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Comparative targeted lipidomics between serum and cerebrospinal fluid of multiple sclerosis patients shows sex and age-specific differences of endocannabinoids and glucocorticoids

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Abstract

Multiple sclerosis (MS) is a complex chronic neuroinflammatory disease characterized by demyelination leading to neuronal dysfunction and neurodegeneration manifested by various neurological impairments. The endocannabinoid system (ECS) is a lipid signalling network, which plays multiple roles in the central nervous system and the periphery, including synaptic signal transmission and modulation of inflammation. The ECS has been identified as a potential target for the development of novel therapeutic interventions in MS patients. It remains unclear whether ECS-associated metabolites are changed in MS and could serve as biomarkers in blood or cerebrospinal fluid (CSF). In this retrospective study we applied targeted lipidomics to matching CSF and serum samples of 74 MS and 80 non-neuroinflammatory control patients. We found that MS-associated lipidomic changes overall did not coincide between CSF and serum. While glucocorticoids correlated positively, only the endocannabinoid (eCB) 2-arachidonoyl glycerol (2-AG) showed a weak positive correlation ($r=0.3$, $p<0.05$) between CSF and serum. Peptide endocannabinoids could be quantified for the first time in CSF but did not differ between MS and controls. MS patients showed elevated levels of prostaglandin E2 and steaorylethanolamide in serum, and 2-oleoylglycerol and cortisol in CSF. Sex-specific differences were found in CSF of MS patients showing increased levels of 2-AG and glucocorticoids in males only. Overall, arachidonic acid was elevated in CSF of males. Interestingly, CSF eCBs correlated positively with age only in the control patients due to the increased levels of eCBs in young relapsing-remitting MS patients. Our findings reveal significant discrepancies between CSF and serum, underscoring that measuring eCBs in blood matrices is not optimal for detecting MS-associated changes in the central nervous system. The identified sex and age-specific changes of analytes of the stress axis and ECS specifically in the CSF of MS patients supports the role of the ECS in MS and may be relevant for drug development strategies.

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Introduction

Multiple sclerosis (MS) is a complex chronic disease of the central nervous system (CNS) characterized by neuroinflammation and axonal demyelination accumulating in neuronal dysfunction and neurodegeneration [12, 91, 92]. MS is a major cause of neurological disability in young adults with an approximately two to three times higher prevalence in women compared to man [12, 89–91]. A hallmark of MS is the CNS compartmentalized inflammation, where acute immune processes with focal lesions are often associated with relapses, while a smoldering disease activity with chronic low-grade inflammation contributes to degenerative processes, which may result in increasing disability [95]. MS can cause a wide spectrum of symptoms including focal impairments, but also more diffuse adverse effects, such as fatigue that could be associated to changes of bioactive lipids like endocannabinoids (eCBs) and prostaglandins [10–12, 54]. Based on the occurrence and progression of the symptoms, MS is clinically classified into relapse-remitting MS (RRMS) and the progressive disease forms secondary-progressive MS (SPMS) and primary progressive MS (PPMS) [12, 92]. Although a cure is still lacking, several disease-modifying therapies, acute attack/relapse treatments and symptomatic therapies are used to reduce the disability burden and to extend the survival of MS patients [19, 35, 77, 90, 92]. Notably, these therapies include Nabiximols (Sativex®), a cannabis extract, approved for the treatment of spasticity and pain of MS patients [26, 35, 54, 61, 73, 75, 94]. Cannabinoids mainly act as agonists on the G-protein coupled cannabinoid receptors (CB1R and CB2R, CB receptors), which are part of the endocannabinoid system (ECS) [54]. The ECS is an essential endogenous lipid signalling system comprised of the primary arachidonic acid (AA) derived eCBs 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoylethanolamine (anandamide, AEA), the CB receptors and the enzymes involved in synthesis and degradation of eCBs [29, 54]. In addition to the lipid eCBs, small CB receptor binding peptides have been recently identified and accordingly named peptide endocannabinoids (pepcans) including RVD-hemopressin (pepcan-12) and its precursor peptide pepcan-23 (Fig. 1a) [6, 36, 74]. In the nervous system the eCBs act as retrograde signalling messengers binding to presynaptic CB1 receptors and modulating neuronal signal transduction, thereby affecting processes like cognition, memory formation and perception of pain [93]. CB2R and other components of the ECS are expressed in peripheral immune cells, as well as in neurons and glia cells in the CNS and are involved in the modulation of inflammatory processes in various inflammation associated diseases including multiple sclerosis [5, 9, 44, 65]. Noteworthy, 2-AG and AEA are connected to the pro-inflammatory arachidonic acid-prostaglandin axis (Fig. 1a), since their degradation, mainly by the enzymes monoacylglycerol lipase (MAGL) for 2-AG and fatty acid amide hydrolase (FAAH) for AEA, generates AA and glycerol or AA and ethanolamine respectively (Fig. 1a) [54]. 2-AG has been shown to be an important source of AA in the brain, as well as peripheral cells and tissues, thereby serving as precursor pool for prostaglandins [1, 27, 32, 63].

In preclinical studies using experimental autoimmune encephalomyelitis (EAE) rodent models mimicking the pathophysiological processes of MS, the possible role of the ECS has been studied extensively [3, 13, 14, 30, 47, 54, 71, 72, 76]. It was shown that a treatment with cannabinoids and ECS modulators can reduce the disease burden in different animal MS models, while the knockout of CB1R aggravates the disease severity [47, 52, 76]. CB1R is involved in the neuroprotective mechanism of the ECS by regulating glutamate homeostasis and reducing glutamate excitotoxicity, which is often associated with neuroinflammation in MS [60, 76]. While CB1R has been demonstrated to regulate spasticity in an MS model [70], CB2R has been shown to be an important modulator of immune cell differentiation, trafficking and accumulation in the CNS [14, 44, 66]. To avoid potential side effects of CB1R agonists like sedation and receptor desensitization, pharmacological strategies to increase eCB concentrations for the treatment of MS patients are widely investigated [54, 72]. Inhibitors of MAGL and FAAH, as well as eCB re-uptake inhibitors have been shown to modulate inflammatory processes and to be neuroprotective, thereby reducing the disease burden in preclinical MS models and some inhibitors are tested in clinical trials for MS and other neuroinflammatory diseases [4, 30, 37, 54, 64, 72].

Given the emerging evidence of the role of the ECS in pathophysiological processes of MS, data on potentially altered eCBs and associated lipids in the CSF and circulation of human MS patients could be valuable to define biomarkers of disease severity and progression. To date, such human data are sparse and in part inconsistent and controversial, and so far it remains unknown whether peripheral eCB levels reflect central eCB changes potentially caused by MS pathophysiological mechanisms [13, 18, 31, 39, 51]. MS has been associated with a dysregulation of the neuroendocrine hypothalamic-pituitary-adrenal (HPA)-axis and cortisol concentrations in human

patients [24, 43, 49, 67, 79, 98]. Glucocorticoids like cortisol are released from the adrenal cortex after prior activation of the HPA-axis and mediate not only a metabolic stress response, but also modulate the immune system [77, 86]. The ECS has been shown to be involved in the regulation of HPA-axis activation and vice versa [57, 59]. To gain a better understanding of potential MS associated changes of eCBs, ECS-associated lipids and glucocorticoids, we performed a retrospective study with targeted lipidomic analysis of CSF and serum samples of MS and non-neuroinflammatory control patients (Fig. 2). By quantifying the analytes in CSF and serum collected from the same patients, we further investigated, whether circulating eCBs and ECS-associated lipids reflect the levels in CSF and may serve as biomarkers for MS associated changes in the CNS. We employed the available patient information including age, sex, MS type, treatment, expanded disability status scale (EDSS) and time since occurrence of first symptoms to identify potential influencing factors on the studied analytes in general and in the context of MS. Finally, we used correlation analysis to explore a potential impact of MS on the association patterns of the analytes.

Materials and methods

Retrospective study design

Serum and cerebrospinal fluid (CSF) were collected from patients with informed consent in the neurology department of the Inselspital in Bern (Switzerland) and processed and stored in a highly standardized procedure at the Liquid Biobank Bern for research purposes (www.biobankbern.ch). We made use of available patient samples in the Liquid Biobank to retrospectively investigate potential changes of eCBs and related lipids, as well as pepcans and glucocorticoids in serum and CSF of patients with multiple sclerosis (MS) compared to patients with non-neuroinflammatory conditions (Fig. 2, Supplementary Table 1). To test the feasibility of the study and to calculate the needed sample size we performed a pilot study quantifying eCBs in the CSF of 20 patients per group (MS and non-neuroinflammatory controls, Supplementary Fig. 1). Since changes of AEA in CSF of MS patients have been reported before [13, 18], we used the CSF AEA levels of the pilot study for a power analysis using the software G*Power 3.1 (HHU). The calculated effect size between MS and control patient AEA levels was d=0.46. We considered a minimum statistical power of 0.8, an equal sample size and an α of 0.05 and performed a sample size calculation with a parametric analysis (t-test, unpaired, two-tailed) and a non-parametric analysis (Wilcoxon-Mann-Whitney test, two-tailed) both resulting in a needed sample size of around 80 patients per group. The selection of MS patients and patients with non-neuroinflammatory conditions as controls was based on the availability of enough serum and CSF samples of each patient, preferably collected on the same day. The exclusion criteria for control patients were MS and other neuroinflammation associated diseases including neuromyelitis optica spectrum disorder, traumatic brain injury, and Alzheimer's disease. An additional aim of the study was to investigate the potential impact of documented patient parameter on the analyte concentrations in serum and CSF. The available patient parameter included age and sex for all patients and additionally MS-type, expanded disability status scale (EDSS), time since first symptoms and reported treatments for MS patients. Furthermore, we aimed to use the recorded information of the pre-analytical sample preparations to access the potential impact of sampling time (time of day) on analyte levels in serum and CSF, as well as the potential impact of the coagulation time (time between blood collection and separation of coagulated blood cells and serum via centrifugation) on the analyte levels in serum.

