

Dynamic regulation of serum aryl hydrocarbon receptor agonists in MS

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ABSTRACT

Objective: Several factors influence the clinical course of autoimmune inflammatory diseases such as MS and inflammatory bowel disease. Only recently, the complex interaction between the gut microbiome, dietary factors, and metabolism has started to be appreciated with regard to its potential to modulate acute and chronic inflammation. One of the molecular sensors that mediates the effects of these environmental signals on the immune response is the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor with key functions in immune cells.

Methods: In this study, we analyzed the levels of AHR agonists in serum samples from patients with MS and healthy controls in a case-control study.

Results: We detected a global decrease of circulating AHR agonists in relapsing-remitting MS patients as compared to controls. However, during acute CNS inflammation in clinically isolated syndrome or active MS, we measured increased AHR agonistic activity. Moreover, AHR ligand levels in patients with benign MS with relatively mild clinical impairment despite longstanding disease were unaltered as compared to healthy controls.

Conclusions: Collectively, these data suggest that AHR agonists in serum are dynamically modulated during the course of MS. These findings may guide the development of biomarkers to monitor disease activity as well as the design of novel therapeutic interventions for MS. *Neurol Neuroimmunol Neuroinflamm* 2017;4:e359; doi: 10.1212/NXI.0000000000000359

GLOSSARY

AHR = aryl hydrocarbon receptor; **CIS** = clinically isolated syndrome; **DMT** = disease-modifying therapy; **HEK** = human embryonic kidney; **IBD** = inflammatory bowel disease; **Kyn** = Kynurenine; **RRMS** = relapsing-remitting MS.

The Aryl hydrocarbon receptor (AHR) is a key regulator of innate and adaptive immune responses relevant to the pathogenesis of autoimmune diseases such as inflammatory bowel disease (IBD) and MS.^{1–4} AHR is a ligand-activated transcription factor, whose function is regulated by small agonists that promote AHR activation, nuclear translocation, and the control of specific transcriptional programs.^{5–14} These agonists are provided by diverse sources, including environmental pollutants, dietary components, microbial products, as well as endogenous metabolites.^{3,6–11,13–17}

The relevance of endogenous AHR ligands during inflammation has been investigated in different experimental paradigms. L-Kynurenine (Kyn), for example, is an AHR agonist generated by endogenous metabolism. Of interest, Kyn is increased in the context of inflammation and dampens proinflammatory T-cell responses, limiting immune-mediated pathology.^{18,19} Similarly, synthetic agonists can also activate AHR to therapeutically modulate the immune response. Laquinimod is an AHR agonist that shows anti-inflammatory and neuroprotective effects in the MS model experimental autoimmune encephalomyelitis probably as a result of the inhibition of NF- κ B activation in mouse and human dendritic cells.^{20–25} Indeed, beneficial effects of laquinimod were also documented in the Benefit-Risk Assessment of Avonex and Laquinimod

Supplemental data
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Table Characteristics of patients with MS and controls tested in luciferase assays						
Figure	Cohorts	Females	Age, y	Disease duration	EDSS	Treatment
1	Controls (26)	16 (61.5)	29.0 (26.0, 30.3)	None	None	None
	RRMS (91)	71 (78)	44.2 (40.0, 48.9)	13.6 (7.8, 17.6)	1.8 (1.0, 2.5)	62 (68.1)
2	Controls (26)	16 (61.5)	31.9 (26.0, 38.0)	None	None	None
	RRMS remission (32)	26 (81.2)	44.7 (40.7, 51.6)	14.8 (12.0, 18.8)	1.8 (1.5, 2.0)	11 (34.4)
	RRMS active (20)	16 (80)	38.3 (31.2, 49.7)	6.6 (3.0, 11.5)	1.6 (1.0, 2.5)	10 (50)
3	Controls (33)	20 (60.1)	36.2 (26.0, 45.5)	None	None	None
	CIS (15)	9 (60)	32.8 (28.1, 34.6)	Onset	1.6 (1.5, 2.0)	0
4	Controls (7)	6 (85.7)	48.9 (42.0, 52.0)	None	None	None
	Benign (11)	10 (90.9)	54.1 (51.0, 56.0)	21.0 (17.0, 28.0)	0.6 (0, 1.5)	2 (18.2)

Abbreviations: CIS = clinically isolated syndrome; RRMS = relapsing-remitting MS.

