data not shown).

A low EDSS correlated with high serum 3HK and xanthurenic acid (Fig. 3). Similarly, low EDSS correlated with low DVR in the NAWM and in thalamus. There were no correlations between EDSS and other measured metabolites. The serum levels of TRP and measured kynurenine pathway metabolites did not correlate with age or disease duration, except for xanthurenic acid, which correlated inversely with age and disease duration (R = -0.3, p = 0.04 and R = -0.38, p = 0.008, respectively). There were no correlations between kynurenine pathway metabolites and volumetric MRI parameters regarding NAWM and thalamus, or the lesion load (data not shown).

Table 1
Demographics, clinical characteristics and volumetric MRI data of the study cohort (n = 48).

Disease type, n RRMS / n SPMS	44 / 4
Sex, n female / n male	37 /11
Age, years	47 (5.8)
Disease duration, years from the first symptoms	13.9 (6.9)
EDSS (median, IQR)	2.75 (2.00–3.13)
MSSS (median, IQR)	2.98 (1.74–4.35)
Disease modifying treatment, n (%)	
no DMT	13 (27 %)
Teriflunomide	14 (29 %)
Interferon beta-1a	6 (12.5 %)
Fingolimod	5 (10 %)
Glatiramer acetate	4 (8 %)
Dimethyl fumarate	3 (6 %)
Natalizumab	3 (6 %)
NAWM volume (cm^3)	457.4 (66.8)
T1 lesion load (cm^3, median, IQR)	2.60 (1.34–5.78)
T2 lesion load (cm^3, median, IQR)	4.93 (2.26–10.54)
GMctx volume (cm^3)	439.3 (40.7)
Thalamus volume (cm^3)	13.6 (1.71)

Variables are presented as mean (SD) unless stated otherwise.
DMT = disease modifying treatment, EDSS = Expanded Disability Status Scale, GMctx = cortical gray matter, IQR = Interquartile range, MSSS = Multiple Sclerosis Severity Score, NAWM = normal appearing white matter, RRMS = relapsing-remitting multiple sclerosis, SPMS = secondary progressive multiple sclerosis

Table 2
Average [^{11}C]PK11195 DVR values in studied region of interests in the brain and serum concentrations of metabolites related to kynurenine pathway.

[^{11}C] PK11195 distribution volume ratio	
NAWM	1.20 (0.048)
T1 lesion	1.14 (0.108)
T2 lesion	1.14 (0.092)
Thalamus	1.33 (0.072)
Serum concentration (nmol/l)	
Tryptophan	59,410 (48,648–65,435)
Kynurenine	1412 (1216–1615)
3-hydroxykynurenine	66.2 (53.3–77.3)
3-hydroxyanthranilic acid	34.3 (27.3–40.9)
Quinolinic acid	502 (396–645)
Kynurenic acid	39.9 (31.0–52.0)
Anthranilic acid	123 (81.9–189.4)
Xanthurenic acid	5.80 (5.28–8.49)
Picolinic acid	123 (81.9–189)

DVR variables are presented as mean (SD) and serum concentrations as median (IQR). DVR = distribution volume ratio, IQR = interquartile range, NAWM = normal appearing white matter