Patients

MS patients were diagnosed according to the 2017 Mc Donald criteria [85]. Although we first could include 80 MS patients and 80 control patients with non-neuroinflammatory conditions with available serum and CSF samples in the Liquid Biobank Bern collected from the same patients, we later had to exclude five of the MS patients who had a coding for a differential MS diagnosis in their documentation but were finally diagnosed with another neuroinflammatory disease. Another MS patient was additionally diagnosed with pancreatic cancer and was therefore excluded. The most common diagnoses and symptoms of the non-neuroinflammatory control patients are summarized in the Supplementary Table 1. We quantified the analytes of interest in serum and CSF of 48 female and 32 male non-neuroinflammatory control patients and 52 female and 22 male patients with MS. The mean age and age range of MS and control patients were comparable (Table 1).

Of the 74 MS patients 58 were diagnosed with RRMS, 9 with SPMS and 4 with PPMS. For three of the MS patients a MS type was not documented (Table 2). 54 of the MS patients had a reported expanded disability status scale (EDSS, mean: 2.5, SD: 1.8) documented maximally three months prior or three months after sample collection (Table 1). The year of the first symptom appearance was documented for 67 MS patients and the time-period between occurrence of the first symptoms to sampling was between 0 and 50 years (Table 1). 23 of the MS patients were untreated, while 49 received a treatment prior to the sample collection. The last documented

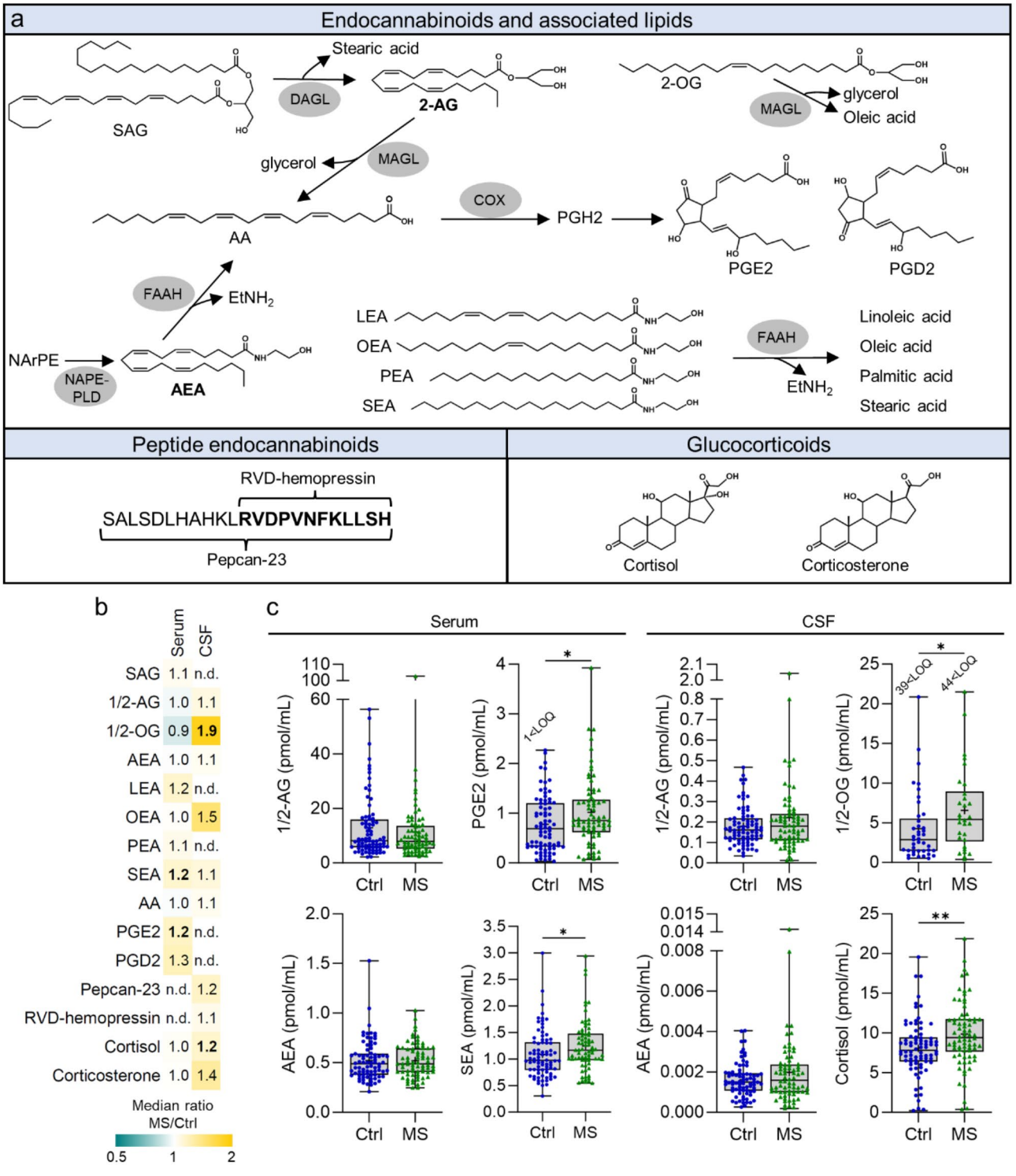

Fig. 1 (See legend on next page.)

treatment prior to the sample collection is summarized in Table 2. Based on the available clinical documentation, it was unclear for two of the MS patients, if they were treated before sample collection.

Sample collection, processing, and storage

The sample collection was part of the clinical routine for diagnostics and performed in non-fasted patients. Blood, for the generation of serum samples, and CSF

(See figure on previous page.)

Fig. 1 Comparison of endocannabinoids and ECS-associated lipids, peptide endocannabinoids and glucocorticoids in serum and CSF between MS and control patients. (**a**) Graphical presentation of analytes with the molecular structure of the endocannabinoids, associated lipids and the glucocorticoids as well as the amino acid sequence of Pepcan-23 (precursor peptide) and RVD-hemopressin. (**b**) Fold change as median ratio of analyte levels in samples of MS patients divided by analyte levels of control (Ctrl) patients in serum and CSF presented in a heatmap. The color-coding legend of the heatmap is shown at the bottom and reaches from 0.5 (blue, indicative for reduction of MS patient analyte level to the half of the analyte level in the Ctrl patients), over 1 (white, comparable analyte levels between MS and Ctrl patients) to 2 (yellow, analyte levels are doubled in MS compared to Ctrl patients). Significant differences between analytes of MS and Ctrl patients are labelled in bold. (**c**) Example graphs of analytes in serum and CSF of Ctrl ($n = 80$) and MS ($n = 74$) patients. Data are presented as box plots with median and 25–75% percentile as box with the range as whiskers. The mean is additionally depicted as + and the analyte level of each patient is represented as one data point (symbol). The number of patients where an analyte level was lower than the limit of quantification (LOQ) is indicated for each group if applicable. For statistical analysis a Mann-Whitney test was performed. Statistically significant differences are indicated as follows: * $p < 0.05$ and ** $p < 0.01$. 2-AG, 2-arachidonoylglycerol; 2-OG, 2-oleoylglycerol; AA, arachidonic acid; AEA, arachidonoylethanolamide; COX, cyclooxygenase; DAGL, Diacylglycerol lipase; EtNH2, Ethanolamine; FAAH, fatty-acid amide hydrolase; LEA, linoleoylethanolamide; MAGL, Monoacylglycerol lipase; NArPE: N-arachidonoyl phosphatidylethanolamine, NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; n.d., not detectable; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGH2, prostaglandin H2; SAG, 1-Stearoyl-2-arachidonoyl-sn-glycerol; SEA, stearoylethanolamide

were collected between 8 am and 7 pm (Table 1) and transported within 60 min to the central laboratory of the clinic for further processing. The coagulation time of the blood samples, which was the time before the samples were centrifuged and the serum collected and frozen, was less than 20 min for most of the samples (Table 1). The time of sample collection and the blood coagulation time was comparable between MS and control patients (see Table 1). The pre-analytical processing and storage is highly standardized at the Liquid Biobank Bern (LBB). After arrival at the LBB the samples were centrifuged (CSF: 2000 g, 10 min, 23 °C; blood: 3100 g, 10 min, 23 °C) and the supernatants were aliquoted and snap-frozen in a controlled nitrogen environment using an ASKION C-line work bench (ASKION GmbH, Germany). The sample aliquots were then stored in an automated robotic sample storage (HAMILTON BIOS, Hamilton Storage, Switzerland) at −80 °C. After retrieval from the LBB, all CSF and serum sample aliquots were kept frozen during transport on dry ice and afterwards stored at −80 °C in the Institute of Biochemistry and Molecular Medicine in Bern until measurement.

Sample preparation for lipid and glucocorticoid analysis

For the quantification of lipids and glucocorticoids in serum and CSF, the samples were slowly thawed on ice and 200 µL of each sample was added into a glass tube containing 0.75 mL of ethyl acetate: hexane (9:1, HPLC grade, VWR Chemicals, Poland) with 0.1% formic acid (Sigma-Aldrich Chemie GmbH, Germany) and 3.5 µL of an internal standard mixture (Table 3) in absolute ethanol (VWR International, France). After addition of 50 µL of 0.1 M formic acid the samples were strongly vortexed and sonicated in a pre-cooled bath for 5 min. Afterwards the samples were centrifuged with 1620 g for 10 min at 4 °C (Centrifuge 5804 R, Eppendorf AG, Germany), the aqueous phase was frozen on dry ice for 10 min and the upper organic phase was collected and evaporated in plastic tubes for 45 min using an Eppendorf Concentrator plus (Eppendorf AG, Germany). The dried pellets were reconstituted in 35 µL of Acetonitrile:H_2O (8:2, HPLC grade, Fisher Scientific, UK) for the injection into the LC-ESI-MS/MS system.