In column "Females," numbers represent absolute numbers, and in parentheses, percentages of females. In columns Age, Disease duration, and Expanded Disability Status Scale (EDSS), numbers represent mean, numbers in parentheses represent 25% and 75% percentiles, respectively. In column "Treatment," numbers represent absolute numbers of treated patients, and in parentheses, percentages of patients under immunomodulatory treatment.

(BRAVO) study, in which laquinimod-treated patients with MS showed a reduction in the rate of cerebral atrophy vs placebo that suggested a neuroprotective role of AHR activation during CNS inflammation.²⁶

Anti-inflammatory AHR ligands are also provided by the diet and commensal bacteria.^{2,9} These ligands have the capability to dampen ongoing inflammation in the colonic mucosa and improve the outcome of experimental colitis.⁶ Moreover, alterations in the composition of the commensal flora as well as genetic polymorphisms detected in IBD patients have been shown to impair the generation of these protective AHR ligands, ultimately contributing to immune dysregulation and disease pathology.⁶

AHR agonists provided by the diet and commensal bacteria also contribute to the control of CNS inflammation. We have recently shown that AHR agonists generated by the interaction of the gut microbiome and host metabolism cross the blood-brain barrier and dampen CNS inflammation by activating AHR in resident cells.⁵ Accordingly, we detected decreased CNS AHR activation in a small set of MS samples, as well as decreased circulating AHR agonists.^{5,6}

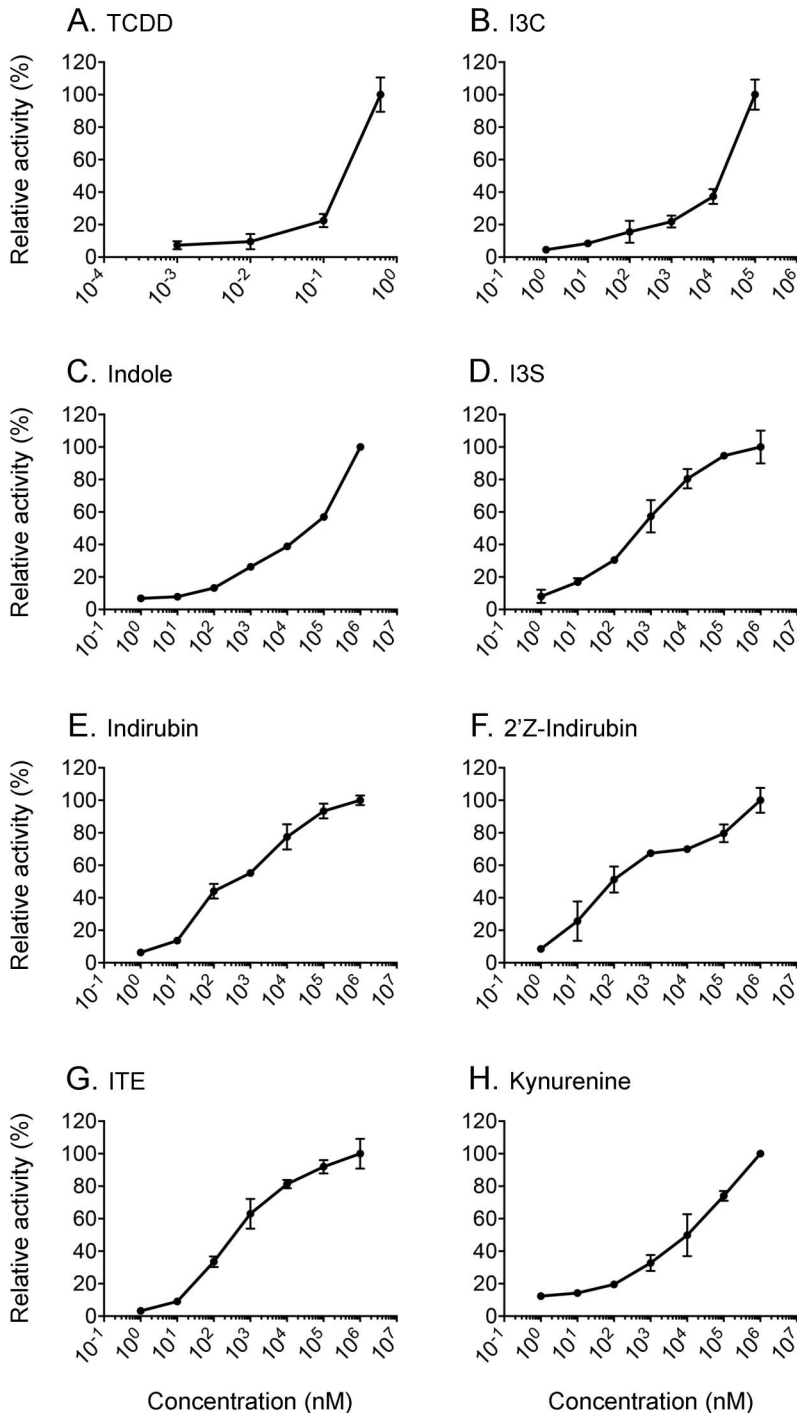
In this study, we analyzed AHR agonists in serum samples from patients with MS and healthy controls. We detected a decrease in serum AHR agonists in relapsing-remitting MS (RRMS) patients.⁵ However, during acute