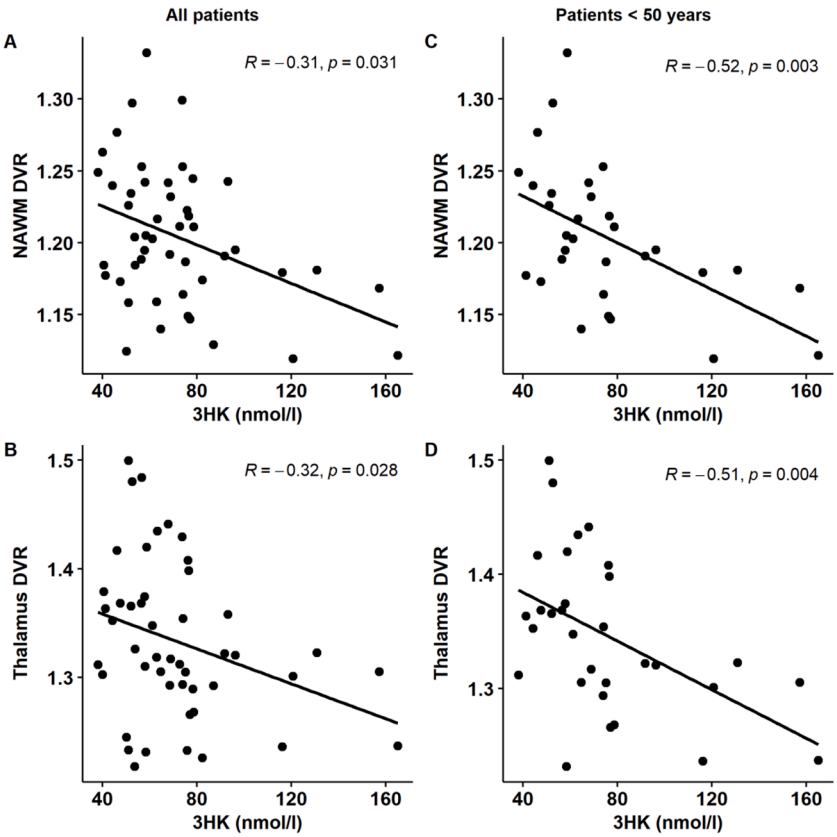

Fig. 2. Correlations between serum 3HK and microglial activation.
Serum concentration of 3HK (nmol/l) correlates with [^{11}C]PK11195 DVR values in the NAWM (A) and thalamus (B) among all MS patients (n = 48). In a more homogenous subgroup of patients younger than 50 years of age (n = 31), similar but stronger correlations were observed (C, D). Shown are Spearman correlation coefficients (R) and p-values. DVR = distribution volume ratio; NAWM = normal appearing white matter; 3HK = 3-hydroxykynurenine.

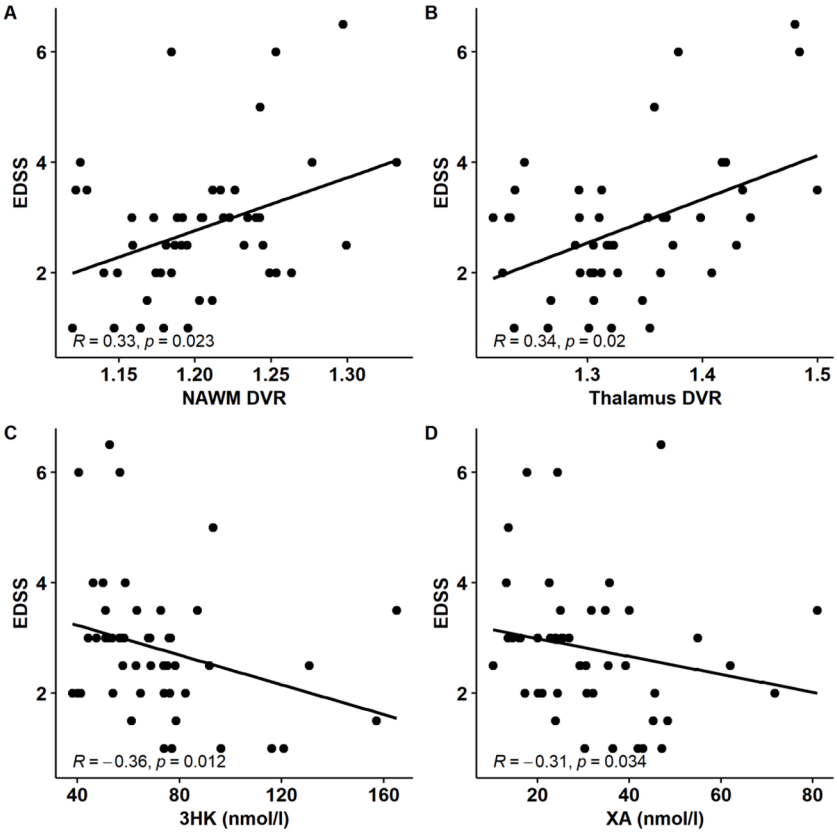

Fig. 3. Correlations of EDSS with microglial activation and serum kynurenine pathway metabolites.
EDSS correlates with [^{11}C]PK11195 DVR values in the NAWM (A) and thalamus (B), and with serum concentrations of 3HK (C) and xanthurenic acid (D). Shown are Spearman correlation coefficients (R) and p-values. DVR = distribution volume ratio, EDSS = expanded disability status scale, NAWM = normal appearing white matter, XA = xanthurenic acid, 3HK = 3-hydroxykynurenine.