LC-ESI-MS/MS analysis of lipids and glucocorticoids

The LC-ESI-MS/MS analysis of lipids and glucocorticoids was performed as described before in Reynoso-Moreno et al. 2021 [46, 72]. In brief, a linear ion trap quadrupole LC-MS/MS mass spectrometer (QTRAP 5500, AB Sciex Instruments, Massachusetts, USA) was operated with the Turbo-Ion Spray interface in positive mode for the analysis of SAG, 2-AG, 2-OG, AEA, LEA, OEA, PEA, SEA, Cortisol and Corticosterone and in negative mode for the analysis of AA, PGE2 and PGD2. For the analysis in positive, as well as in negative mode, 10 µL of the reconstituted samples were injected into the system and separated with a ReproSil-Pur Basic-C18 column (3 µm particle size; 2×50 mm, Dr. A. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) at a maintained temperature of 40 °C. For the operation in positive mode the mobile phase was a composition of (A) 2 mM ammonium acetate (Sigma-Aldrich Chemie GmbH, Germany) with 0.1% formic acid and (B) methanol (HPLC grade, Fisher Scientific, UK) with 2 mM ammonium acetate running with a flow rate of 0.35 mL/min. A gradient elution protocol was applied as follows. After a start with 85% A and 15% B for 0.5 min the concentration of B was linearly increased to 70% at 3.5 min running time. The concentration of B was further increased to 99% B at 8 min and kept constant for another 4 min. From 12 min to 14 min running time B was linearly reduced to 15%, which was afterwards kept constant for re-equilibration for 1 min. For the operation in negative mode the mobile phase was a composition of (A) 2 mM ammonium acetate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid running with a flow rate of 0.3 mL/min. The gradient elution protocol started with 95% A and 5% B with B linearly increasing to 40% until 3 min running time. This was followed by a slower increase of B to 65% until 9 min and a more rapid increase to 95% of B until 10 min running time. 95% of B

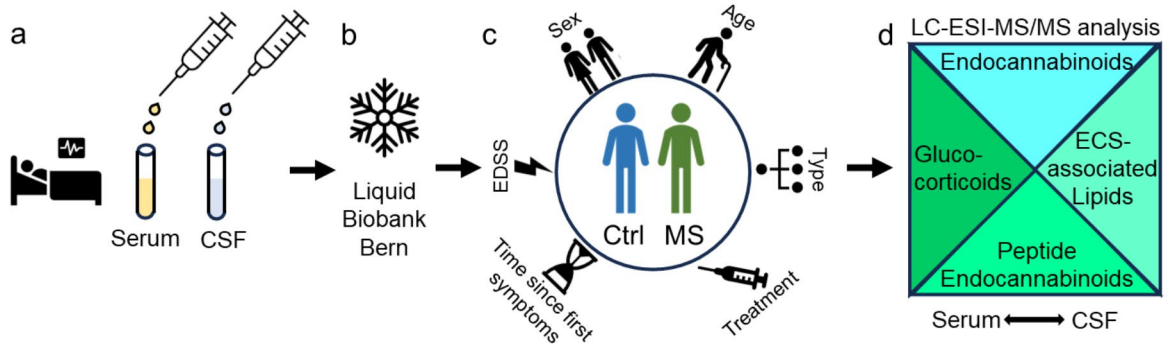

Fig. 2 Retrospective study design. (**a**) Standardized collection of serum and cerebrospinal fluid (CSF) of patients in the neurology department of the Inselspital in Bern, Switzerland. (**b**) Standardized processing and storage of frozen samples for research purposes at the Liquid Biobank in Bern, Switzerland. (**c**) Retrospective selection of multiple sclerosis (MS) and non-neuroinflammatory control patients (Ctrl) with available subject matching serum and CSF samples. Available patient data included sex, age, disease type, treatments, time since first symptoms and the expanded disability status scale (EDSS). (**d**) Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis of endocannabinoids, ECS-associated lipids, peptide endocannabinoids (pepcans) and glucocorticoids in subject matching serum and CSF samples of MS and Ctrl patients

Table 1 Available information of patients and sampling conditions

Parameter	Patient group	No.	Mean (SD)	Median	25–75% Percentile	Min-max	Range
age (years)	Ctrl	80	44.7 (16.1)	43	30.0–57.8	17–76	59
	MS	74	43.6 (14.1)	42	33.8–53.0	19–79	60
daytime of blood collection (hours)	Ctrl	80	13.4 (1.8)	14.0	12.0–14.8	8.4–16.5	8.1
	MS	74	13.14 (1.8)	12.6	11.6–14.7	8.5–18.8	10.3
daytime of CSF collection (hours)	Ctrl	80	13.6 (1.6)	14.1	12.1–14.8	10.4–16.3	5.9
	MS	74	13.1 (1.7)	12.6	11.5–14.5	10.5–18.7	8.2
coagulation time (minutes)	Ctrl	79*	18.2 (3.2)	17	16–19	14–32	18
	MS	73*	19.0 (6.9)	17	16–20	12–67	55
EDSS	MS	54*	2.5 (1.8)	2	1.5–3.5	0–6.5	6.5
years since first symptoms	MS	67*	9.1 (10.3)	6	0–14	0–50	50

*information available

Table 2 MS type and last documented treatment of MS patients

		All (*n* = 74) no.	Females (*n* = 52) no.	Males (*n* = 22) no.	All (*n* = 74) (%)	Females (*n* = 52) (%)	Males (*n* = 22) (%)
MS type	RRMS	58	42	16	78.4	80.8	72.7
	SPMS	9	6	3	12.2	11.5	13.6
	PPMS	4	2	2	5.4	3.8	9.1
	undefined	3	2	1	4.1	3.8	4.5
last documented treatment	untreated	23	16	7	31.1	30.8	31.8
	Fingolimod	7	4	3	9.5	7.7	13.6
	Dimethyl fumarate	7	4	3	9.5	7.7	13.6
	Interferon β 1-a	1	1	0	1.4	1.9	0.0
	4-Aminopyridin	1	1	0	1.4	1.9	0.0
	Glatirameracetat	1	1	0	1.4	1.9	0.0
	Leflumonid	1	1	0	1.4	1.9	0.0
	Glucocorticoids	9	6	3	12.2	11.5	13.6
	Glucocorticoids+ Plasmapheresis	4	4	0	5.4	7.7	0.0
	Natalizumab	12	9	3	16.2	17.3	13.6
	Ocrelizumab	4	2	2	5.4	3.8	9.1
	Rituximab	2	2	0	2.7	3.8	0.0
	unknown	2	1	1	2.7	1.9	4.5

Table 3 Monitored MRM transitions of the analytes and the used internal standards

Analyte (m/z)	Internal standard (m/z)
SAG (667.4 → 327.1)	2-AG-*d5* (384.1 → 287.2)
2-AG (379.1 → 287.2)	2-AG-*d5* (384.1 → 287.2)
2-OG (357.3 → 265.3)	2-AG-*d5* (384.1 → 287.2)
AEA (348.3 → 62.1)	AEA-*d4* (352.2 → 66.0)
LEA (324.2 → 66.9)	LEA-*d4* (328.1 → 66.0)
OEA (326.3 → 309.2)	OEA-*d4* (330.3 → 66.0)
PEA (300.2 → 282.2)	PEA-*d4* (305.0 → 62.0)
SEA (328.0 → 311.0)	AEA-*d4* (352.2 → 66.0)
AA (303.1 → 59.0)	AA-*d8* (311.1 → 59.0)
PGE2 (351.1 → 271.1)	PGE2-*d4* (355.0 → 319.0)
PGD2 (351.1 → 189.0)	PGE2-*d4* (355.0 → 319.0)
Pepcan-23 (520.6 → 611.2)	RVDPVNFKFL-*d3*SH (488.1 → 371.2)
RVD-hemopressin (475.8 → 371.0)	RVDPVNFKFL-*d3*SH (488.1 → 371.2)
Cortisol (363.0 → 97.0)	Corticosterone-*d4* (350.9 → 314.8)
Corticosterone (347.0 → 121.0)	Corticosterone-*d4* (350.9 → 314.8)

was kept constant until 14 min and then linearly reduced to 5% until 15 min running time. For re-equilibration the mobile phase was kept constant at 95% A and 5% B for 2 min. The MS parameters of the ESI source were used as described before (see [72]). The monitored MRM transitions used for quantification are summarized for all analytes with the used internal standards in Table 3. For the quantification a calibration was prepared with increasing amounts of analyte standards in a matrix of artificial CSF (148.2 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2{\cdot}2H_2O$, 0.8 mM $MgCl_2{\cdot}6H_2O$, 0.8 mM $Na_2HPO_4{\cdot}2H_2O$, 0.2 mM $NaH_2PO_4{\cdot}2H_2O$) with 0.2% bovine serum albumin (BSA, fatty acid free, Sigma-Aldrich, Missouri, USA). The calibration samples were processed and measured in the exact same way as the serum and CSF samples. With the software Analyst 1.6.3 (AB Sciex, Massachusetts, USA) a linear calibration curve for each analyte was generated by linear regression with at least 5 calibration points using the quantified area ratio (analyte peak area/ internal standard peak area) and the known analyte concentration for each calibration sample. For the linear calibration curve the slope, intercept and regression coefficient were calculated. The slope and intercept were used to calculate the analyte concentration and the full amount of each analyte in the sample preparations. The quantified amount of each analyte was then normalized to the volume of sample used at the beginning for the extraction to determine the analyte concentration of the serum and CSF samples. As reported earlier, different extraction and storage methods can lead to an isomerisation of 2-AG and 2-OG to 1-AG and 1-OG [38]. In the chromatography 1-AG and 1-OG are released at a slightly different retention time leading to a second peak in the chromatogram (see Supplementary Fig. 9) [38]. To assess the biological levels of 2-AG and 2-OG as accurate as possible, both peaks of 2-AG and 1-AG, as well as 2-OG and 1-OG were quantified together and represented as 1/2-AG and 1/2-OG respectively.