CNS inflammation in clinically isolated syndrome (CIS) or patients with RRMS, we detected increased AHR agonist levels as compared to healthy controls or clinically stable patients with RRMS. Serum AHR agonists in patients with benign MS with relatively mild clinical impairment despite longstanding disease, however, exhibited unaltered AHR ligand levels as compared to healthy controls. Collectively, these findings suggest that serum AHR agonists are dynamically modulated during the course of MS. Low basal levels of circulating AHR agonists are detected in patients with MS, probably reflecting deficits associated not only with the diet and commensal flora but also in the pathways that control the production and degradation of AHR agonists. Inflammation increases AHR agonists in serum, probably by promoting the production of endogenous anti-inflammatory metabolites such as Kyn. Finally, a fraction of patients with MS maintains control levels of circulating AHR agonists concomitant with a more benign disease course, suggesting a protective role of AHR ligands in later stages of MS in the absence of acute inflammation. These observations might guide the development of novel therapeutics for MS and biomarkers for risk stratification and treatment selection in patients with MS.

METHODS Determination of AHR agonistic activity.

Fifteen thousand human embryonic kidney (HEK)-293 cells per well were plated in 96-well plates (flat bottom). Twenty-four hours after plating, cells were transfected with equal amounts of

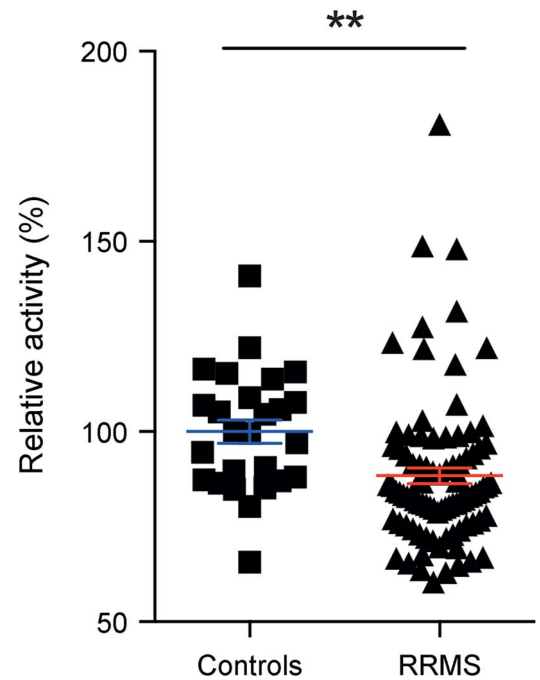
Figure 1 Detection of aryl hydrocarbon receptor ligands from different sources