4. Discussion

The results from the present study demonstrate that in MS patients **low** serum levels of the kynurenine pathway product 3HK associate with **enhanced** microglial activation both in the NAWM and in thalamus. Compared to healthy controls, MS patients display increased TSPO-PET-measurable microglial activation in the NAWM and in thalamus (Rissanen et al., 2018). This phenomenon is more pronounced in advanced MS, and as increased TSPO-binding also predicts later MS progression (Sucksdorff et al., 2020), the TSPO-PET measurable activated microglial phenotype is considered detrimental in MS pathogenesis. The mechanisms fostering the prolonged pro-inflammatory innate immune cell activation state within MS brain are presently poorly understood.

The finding of low serum 3HK associating with increased microglial activation is surprising at first glance considering that under experimental conditions, 3HK promotes apoptosis of neurons, and enhances oxidative stress (Heyes et al., 1997; Amori et al., 2009). Moreover, its microglia-produced downstream metabolite QUIN enhances production of pro-inflammatory mediators in astrocytes (Guillemin et al., 2003), and promotes neurotoxicity via excitatory mechanisms (Guillemin, 2012; Müller and Schwarz, 2007) and via activation of microglia through NMDA receptors, a pathway shown to trigger neuronal cell death (Kaindl et al., 2012). The association of low serum 3HK and high microglial activation might however be explained by the large variability of the kynurenine pathway enzymes and the respective metabolites in various compartments of the body. Levels of enzymes are much higher in the periphery compared to the CNS (Schwarcz et al., 2012). There is also significant heterogeneity how the different metabolites cross the BBB (Fig. 1). TRP, KYN and 3HK are efficiently transported across the BBB, whereas KYNA and QUIN penetrate the BBB poorly (Fukui et al., 1991). A high proportion of KYN in the CNS is considered to be of exogenous origin under physiological conditions, due to active transportation by the large neutral amino acid transporter (Fukui et al., 1991; Kita et al., 2002). Following systemic immune activation, this may increase to close to 100% (Kita et al., 2002). It would have been interesting to measure 3HK in the CSF of the patients, but unfortunately this was not possible due to ethical considerations. This is a clear weakness of the study. Measurement of the metabolite levels in both serum and CSF could have provided important insight about the potential variabilities in 3HK concentration in blood vs. CNS. Other studies have shown a positive correlation between blood and CSF for certain kynurenine pathway metabolites for example in un-medicated depressed patients, and also in a small cohort of MS patients, but here 3HK was not included in the measurements (Haroon et al., 2020; Lim et al., 2017).

Our observation of a cooperation between low serum 3HK concentration, high PET-measurable innate immune cell activation in the brain, and high clinical disability status suggests a potential role for 3HK in regulating the pathogenesis of MS, potentially via mechanisms involving microglia. TRP metabolites seem to limit CNS inflammation via microglial and astrocytic aryl-hydrocarbon receptor (AHR) activation, which brings further complexity to the association between the kynurenine pathway and MS progression (Rothhammer et al., 2018). It would be tempting to hypothesize that AHR might be a link between increased serum 3HK and decreased microglial activation. However, further *in vitro* studies are needed to clarify whether 3HK can act as an AHR ligand and consequently dampen microglial response. According to the current knowledge, the critical AHR-binding metabolites are downstream metabolites of the KYN-KYNA branch of the pathway, as KYNA effects in the CNS have been shown to be neuroprotective, possibly via AHR signaling (Foster et al., 1984; Opitz et al., 2011). Furthermore, 3HK is one of the three kynurenine pathway metabolites that may suppress T cell responses by a time-dependent cytotoxic action of activated T cells, but also B cells and natural killer cells, and thereby low serum 3HK may directly contribute to enhanced inflammation also in the CNS (Terness et al., 2002).