Sample preparation for peptide endocannabinoid analysis

For the quantification of pepcans, a second aliquot of each serum and CSF sample from the same MS and Ctrl patients were transported and stored like the first aliquots (see above). The sample preparation was performed as described before by Glasmacher and Gertsch using a gentle extraction method consisting of protein precipitation (PPT) and solid-phase extraction (SPE) with minor modifications [29]. In brief, the samples were slowly thawed and kept on ice during the sample preparation. 220 µL of each serum or CSF sample were transferred into a plastic reaction tube and 1.25 µL of internal standard (0.2 µg/mL deuterated rat RVD-hemopressin in DMSO, custom made peptide from SHBC, Shanghai, China) were added and vortexed. For the PPT 210 µL of pre-cooled acetonitrile were added and vortexed, followed by 5 min incubation on ice and 10 min sonication in a pre-cooled bath. After a centrifugation for 10 min with 16,000 g at 4 °C the supernatant was transferred into a new tube and mixed with 1650 µL 50 mM Tris-HCl buffer (pH 9.0) containing 5 mM $MgCl_2$ and 2.5 mM EDTA. For the SPE the samples were loaded on Oasis® HLB 1 cc (30 mg) extraction cartridges (Waters, Ireland), which were earlier activated with 1 mL of methanol and equilibrated with 1 mL of 10% acetonitrile in H_2O. The cartridges with loaded samples were washed with 500 mL H_2O. The bound samples were eluted with 800 µL of 10% acetonitrile in H_2O and evaporated overnight using an Eppendorf Concentrator plus (Eppendorf AG, Germany). For the LC-ESI-MS/MS analysis the dried sample pellets were reconstituted in 25 µL of 10% acetonitrile in H_2O.

LC-ESI-MS/MS analysis of peptide endocannabinoids

The LC-ESI-MS/MS analysis of pepcans was described and validated before (see [29]) and used with a minor modification of the liquid chromatography protocol. 15 µL of the reconstituted samples were injected and separated with a ReproSil-Pur Basic-C8-2 column (3 µm particle size; 2×50 mm, Dr. A. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) at a maintained temperature of 40 °C. The mobile phase was a composition of (A) H_2O with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid running with a flow rate of 0.3 mL/min. A gradient elution protocol was used starting with 98% A and 2% B for 1 min. In a first fast linear gradient B was increased to 9% until 2 min running time followed by a slower linear gradient with an increase to 30% of B over a

period of 14 min. Afterwards B was linearly increased to 98% within 0.5 min and kept at 98% for 0.5 min. This was followed by a reduction of B back to 2% within 0.5 min and an equilibration of the column for 1.5 min with 98% A and 2% B. The MS parameters of the ESI source were used as described before (see [29]) and monitored MRM transitions used for the quantification of pepcan-23 and RVD-hemopressin, as well as the used internal standard are shown in Table 3. Calibration curves were prepared for pepcan-23 and RVD-hemopressin with increasing analyte concentrations and the calibration samples were processed and measured with the same protocol used for the CSF and serum samples. As calibration matrix a 50 mM Tris-HCl buffer with pH 9.0, containing 5 mM $MgCl_2$ and 2.5 mM EDTA was used with 0.05% BSA for pepcan-23 and 0.005% BSA for RVD-hemopressin. With the software Analyst 1.6.3 (AB Sciex, Massachusetts, USA) the analyte peak area and internal standard peak area were quantified for each calibration concentration and the area ratio (analyte peak area/ internal standard peak area) was used to generate a linear calibration curve for each analyte by linear regression with at least 5 calibration points. The slope and intercept were used to calculate the analyte concentration and the full amount of each analyte in the sample preparations. The quantified amounts of each analyte were then normalized to the volume of sample used at the beginning for the extraction to determine analyte concentrations in serum and CSF samples.

Statistical analysis

The generation of graphs and the statistical analysis was performed with the software GraphPad Prism 10 (GraphPad Software, Massachusetts, USA). Since several of the analytes had a non-parametric distribution (Shapiro-Wilk test) we mainly employed statistical analyses, where a parametric distribution is not required. For the statistical comparison of two groups, we used a two-tailed Mann-Whitney test with a definition of statistical significance of $p<0.05$. For a statistical comparison of more than two groups we employed a Kruskal-Wallis test with Dunn's multiple comparisons test ($\alpha=0.05$). For the statistical analysis of potential associations between the analytes and between the analytes and the available patient information, as well as sampling parameters we employed Spearman correlations (two-tailed, confidence interval: 95%). For graphical representation of the correlations the Spearman r values are shown in heatmaps generated with Excel (Microsoft 365, Microsoft Corporation, Washington, USA). Simple linear regressions were additionally used to assess the impact of patient parameters on specific analyte levels. In case prior analyses revealed significant associations of an analyte level with more than one of the independent patient parameters a multiple linear regression analysis was applied to assess the potential impact of the different covariates.

Results

Differential targeted lipidomics and pepcan analysis between MS and control patients in CSF and serum

We employed validated multiple-reaction monitoring (MRM) based LC-ESI-MS/MS methods to quantify 13 lipids and two pepcans [29, 46, 72]. The absolute concentrations of the eCBs AEA and 2-AG in CSF (mean [SD], AEA: 0.0018 [0.0015] pmol/mL, 2-AG: 0.2 [0.19] pmol/mL) and serum (mean [SD], AEA: 0.52 [0.19] pmol/mL, 2-AG: 12.1 [12.0] pmol/mL) samples were in line with earlier reports [40, 45]. In a group comparison between MS and control patients we could not detect significant differences of 2-AG and AEA in serum and CSF (Fig. 1) and most of the other analytes were found at comparable concentrations in MS and control patients (Fig. 1, Supplementary Fig. 2). However, we identified a significant increase of stearoylethanolamide (SEA) and prostaglandin E2 (PGE2) in serum, as well as of 2-oleoylglycerol (2-OG) and cortisol in CSF of MS patients in comparison to the control patients (Fig. 1). All analytes detectable in serum and CSF were found in a higher concentration in serum compared to CSF, with 2-AG about 60 times and AEA approximately 300 times higher concentrated in serum (Supplementary Fig. 3).

Impact of MS type, EDSS and time since occurrence of first symptoms on analytes in MS patients

When subdividing the MS patients according to the MS type (Table 2) elevated cortisol levels in CSF of patients with progressive MS forms (SPMS, PPMS) could be identified compared to RRMS and control patients (Supplementary Fig. 4). Accordingly, corticosterone was significantly increased in CSF of MS patients with a progressive form compared to the levels in the CSF of control patients. A similar tendency for the serum cortisol levels could be observed (Supplementary Fig. 4). Importantly, there was no significant difference in the time of CSF sample collection between the control, the RRMS and progressive MS patients (mean [SD]: Ctrl=13.6 [1.6] hours, RRMS=13.3 [1.7] hours, SPMS+PPMS=12.8 [1.5] hours, Kruskal-Wallis test: $p=0.113$). For all other analytes no differences in serum and CSF between the patients with RRMS and progressive MS forms were found (Supplementary Fig. 4). 23 MS patients were untreated at the time of sample collection, while 49 MS patients were already treated with various treatment strategies. The last reported treatments before sample collection are summarized in Table 2. Cortisol was increased in the CSF of untreated MS patients compared to the controls. For all other analytes we could not find a difference between the untreated MS and control

patients (Supplementary Fig. 5). In serum of MS patients a weak positive correlation of SEA and a weak negative correlation of cortisol with the time since the occurrence of the first symptoms was observed and the associations were confirmed by simple linear regression analyses (Supplementary Fig. 6, Table 1). Although the coefficients of determination were small (see Supplementary Fig. 6), these findings might indicate an increase of cortisol also in the circulation of MS patients in the early phase, while SEA might be increasing with the progression of the disease. We further investigated if the analyte levels would be associated with disease severity. 54 MS patients had a reported EDSS documented in a period of maximally 3 months prior or after sample collection (Table 1). The performed Spearman's correlation and simple linear regression analyses between the EDSS and the analyte levels in serum and CSF did not reveal any significant impact of the EDSS on the analyte levels (Supplementary Fig. 7).

Potential effects of sampling time and ex-vivo coagulation on analyte concentrations

In an earlier published study it was reported that circulating 2-AG and the structurally related lipid 2-oleoylglycerol (2-OG) are showing a circadian rhythm in humans [33]. The time of sample collection of the patients included in our study was between 8 am and 7 pm and the range and distribution of the sampling time was comparable between MS and control patients (Table 1, Supplementary Fig. 8a). We analysed whether the analytes in serum and CSF were associated with the time of sample collection. As expected, cortisol negatively correlates with the sampling time, showing higher cortisol levels in samples collected in the morning and a reduction of cortisol during the rest of the day (Supplementary Fig. 8b, c) [15]. We further observed a noteworthy weak negative correlation of serum 2-AG and 2-OG and a weak positive correlation of PEA with the sampling time in the MS, but not the control patients (Supplementary Fig. 8b, c). As recently reported by Kratz et al., the serum concentration of 2-AG and the isomerisation from 2-AG to 1-AG can significantly increase with prolonged blood coagulation times (≥40 min) [45]. The coagulation time used for serum generation in our study was, with the exception of one sample of one MS patient, shorter than 40 min and the majority of the blood samples were coagulating for a little less than 20 min (Table 1, Supplementary Fig. 9b). Importantly, the reported coagulation times were comparable between the MS and control patient samples (Table 1, Supplementary Fig. 9b). A shift to the 1-AG isomer could be observed in the chromatograms of the serum samples (Supplementary Fig. 9a). We therefore quantified the 2-AG and 1-AG chromatogram peaks separately and calculated the 1-AG/2-AG ratios (Supplementary Fig. 9a, b). The comparison of the 1-AG/2-AG ratios revealed no differences in the pre-analytical isomerisation of 2-AG to 1-AG in serum samples of MS and control patients (Supplementary Fig. 9a, b). Furthermore, the correlation of the serum analyte concentrations with the coagulation time did not show an association of 2-AG levels with the time of blood coagulation (Supplementary Fig. 9c, d). A weak negative correlation of serum PEA levels in the control patients and weak positive correlations of 2-OG and LEA levels in the serum of MS patients were observed with the coagulation time (Supplementary Fig. 9c, d). In additional simple linear regression analyses only the coefficient of regression (slope) of the regression of LEA with the coagulation time was significantly non-zero (Supplementary Fig. 9d).