Aryl hydrocarbon receptor (AHR) agonistic activity was measured for a collection of AHR ligands from exogenous and exogenous sources, including the pollutant 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (A), the diet-derived ligand Indole-3-carbinol (I3C) (B), ligands derived from microbial and host tryptophan metabolism Indole (C), Indoxyl-3-sulfate (I3S) (D), Indirubin (E), and 2'Z-Indirubin (F), the mucosal ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (G), and the endogenous metabolite Kynurenine (H). Data are normalized to 100% (maximum activity per ligand) and are representative of 2 independent experiments.

pGud-Luc (Firefly luciferase under control of AHR-responsive promoter element²⁷) and pTK-Renilla (Renilla luciferase under control of constitutively active thymidine kinase promoter; Promega, Madison, WI) using Fugene Transfection Reagent

Figure 2 Aryl hydrocarbon receptor ligand levels are decreased in patients with relapsing-remitting MS



Aryl hydrocarbon receptor (AHR) agonistic activity in serum samples of healthy controls (controls, n = 26) and relapsing-remitting MS (RRMS) patients (RRMS, n = 91) was assessed in duplicates using an AHR ligand-sensitive luciferase assay. Relative activity was calculated by dividing firefly luciferase activity (pGud-Luc) by Renilla luciferase activity (pTK-Renilla). Values are means of duplicate measurements. Lines represent mean and error bars standard error of the mean (SEM). Significance levels were derived using the Student t test. **p < 0.01.

(Promega) as suggested by the manufacturer. After 24 hours, transfected cells were incubated with Dulbecco's modified eagle medium (DMEM) supplemented with 10% of patient serum in duplicates. Luciferase activity was analyzed 24 hours later using the Dual-Luciferase Reporter System (Promega). Firefly luciferase activity was divided by Renilla luciferase activity and normalized to their respective control levels, which were set as 100%.

The study was approved by the Institutional Review Board of Brigham and Women's Hospital, and all participants provided written informed consent.

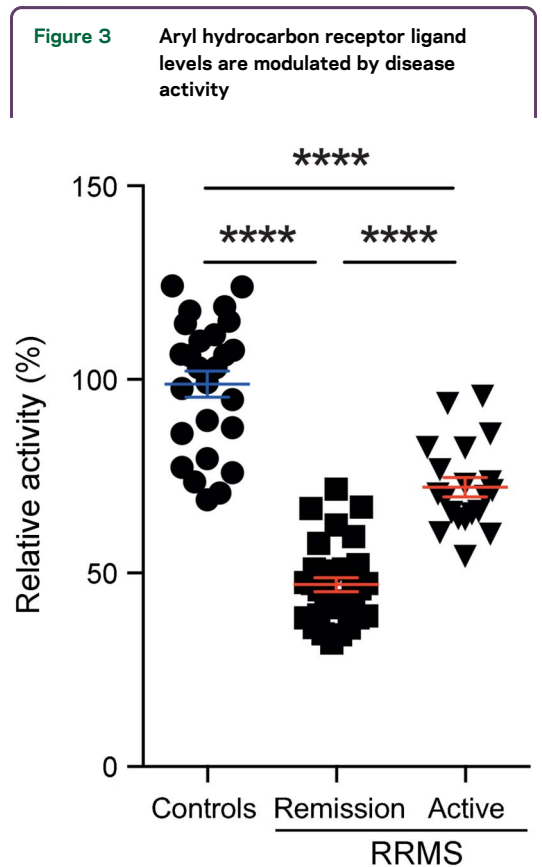
Statistical analysis. Statistical analyses were performed with Prism software (GraphPad, San Diego, CA), using the statistical tests indicated in the individual figure legends. No samples were excluded. The investigators were blinded as to sample cohorts when performing AHR ligand level measurement and samples were run in duplicates. p Values of < 0.05 were considered significant. All error bars represent SEM.