Kynurenine pathway metabolites, especially TRP and KYN, have been analyzed in certain MS-related settings also previously. These studies are heterogeneous and the results are somewhat conflicting (Supplement 1). In the present study, we analyzed serum 3HK concentration also in a more homogenous group of MS patients with shorter disease duration, less pronounced disability and younger age, and found a yet stronger association between both 3HK and microglial activity, and 3HK and EDSS compared to older, more disabled patients with a longer disease duration. This could partly explain why our results were not in line with findings from a previous study, in which a positive correlation between EDSS and serum levels of 3HK and QUIN were observed, as in the study of Lim et al., almost half of the patients had progressive disease course and were heavily disabled (Lim et al., 2017). Interestingly, in another subgroup analysis serum 3HK levels were slightly higher among women than men [75.8 (29.2) vs. 57.2 (16.3) nmol/l, $p = 0.048$, data not shown]. This does not contrast previous studies reporting a more inflammatory phenotype in microglial cells among males (Villa et al., 2018). Other studies have indicated a negative association between EDSS and CSF levels of TRP and KYNA, and a positive association between EDSS and QUIN (Rajda et al., 2020), but not with KYN (Herman et al., 2019). No correlation have been observed between CSF KYN and KYNA levels and MS disease duration, or with number of T1 or T2 lesions (Herman et al., 2019). In pediatric MS patients low serum TRP levels predicted increased risk of MS, and KYN predicted risk of relapse (Nourbakhsh et al., 2018).

The present study has some important limitations. Our study population was relatively small and heterogeneous regarding the age, treatment status and sex, which may have affected the strength and significance of the correlations between microglial activation and kynurenine pathway metabolites. On the other hand, the study population consisted mainly of RRMS patients, which might limit the generalizability of our results. Thus, larger studies are needed to confirm and expand our results. The lack of adequate controls is another major limitation of the present study. Therefore, it remains to be seen whether the observed association is specific to MS. Under certain conditions, TRP availability may be highly influenced by the gut microbiome, which transforms TRP to indole metabolites (Cervenka et al., 2017). Unfortunately, the measurement of microbiome-derived indole metabolites was out of the scope of the present study, and thus further studies are needed to elucidate the association of gut microbiota derived TRP metabolites with microglial activation in MS. In addition, due to the exploratory nature of the study, the results were not corrected for multiple testing. Hence, the possibility of false positive results cannot be entirely ruled out. However, the significance of the main finding of our study, i.e. the negative correlation between 3HK and microglial activation, was corroborated in a multiple regression analysis. More importantly, the fact that 3HK correlated negatively also with EDSS, emphasizes the true nature of this finding.

In summary, our study has identified a connection between low serum 3HK and high PET-measurable microglial activation in brain regions relevant for MS progression, such as the NAWM and thalamus. In addition, low serum 3HK levels associated with high EDSS scores. To our knowledge, ours is the first study addressing the impact of kynurenine pathway metabolites on glial cell activation in MS patients *in vivo*. Assessing the mechanisms of the potential impact of blood TRP metabolites on brain cell pathology is complex, and thus further investigations are needed to confirm our preliminary results, and to explore the biological mechanisms, through which kynurenine pathway might function as a plausible link between tryptophan intake, microglial activation and MS disease progression.

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CRediT authorship contribution statement

Maija Saraste: Investigation, Writing – original draft, Writing – review & editing. **Markus Matilainen:** Formal analysis, Data curation. **Cecilia Rajda:** Investigation, Writing – review & editing. **Zsolt Galla:** Investigation, Writing – original draft, Writing – review & editing. **Marcus Sucksdorff:** Resources, Investigation. **László Vécsei:** Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing. **Laura Airas:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

M.Sa, M.M, C.R, Z.G and L.V have no declaration of interests. M.Su has served on advisory boards for Sanofi-Aventis and Roche, has received speaker honoraria from Merck Serono and travel honoraria from Orion, Roche, Biogen and Sanofi-Aventis, and received research support from The Finnish Medical Foundation, The Finnish MS Foundation and from The Finnish Medical Society (Finska Läkaresällskapet). L.A has received honoraria from Biogen, Roche, Genzyme, Merck Serono and Novartis, and institutional research grant support from Finnish Academy, Sanofi-Genzyme and Merck Serono.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2022.103667.

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