Impact of age and sex on analyte levels

Since age and sex related differences of the ECS have been described earlier [2, 16], we analysed the potential impact of age and sex on the analyte concentrations. The mean age, as well as the age range of MS and control patient groups were comparable (see Table 1). To assess the potential influence of the patients age on the analytes, we performed a Spearman correlation analysis (Fig. 3, Supplementary Fig. 10). A weak to moderate positive association of both eCBs AEA and 2-AG in CSF with age was found for control-, but not MS patients (Fig. 3a, b). In addition, a positive correlation of CSF AA levels with age was identified, which was moderate in control and weak in MS patients (Fig. 3a, b). Accordingly, the serum/CSF ratios of AA were negatively correlated with the age of the patients (Supplementary Fig. 3c). In serum of the control patients 2-AG and 2-OG correlated weakly positive with increasing age. Although there was a comparable trend for 2-AG and 2-OG in serum of the MS patients, the correlation with age did not reach significancy (Fig. 3a, Supplementary Fig. 10). In agreement with the previously described weak positive correlation of SEA in serum of MS patients with the time since the occurrence of the first symptoms (Supplementary Fig. 6), we observed a weak positive correlation of SEA serum levels and the SEA serum/CSF ratio with the increasing age of the MS patients (Fig. 3a, Supplementary Fig. 3c, Supplementary Fig. 10). Since the patients age and the time since the occurrence of the first symptoms were positively correlated (Supplementary Fig. 3c), a clear differentiation between the potential impact of disease duration and age on the SEA serum levels of MS patients is not possible. Finally, in serum of both patient groups the concentrations of cortisol and corticosterone were found to correlate weakly negative with age (Fig. 3a, Supplementary Fig. 10). In accordance, we observed a negative correlation between the cortisol and corticosterone serum/CSF ratios and the patients age (Supplementary Fig. 3c).

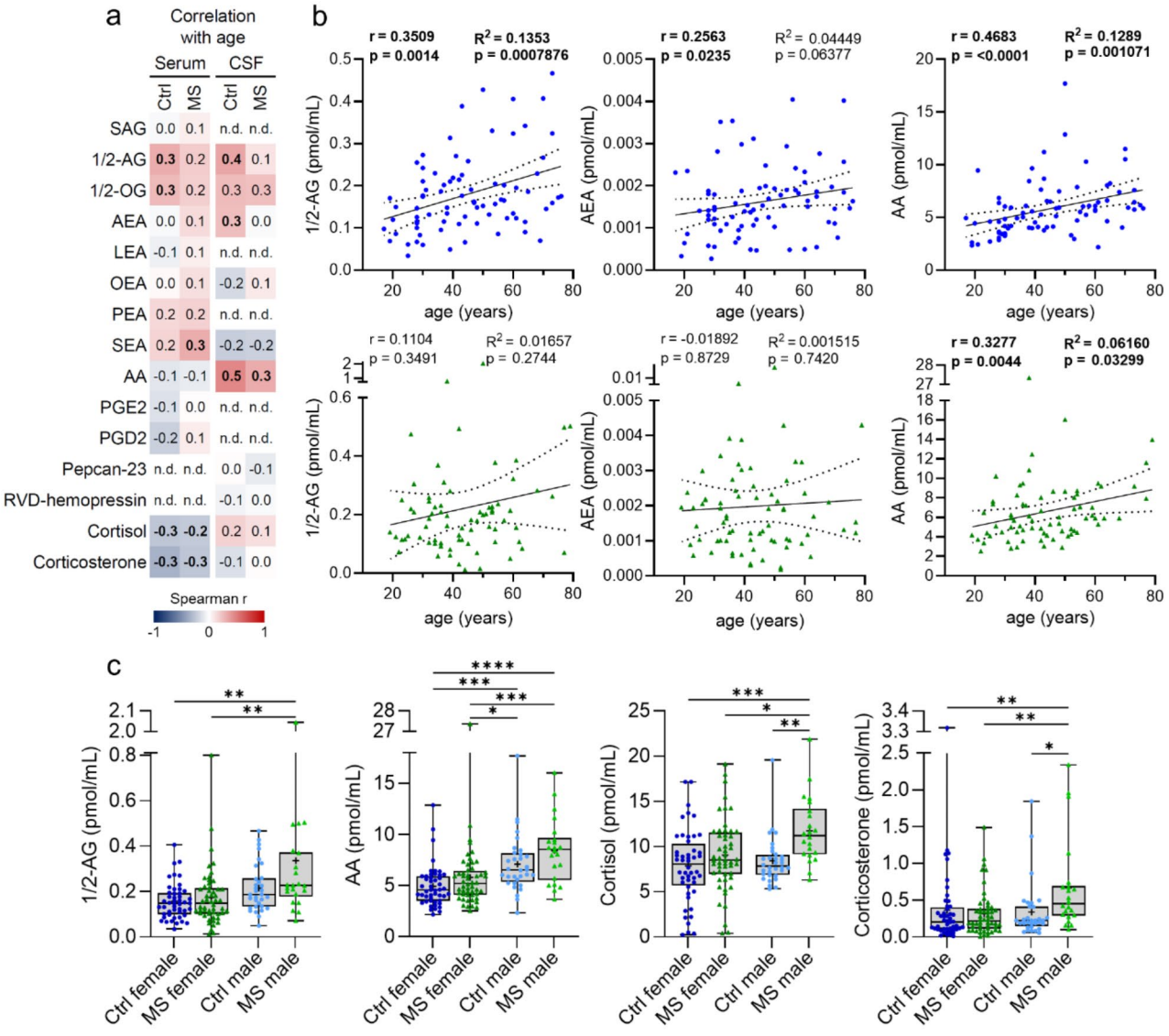

Fig. 3 Impact of age and sex on quantified analytes in serum and cerebrospinal fluid of control and multiple sclerosis patients. (**a**) Spearman correlation heatmap of analytes in serum and CSF of control (Ctrl) and multiple sclerosis (MS) patients with age. For each pairwise correlation the Spearman r is shown in the heatmap. The color-coding legend of the Spearman r values is presented at the bottom of the heatmap and reaches from −1 (strong negative correlation, blue) over 0 (no correlation, white) to 1 (strong positive correlation, red). Significant correlations ($p < 0.05$) are highlighted with bold Spearman r values. The patient number, p-value and confidence interval of each pairwise Spearman correlation are presented in Online Resource 2. (**b**) Example scatterplots of the Spearman correlations between analyte CSF levels and age of MS and Ctrl patients. The Spearman r and the p-values of the correlation are indicated in the top left of the graphs and labelled bold if significant ($p < 0.05$). Additionally, a simple linear regression is shown with 95% confidence bands of the best-fit line in each graph with the coefficient of determination (R^2) indicated in the top right of each graph and labelled bold in case the slope is significantly non-zero ($p < 0.05$). (**c**) Example graphs of analyte levels in CSF of Ctrl and MS patients sorted by sex (Ctrl females $n = 48$, MS females $n = 52$, Ctrl males $n = 32$, MS males $n = 22$). Data are presented as box plots with median and the 25–75% percentile as box with the range as whiskers. The mean is additionally depicted as + and the analyte level of each patient is represented as one data point (symbol). For statistical analysis a Kruskal-Wallis test with Dunn's multiple comparisons test was performed. Statistically significant differences are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$

We supplemented the identified significant correlation results with simple linear regression analyses, revealing coefficients of regression (slopes) to be significantly non-zero for most of the analytes (Fig. 3b, Supplementary Fig. 10). Overall, we analysed serum and CSF samples of 48 female and 32 male control patients and 52 female and 22 male patients with MS (Supplementary Table 2). Although the mean age of the male MS and control patients were slightly higher compared to the mean age of the female MS and control patients (see Supplementary Table 2), the age differences were not statistically significant (Kruskal-Wallis test, $p = 0.2079$). A subgrouping

of the patients by sex revealed no differences in the analyte concentrations in serum of MS and control patients (Supplementary Fig. 11). In contrast, sex specific changes of several analytes were observed in the CSF samples (Fig. 3c, Supplementary Fig. 11). 2-AG levels in CSF of male MS patients were significantly higher compared to female MS and female control patients (Fig. 3c, Supplementary Fig. 11). A similar trend was observed for 2-AG levels in the CSF of male control patients compared to female control patients but was not reaching significance ($p=0.0712$, Kruskal-Wallis test with Dunn's multiple comparisons test; Fig. 3c, Supplementary Fig. 11). Further, cortisol and corticosterone were found to be significantly higher in the CSF of male MS patients compared to female MS patients, as well as to male and female control patients (Fig. 3c, Supplementary Fig. 11). On the other hand, we observed AA concentrations to be in general significantly higher in the CSF of males compared to females (Fig. 3c, Supplementary Fig. 11). Consistently, significantly lower AA serum/CSF ratios were found in male compared to female patients (Supplementary Fig. 3d). Since we identified age and sex as potentially impacting factors on CSF 2-AG and AA levels, we performed a multiple linear regression analysis of the combined patient groups with MS, age and sex as covariates. The regression model for the 2-AG CSF levels was significant ($F(3, 150)=5.639$, $p=0.0011$, $y=0.067+0.054$ * MS (no, yes [yes]) $+0.002$ * age (years) $+0.096$ * sex (females, males [males])). Only sex was a significant predictor in the model ($t(150)=3.090$, $p=0.0024$) and the regression model did only weakly fit the data ($R^2=0.1013$), indicating that the 2-AG levels in the CSF are clearly impacted by other factors as well. For the AA CSF levels the multiple linear regression model was significant ($F(3, 150)=12.25$, $p<0.0001$, $y=2.788+1.052$ * MS (no, yes [yes]) $+0.049$ * age (years) $+2.089$ * sex (females, males [males])). In the regression model MS ($t(150)=2.277$, $p=0.0242$), age ($t(150)=3.163$, $p=0.0019$) and sex ($t(150)=4.255$, $p<0.0001$) are significant predictors for the CSF AA levels. However, the R^2 of 0.197 indicates that also other factors impact the levels of AA in the CSF.