RESULTS AHR agonistic activity in serum is decreased in stable RRMS. To study circulating AHR agonistic activity in MS samples, we first analyzed sera from a cohort of patients with RRMS and compared these to sera from healthy controls (table). In these studies, we used a reporter assay based on HEK-

293 cells cotransfected with a plasmid containing an AHR-responsive promoter element (xenobiotic response element) driving firefly luciferase expression (pGud-Luc²⁷), and a thymidine kinase promoter-driven Renilla luciferase construct (pTK-Renilla) to control for transfection efficiency.⁵ Following transfection, the reporter cells were incubated with patient serum, and relative luciferase activities (pGud-Luc/pTK-Renilla) were determined after 24 hours using a commercial dual-luciferase assay. This assay detected AHR activation in response to a broad range of AHR agonists, including the pollutant 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the dietary ligand Indole-3-carbinol (I3C), ligands derived from microbial and host tryptophan metabolism such as Indole, Indoxyl-3-sulfate (I3S), Indirubin, and 2'Z-Indirubin, the mucosal ligand 2-(1'H-indole-3'-carboxyl)-thiazole-4-carboxylic acid methyl ester (ITE) and the endogenous metabolite Kyn (figure 1). Using this approach, we detected a global decrease in AHR agonistic activity in RRMS patient sera as compared to healthy controls (figure 2). Note that some patients displayed higher serum AHR agonistic activity than healthy controls, suggesting that additional disease-linked mechanisms may increase AHR agonists in patients with MS. However, patient age, disease duration, or the prevalence of disease-modifying therapy (DMT) were not associated with the detected AHR agonistic activity (figure e-1 at Neurology.org/nn).

Circulating AHR agonists are increased during acute CNS inflammation. AHR ligands are generated during acute inflammation by different mechanisms including the enzymatic activity of indoleamine 2,3-dioxygenase (IDO) which produces anti-inflammatory Kyn.¹⁻³ Thus, we speculated that acute CNS inflammation such as that linked to MS relapses might modulate AHR agonists in serum. To test this hypothesis, we analyzed an additional cohort of patients with MS with active CNS inflammation as determined by the presence of cerebral gadolinium-enhancing lesions in MRI at the time of sample acquisition and compared them to a group of patients with RRMS with nonactive disease (table). While we still detected a global decrease in AHR ligand levels in comparison to healthy controls, RRMS active patients displayed increased AHR serum levels as compared to the samples from the RRMS remission cohort (figure 3).

To further validate these findings, we used an independent cohort of patients who had been recently diagnosed with CIS, the first clinical manifestation of autoimmune CNS inflammation (table). CIS does not fulfill anamnestic or MRI tomographic criteria for MS and does not always convert into clinically definitive



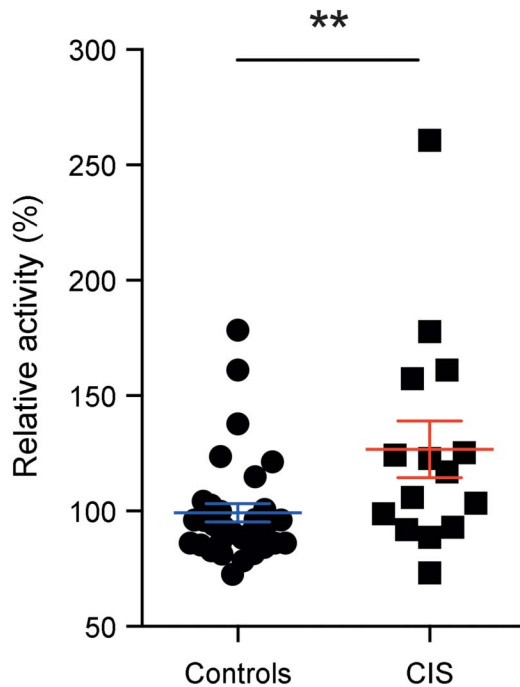
Aryl hydrocarbon receptor (AHR) agonistic activity in serum samples of healthy controls (controls, n = 26), relapsing-remitting MS (RRMS) patients during remission (RRMS remission, n = 32), and patients with RRMS with active disease (RRMS active, n = 20) was assessed in duplicates using an AHR ligand-sensitive luciferase assay. Values are means of duplicate measurements. Lines represent mean and error bars standard error of the mean (SEM). Significance levels were derived using 1-way analysis of variance followed by the Tukey multiple comparisons test. ****p < 0.0001.

MS, the risk of which can be assessed by evaluating additional biomarkers, such as MRI, CSF composition, or electrophysiologic studies, among others.^{28,29}

Sera from CIS patients displayed increased AHR agonistic activity as compared to healthy controls (figure 4). Together with our findings on patients with RRMS during a disease relapse, these findings suggest that acute CNS inflammation results in increased serum AHR agonist levels.

Unaffected AHR agonist levels in patients with benign MS. Patients with benign MS present a relatively mild disease course, despite long disease duration and limited use of DMTs.³⁰ Based on the anti-inflammatory effects of AHR in several experimental models of autoimmunity^{2,3,13} and potentially MS,^{21,22} we analyzed circulating AHR agonist levels in a cohort of patients with benign MS characterized by mild clinical impairment despite longstanding RRMS (“Benign MS,” table). We found that serum samples from patients with benign MS showed AHR

Figure 4 Aryl hydrocarbon receptor ligand levels are increased in clinically isolated syndrome as compared to healthy controls



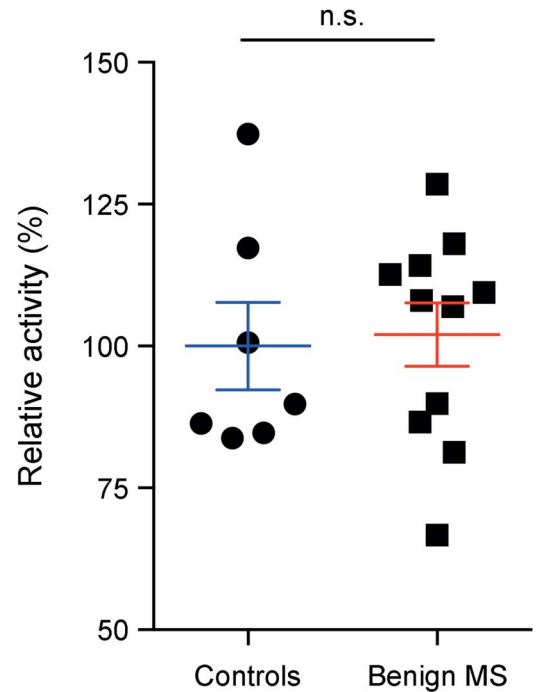
Aryl hydrocarbon receptor agonistic activity in serum samples of healthy controls (controls, $n = 33$) and patients with clinically isolated syndrome (CIS, $n = 15$) was assessed in duplicates. Values are means of duplicate measurements. Lines represent mean and error bars standard error of the mean (SEM). Significance level was derived by the Student t test. $**p < 0.01$.

agonist levels comparable to those detected in controls (figure 5).