Comparison of analytes in serum and CSF of RRMS patients with an age and sex matched control group

Since we observed a potential impact of age and sex on specific analytes, we focused on the 58 patients with RRMS and selected 58 pairwise age and sex matched patients of the control group. Both patient groups (RRMS and matched controls) included 42 females and 16 males with a maximal age gap of 7 years between paired MS and control patients. The mean age and age distribution, as well as the documented sampling parameters were comparable between the RRMS and the matched control patients (Table 4, Supplementary Table 3). As observed for all MS patients (Fig. 1), RRMS patients showed increased concentrations of PGE2 in serum and of cortisol in CSF compared to the age and sex matched control patients (Supplementary Fig. 12). An EDSS was documented for 42 of the 58 RRMS patients (Table 4, Supplementary Table 3). A mild negative association of serum cortisol, as well as of CSF corticosterone with the reported EDSS of the RRMS patients was observed (Supplementary Fig. 13b). The cortisol levels in serum of patients with RRMS were weakly correlating with the time since the occurrence of the first symptoms (Table 4, Supplementary Fig. 13c). Sorting by sex of the RRMS and matched control patients (Supplementary Fig. 14) revealed similar sex differences of analytes in CSF as earlier described for all MS and control patients (Fig. 3, Supplementary Fig. 11). Further, the correlation patterns of the analytes with the age of the RRMS and matched control patients (Supplementary Fig. 13a) were comparable to those of all MS and control patients (Fig. 3). This included the weak to moderate positive correlation of AEA and 2-AG in CSF with age of the matched control group but not in the CSF of the RRMS patients (Fig. 3, Supplementary Fig. 13a). A similar correlation was

Table 4 Available patient information and sampling conditions of RRMS and age and sex matched non-neuroinflammatory control patients

Parameter	Patient group	No.	Mean (SD)	Median	25–75% percentile	Min-max	Range
age (years)	Ctrl	58	40.8 (13.8)	39.0	28.8–53.3	17–73	56
	RRMS	58	40.5 (12.3)	39.0	32.0–49.3	19–77	58
daytime of blood collection (hours)	Ctrl	58	13.4 (1.8)	13.9	11.9–14.8	8.6–16.5	7.9
	RRMS	58	13.3 (1.7)	12.8	11.7–14.7	11.0–18.8	7.9
daytime of CSF collection (hours)	Ctrl	58	13.4 (1.6)	14.0	12.0–14.8	10.4–16.3	5.9
	RRMS	58	13.2 (1.7)	12.7	11.6–14.5	10.9–18.7	7.8
coagulation time (min)	Ctrl	57*	18.4 (3.3)	18.0	16–19	14–32	18
	RRMS	58	19.3 (7.6)	17.5	16–20.3	12–67	55
EDSS	RRMS	42*	2.0 (1.3)	2	1.4–2.6	0–5.5	5.5
years since first symptoms	RRMS	55*	8.0 (8.5)	6	0–13	0–39	39

*information available

observed for AA, the downstream fatty acid of the eCBs metabolism (Supplementary Fig. 13a, Fig. 1).

Increased endocannabinoid levels in CSF of young but not old RRMS patients compared to age and sex matched control patients

To further analyse the age correlation patterns of the lipid eCBs and AA in CSF, we grouped the RRMS and matched control patients by median age (39 years, see Table 4) into young and old patient groups (Fig. 4, Supplementary Table 4). In the young patient groups (<39 years), we found significant higher levels of 2-AG and AEA in the CSF of RRMS patients compared to matched control patients (Fig. 4). In contrast, the CSF concentrations of both lipid eCBs were comparable between RRMS and matched control patients with older age (age≥39 years; Fig. 4). A similar trend was observed for AA concentrations in CSF of the young patient groups, however not reaching significance (p-value=0.0575, Mann-Whitney test; Fig. 4). In the young patient groups, SEA serum levels and CSF cortisol levels of RRMS patients were significantly increased compared to the matched controls (Fig. 4a, Supplementary Fig. 15). Whereas elevated levels of PGD2 in serum and 2-OG in CSF of older RRMS patients could be observed compared to the matched control patients (Fig. 4a, Supplementary Fig. 16).

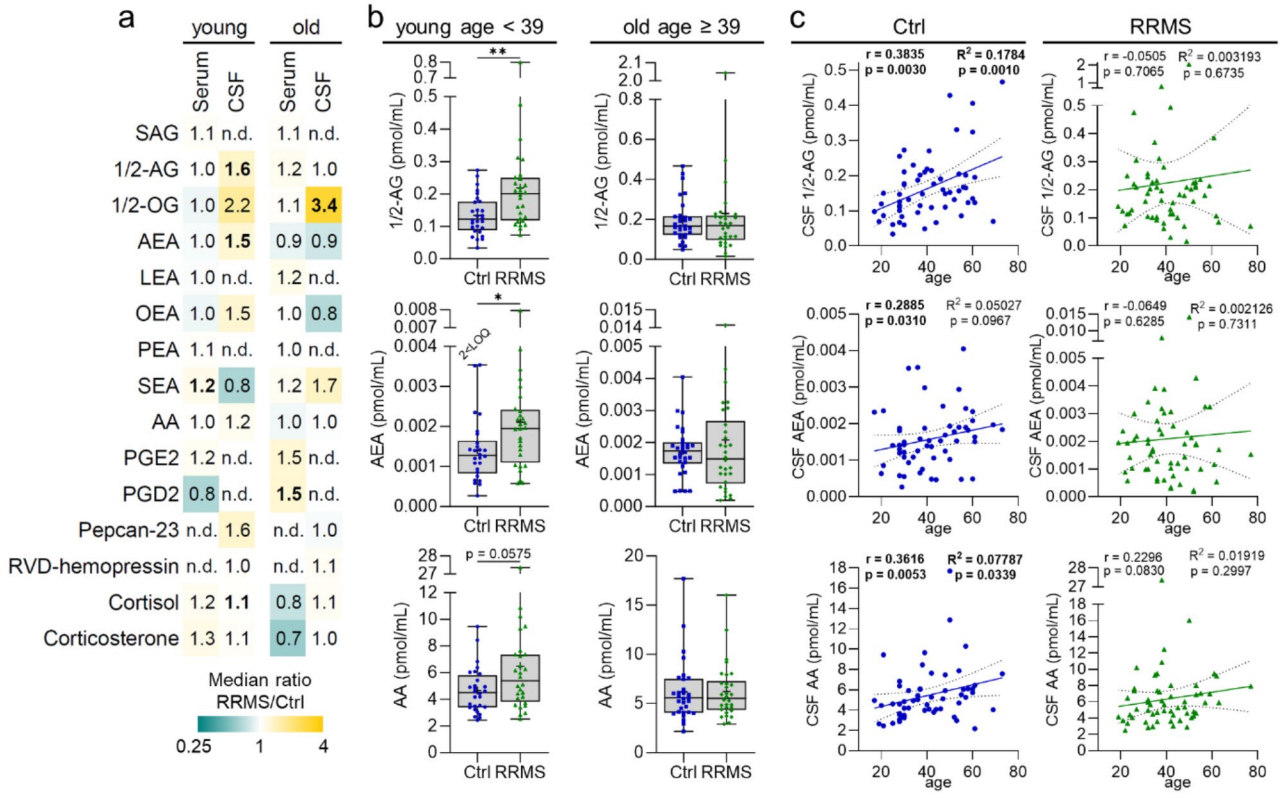

Fig. 4 Increased endocannabinoid levels in cerebrospinal fluid of young but not old relapsing-remitting MS patients compared to age and sex matched control patients. Comparison of analytes in serum and CSF of relapsing-remitting (RRMS) patients with age and sex matched controls sorted by the median age of 39 years into young and old patient populations. (**a**) Analyte levels are represented as fold change with the median ratio of analyte levels in samples of RRMS patients divided by analyte levels of age and sex matched control (Ctrl) patients in serum and CSF presented in a heatmap. The color-coding legend of the heatmap is shown at the bottom and reaches from 0.25 (blue, indicative for reduction of MS patient analyte level to a quarter of the analyte level in the Ctrl patients), over 1 (white, comparable analyte levels between MS and Ctrl patients) to 4 (yellow, analyte levels are four times higher in MS compared to Ctrl patients). Significant differences between analytes of RRMS and Ctrl patients are labelled in bold (n.d., not detectable). (**b**) Example graphs of analytes in CSF of young Ctrl patients (21 females, 7 males), young RRMS patients (21 females, 7 males), old Ctrl patients (21 females, 9 males) and old RRMS patients (21 females, 9 males). Data are presented as box plots with median and the 25–75% percentile as box with the range as whiskers. The mean is additionally depicted as + and the analyte level of each patient is represented as one data point (symbol). The number of patients where an analyte level was lower than the limit of quantification (LOQ) is indicated for each group if applicable. For statistical analysis a Mann-Whitney test was performed. Statistically significant differences are indicated as follows: * $p < 0.05$ and ** $p < 0.01$. (**c**) Example scatterplots of the Spearman correlations between CSF analytes and age of RRMS patients and matched control patients (Ctrl). The Spearman r and the p-values of the correlation are indicated in the top left of the graphs and labelled bold if significant ($p < 0.05$). Additionally, a simple linear regression is shown with 95% confidence bands of the best-fit line with the coefficient of determination (R^2) indicated in the top right and labelled bold in case the slope is significantly non-zero ($p < 0.05$)

Analyte correlations in serum and in CSF of RRMS and age and sex matched control patients