DISCUSSION In this work, we analyzed serum levels of AHR agonists in patients with MS. Our data suggest that AHR agonist levels are dynamically modulated during the course of MS: in acute inflammation, such as the first relapse in CIS or during relapses in RRMS, AHR agonistic activity is increased as compared to controls or patients with RRMS with stable disease, respectively. By contrast, during stable disease, AHR ligand levels negatively correlate with disease severity, since patients with benign MS exhibit higher levels of AHR agonistic activity than patients with MS suffering from more severe disease (figure 6).

Several factors might contribute to the decrease in circulating AHR agonists detected in patients with MS. It has become clear in recent years that genetic polymorphisms correlate with an increased risk of developing MS. While most of these polymorphisms have been linked to the immune system,^{31–34} metabolic pathways relevant to the uptake, activation, or degradation of AHR ligands are also affected, as indicated by reported alterations in enzymes that catalyze

Figure 5 Aryl hydrocarbon receptor ligand levels in patients with benign relapsing-remitting MS are unchanged as compared to healthy controls

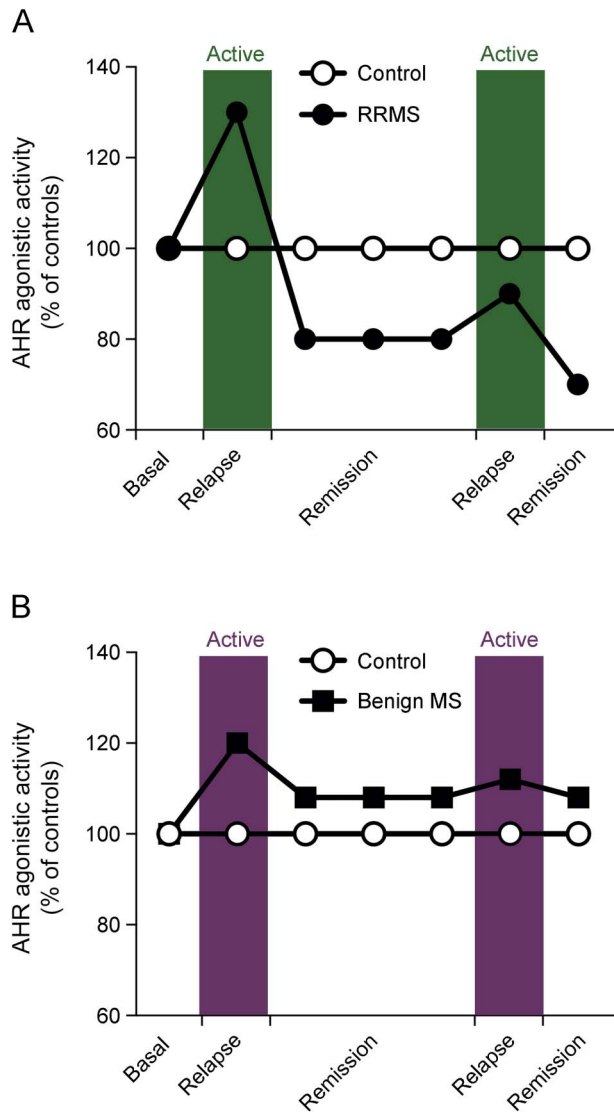


Aryl hydrocarbon receptor agonistic activity in serum samples of healthy controls (controls, $n = 7$) and patients with benign relapsing-remitting MS as defined by low EDSS scores despite longstanding disease (benign MS, $n = 11$) was assessed in duplicates. Values are means of duplicate measurements. Lines represent mean and error bars standard error of the mean (SEM). Significance level was derived by the Student t test. n.s. = not significant.

the generation of AHR ligands from dietary tryptophan.^{35–37} Moreover, genetically defined factors have the potential to influence the composition of the gut microbiome, for example, via the production of microRNAs or altered cytokine signaling.^{6,38,39} Finally, the genetic background of patients with MS may impair the uptake of microbiota-produced AHR agonists and their precursors, as well as their activation into potent AHR agonists. Collectively, these factors may influence AHR-dependent immunoregulation in MS.