To assess potential changes of the analyte association patterns in serum and CSF of RRMS patients in comparison to the age and sex matched control patients we performed Spearman correlation analyses and presented the Spearman r values in heatmaps (Fig. 5, Supplementary Figs. 17, 18). In serum of both patient groups, we observed a strong positive correlation of the structurally related lipids 2-AG and 2-OG, which can be hydrolysed

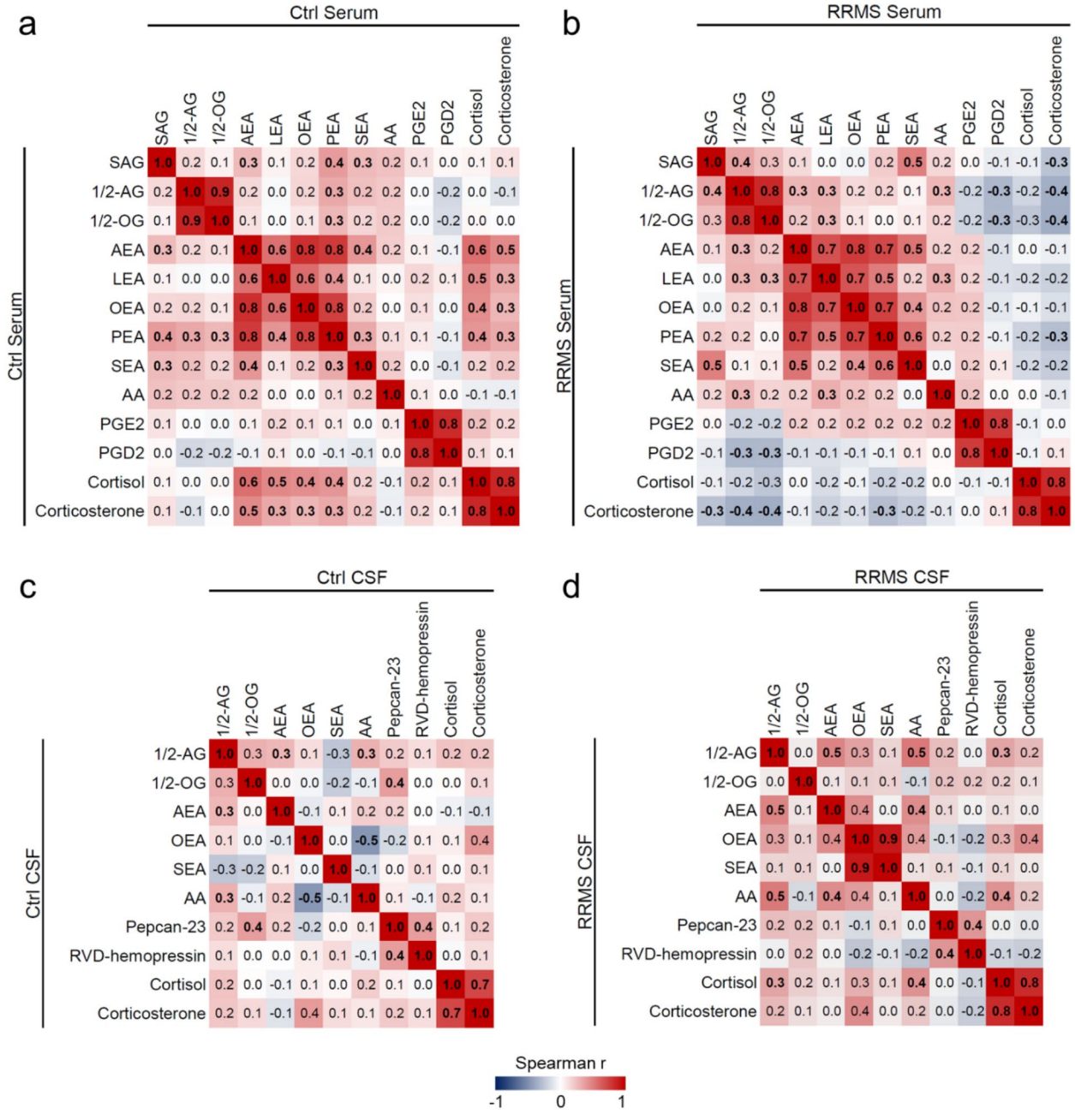

Fig. 5 Correlations of analytes in serum and in cerebrospinal fluid of RRMS and age and sex matched control patients. Spearman correlation heatmaps of analytes of age and sex matched control (Ctrl; **a**, **c**) and relapsing remitting MS (RRMS; **b**, **d**) patients in serum (**a**, **b**) and in cerebrospinal fluid (CSF; **c**, **d**). For each pairwise correlation the Spearman r is shown in the heatmaps. The color-coding legend of the Spearman r values is presented at the bottom and reaches from − 1 (strong negative correlation, blue) over 0 (no correlation, white) to 1 (strong positive correlation, red). Significant correlations ($p < 0.05$) are highlighted with bold Spearman r values. The patient number, p-value and confidence interval of each pairwise Spearman correlation are presented in the Online Resource 2

by the same enzymes including the monoacylglycerol lipase (MAGL; Figs. 1 and 5a and b, Supplementary Fig. 17). In contrast, no significant correlations of 2-AG and 2-OG could be observed in the CSF samples (Fig. 5c, d). Further, robust positive associations between the *N*-acylethanolamines were found in serum samples of RRMS and control patients (Fig. 5a, b, Supplementary Fig. 17). In accordance with our earlier report presenting pepcan-23 as precursor peptide of RVD-hemopressin [29], we observed a moderate positive correlation between both peptides in the CSF samples (Fig. 5c, d). In addition, a strong positive correlation between both glucocorticoids, cortisol and corticosterone in serum and CSF was identified (Fig. 5, Supplementary Figs. 17, 18). In serum samples both analysed prostaglandins were found to be strongly positive associated with each other (Fig. 5a, b, Supplementary Fig. 17). Interestingly, 2-AG correlated weakly to moderately positive with its precursor lipid SAG and the downstream AA in serum of RRMS, but not of control patients (Fig. 5a, b). On the other hand, in the CSF 2-AG was found to be positively associated with AA in both patient groups with a moderate correlation in RRMS patients and a weak correlation in the controls (Fig. 5c, d). This association between 2-AG and AA in the CSF was observed in young and old RRMS patients, however in age and sex matched control patients it was only observed for young but not old patients (Supplementary Fig. 18). In serum samples the eCB AEA and the downstream AA were found to be significantly associated in young RRMS and control patients but not in the old patient groups (Supplementary Fig. 18). In CSF a weak to moderate positive correlation of AEA and AA was observed in RRMS patients, but not in the CSF of the age and sex matched control patients (Fig. 5c, d, Supplementary Fig. 18). Of note, in the CSF samples a significant positive association was found for both eCBs, 2-AG and AEA, with a moderate positive correlation in RRMS patients and a weak positive correlation in the control patients (Fig. 5c, d). However, in the analysed serum samples both eCBs only correlated weakly positive in RRMS, but not control patients (Fig. 5a, b). A splitting of the RRMS patient cohort by age revealed an age difference for this observation with a moderate positive correlation of 2-AG and AEA in serum of young (<39 years of age), but not old (≤39 years of age) RRMS patients (Supplementary Fig. 17). Further differences in the analyte association patterns of RRMS and control patients occurred. In serum of RRMS patients 2-AG and 2-OG correlated weakly negative with the PGD2 and corticosterone, which was not the case for the control patients (Fig. 5a, b). Weak to moderate positive associations of cortisol, as well as corticosterone could be observed with the *N*-acylethanolamines AEA, LEA, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) in serum samples of control patients but were absent in the RRMS patients (Fig. 5a, b). On the other hand, cortisol was positive associated with 2-AG and AA in CSF of RRMS patient but not in the CSF of the control patients (Fig. 5c, d). Further, a moderate negative correlation between AA and RVD-hemopressin was observed in CSF of young RRMS patients (Supplementary Fig. 18).

Analyte correlations between serum and CSF samples of RRMS and age and sex matched control patients

Next, we investigated whether MS impacts the analyte correlations between CSF and serum (Fig. 6, Supplementary Fig. 19). The time gap between sample collection of CSF and serum was less than 10 min for the majority of the samples (Supplementary Fig. 8a). One of the control patients was excluded from this analysis since the sample collection of CSF and serum was performed three days apart. In control and RRMS patients only 2-AG but not AEA levels correlated weakly positive between serum and CSF (Fig. 6b, Supplementary Fig. 19). As expected, cortisol and corticosterone levels correlated positively with itself and each other (Fig. 6, Supplementary Fig. 19). In RRMS patients a weak positive correlation could be observed between serum 2-AG and CSF AA, while AA in serum did not correlate with AA in CSF and CSF 2-AG levels were not associated with serum AA levels, in both RRMS and matched control patients (Fig. 6). Notably, a moderately positive association of serum 2-AG with CSF AA levels was found in young, but not old RRMS patients (Supplementary Fig. 19). In addition, the concentrations of the 2-AG precursor SAG in serum of RRMS patients were weakly positively associated with AA in CSF (Fig. 6b). This was however not the case for non-neuroinflammatory control patients (Fig. 6a). On the other hand, as observed in the association patterns in serum of the control patients (Fig. 5a), the CSF cortisol levels were positively associated with the serum levels of *N*-acylethanolamines AEA, LEA, OEA and PEA, as well as with the serum prostaglandins PGE2 and PGD2 (Fig. 6a). These association patterns were not observed in RRMS patients (Fig. 6). In contrast, the circulating cortisol levels did not correlate with CSF levels of AEA, or OEA in RRMS and control patients (Fig. 6).