Inflammation seems to increase circulating AHR agonists in MS. Inflammation has profound effects on metabolism. Indeed, it has been reported that the AHR agonist Kyn is produced by the metabolism during inflammation.^{18,19} Thus, together with additional AHR agonists that may be generated during inflammation, Kyn may participate in a negative feedback loop aimed at limiting immunopathology. This anti-inflammatory mechanism may cross-talk with additional immunoregulatory pathways⁴⁰ and/or DMTs. Type I interferons, for example, modulate Kyn levels⁴¹ in patients with MS.

Figure 6 Dynamic modulation of aryl hydrocarbon receptor ligand levels in patients with relapsing-remitting MS



(A) Serum aryl hydrocarbon receptor (AHR) agonistic activity is increased during the first relapse of the disease but decreases below control levels in remission. Further relapses increase AHR ligand levels during the relapse. (B) Benign courses of MS have control levels of AHR agonistic activity and might be increased during relapses. RRMS = relapsing-remitting MS.

Several limitations and potential confounding factors have to be taken into consideration when assessing AHR agonist levels in human samples in our study. First, some of our cohorts were limited in patient numbers and exhibited imperfect matching of age, disease duration, or prevalence of DMT. Although we did not detect systematic changes when analyzing the correlation of these factors with agonistic activity (figure e-1), additional potentially unknown variables, such as preanalytical sample processing, storage conditions, or selective AHR ligand degradation or enrichment during sample preparation cannot be excluded. Also, cohort-specific differences, including dietary factors, changes in the gut flora, and

potential effects of specific therapies, might constitute additional confounding factors. Indeed, some patients showed an increased activity of serum AHR ligands, the reasons for which are not clear as of now. Future longitudinal studies may be helpful in determining the clinical relevance of this observation. Moreover, our assay determines the net agonistic activity of AHR ligands in biological samples. Thus, relative changes in specific agonistic or inhibitory AHR ligand levels could be masked or missed by our approach. Finally, technical aspects need to be taken into consideration, since AHR ligand binding and activation has been shown to be species and cell line specific.^{42–45} Thus, the use of different cell lines or transfection techniques (e.g., stable vs transient transfection) may lead to varying results in individual assay systems.

Based on our observations, it is tempting to speculate that different sources of AHR agonists drive chronic and acute AHR activation in MS. Chronic AHR activation may be controlled by the genetic background, diet, and/or the commensal flora, with potential confounding effects provided by environmental factors such as sun exposure and daylight that may differentially influence specific cohorts of patients with MS and controls.^{31,46} Acute AHR activation may be controlled by AHR-activating metabolites, such as Kyn, produced in the context of inflammation to limit immunopathology. The integration of these multiple sources of AHR agonists determines the contribution of AHR signaling to immune modulation. Longitudinal studies based on metabolomic approaches are therefore needed to analyze the correlation between specific AHR agonists, their sources, and disease activity in MS and, potentially, other conditions such as IBD. More importantly, given the potential of AHR agonists to cross the blood-brain barrier and modulate CNS inflammation,⁵ AHR activation could represent a novel therapeutic avenue for MS.

AUTHOR CONTRIBUTIONS

Veit Rothhammer: AHR ligand measurement, data analysis, data interpretation, and manuscript writing and revision. Davis M. Borucki: AHR ligand measurement, data analysis, and manuscript revision. Maria Antonietta Mazzola, Christopher C. Hemond, Anu Paul, Maria Isabel Garcia Sanchez, Guillermo Izquierdo, Keren Regev, Pia Kivisäkk, Rohit Bakshi, and Howard L. Weiner: providing of patient samples and clinical data. Francisco J. Quintana: design and supervision of the study, data analysis and interpretation, and manuscript writing, revision, and editing.

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DISCLOSURE

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