Discussion

Cannabinoids are already in clinical use to alleviate the burden of spasticity in MS patients and further potential treatment strategies targeting the ECS are currently developed, supporting a role of the ECS in MS patients [26, 35, 54, 61, 73, 94]. However, data on potential alterations of eCBs and ECS-associated lipids in human MS patients is still sparse and in part inconsistent, which may also be due to pre-analytical issues [13, 18, 31, 39, 45, 51]. To date, using an analytical fluorescence-based

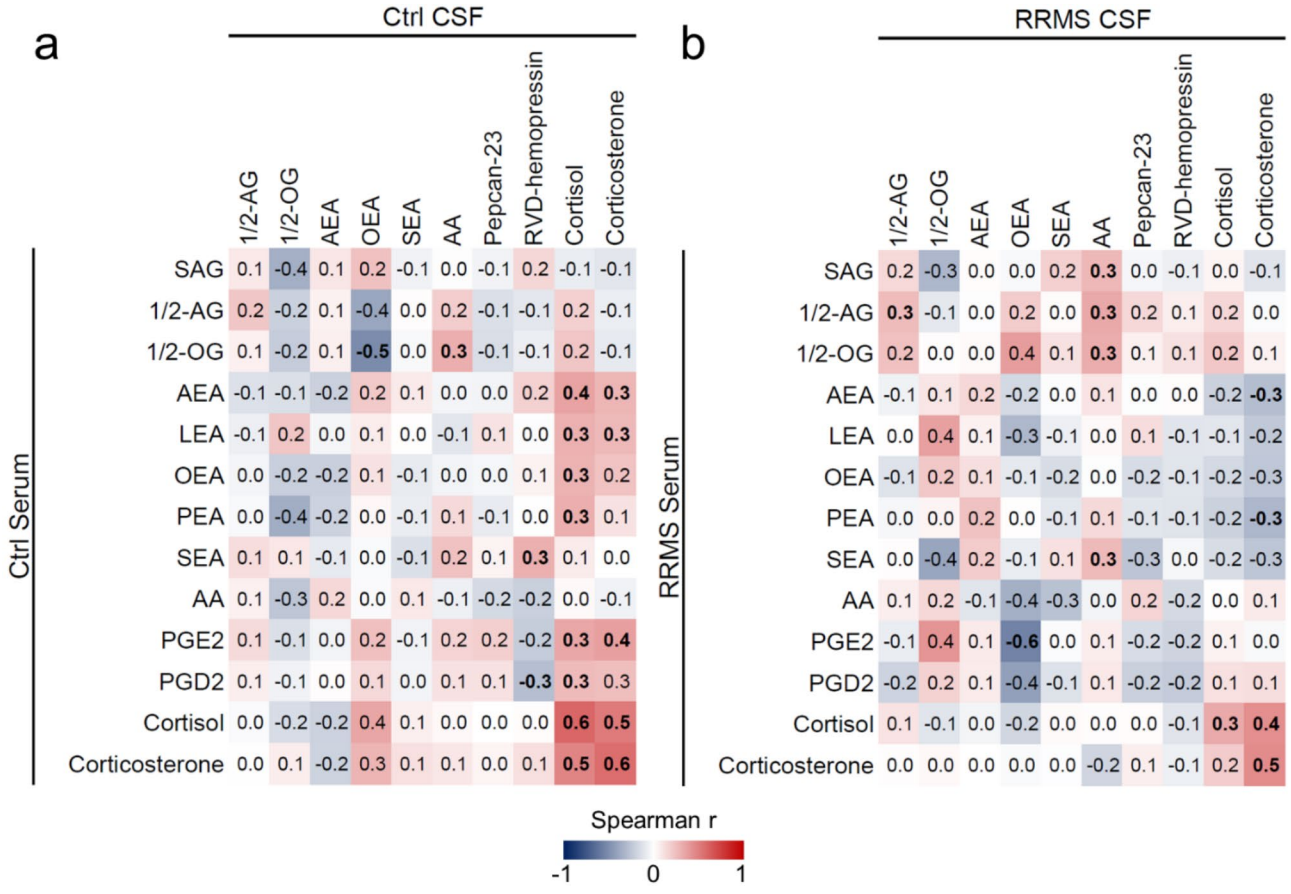

Fig. 6 Correlations of analytes between serum and cerebrospinal fluid of relapsing-remitting MS and age and sex matched control patients. Spearman correlation heatmaps of analytes between serum and CSF of age and sex matched control (Ctrl; **a**) and relapsing-remitting MS (RRMS; **b**) patients. For each pairwise correlation the Spearman r is shown in the heatmaps. The color-coding legend of the Spearman r values is presented at the bottom and reaches from −1 (strong negative correlation, blue) over 0 (no correlation, white) to 1 (strong positive correlation, red). Significant correlations ($p < 0.05$) are highlighted with bold Spearman r values. The patient number, p-value and confidence interval of each pairwise Spearman correlation are presented in Online Resource 2

HPLC method that was not validated in CSF, Centonze et al. reported that AEA, but not 2-AG levels were increased in CSF of 11 RRMS patients during a current relapse compared to 11 healthy controls [13]. AEA in the CSF was found to be positively associated with the number of observed Gd-enhancing lesions on MRI as a proxy of focal blood-brain barrier disruption [13]. In contrast, in a GC-MS based method which was validated in CSF, Di Filippo et al. observed a general reduction of 2-AG, AEA and the AEA related *N*-acylethanolamines oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) in the CSF of 50 patients with MS compared to 20 control patients [18]. Notably, a subgroup comparison of the MS patients revealed significantly higher levels of AEA and PEA with the same tendency of higher 2-AG and OEA levels in the CSF of RRMS patients with a current relapse compared to RRMS patients with a stable disease status [18]. In accordance with the observation by Centonze et al. in 2007 [13], AEA CSF levels were reported to be positively associated with the appearance of Gd-enhancing lesions of RRMS patients [18]. eCBs are highly lipophilic molecules and therefore able to equilibrate from organs into the circulation, where they may be useful as indirect biomarkers for tissue eCB changes, as reviewed by Hillard in 2017 [38]. So far, only few previous studies investigated potential alterations of eCBs in the circulation of MS patients with inconsistent results [31]. In 2009, Jean-Gilles et al. observed increased AEA levels in plasma of RRMS ($n=10$), SPMS ($n=8$) and PPMS ($n=6$) patients in comparison to 17 healthy controls. They further reported, elevated levels of PEA in RRMS and SPMS patients and OEA in SPMS patients, while 2-AG levels were unchanged and comparable between all groups [39]. In contrast, Lötsch and colleagues reported a reduction or a tendency for a reduction of AEA, OEA and PEA in the serum of 102 MS patients compared to serum of 301 healthy control subjects, while the 2-AG levels again were not impacted by MS [51]. On the other hand, in a

recent study of Gustavsen and colleagues no alterations of AEA, PEA, OEA and 2-AG were found in plasma of 66 untreated newly diagnosed MS patients compared to 46 healthy controls [31]. Therefore, it remains unclear whether the concentrations of eCBs and ECS-associated lipids are modified in the circulation of MS patients and could serve as indirect biomarkers reflecting potential eCB changes in the CSF. The 2-AG and AEA levels found in the circulation and CSF of MS and control patients reported so far are summarized, in comparison to the findings of this study, in Table 5.

In this retrospective study we investigated eCBs, ECS-associated lipids and pepcans, as well as glucocorticoids in matching CSF and blood serum samples of 74 MS patients and 80 non-neuroinflammation control patients. Although we could not identify MS related changes of

Table 5 Summary of articles analysing 2-AG and AEA in CSF or Serum/Plasma in comparison to the findings of this study

Article	Body fluid	Patients (no.)	2-AG	AEA	Method
Centonze et al. 2007 [14]	CSF	Ctrl (2-AG: 5, AEA: 11)	Mean: ~ 68 (SEM: 29) pmol/mL	Mean: 2.4 (SEM: 1.3) pmol/mL	HPLC with fluorescence detection
		MS (2-AG: 5, AEA: 11)	Mean: ~ 92 (SEM: 38) pmol/mL	Mean: 20.3 (SEM: 15.7) pmol/mL	
Di Filippo et al. 2008 [18]	CSF	Ctrl (20)	Mean: 1.07 (SD: 0.12) pmol/mL	Mean: 0.0085 (SD: 0.0019) pmol/mL	GC-MS
		RRMS stable (20)	Mean: 0.82 (SD: 0.1) pmol/mL	Mean: 0.0048 (SD: 0.0009) pmol/mL	
		RRMS relapse (15)	Mean: 1.0 (SD: 0.13) pmol/mL	Mean: 0.0068 (SD: 0.0009) pmol/mL	
		SPMS (15)	Mean: 0.76 (SD: 0.1) pmol/mL	Mean: 0.0036 (SD: 0.0006) pmol/mL	
In this study	CSF	Ctrl (2-AG: 80, AEA: 78)	Median: 0.16 (IQR: 0.12–0.22) pmol/mL	Median: 0.0015 (IQR: 0.0011–0.0019) pmol/mL	LC-ESI-MS/MS
		MS (74)	Median: 0.18 (IQR: 0.11–0.24) pmol/mL	Median: 0.0016 (IQR: 0.001–0.0024) pmol/mL	
		young matched Ctrl (2-AG: 28, AEA: 26)	Median: 0.12 (IQR: 0.09–0.18) pmol/mL	Median: 0.0013 (IQR: 0.0008–0.0016) pmol/mL	
		young RRMS (28)	Median: 0.2 (IQR: 0.12–0.25) pmol/mL	Median: 0.002 (IQR: 0.0011–0.0024) pmol/mL	
		old matched Ctrl (30)	Median: 0.17 (IQR: 0.12–0.22) pmol/mL	Median: 0.0017 (IQR: 0.0013–0.002) pmol/mL	
		old RRMS (30)	Median: 0.17 (IQR: 0.1–0.22) pmol/mL	Median: 0.0015 (IQR: 0.0007–0.0027) pmol/mL	
Jean-Gilles et al. 2009 [39]	Plasma	Ctrl (17)	Mean: ~ 21 (SEM: 3) pmol/mL	Mean: ~ 0.8 (SEM: 0.07) pmol/mL	LC-ESI-MS/MS
		RRMS (10)	Mean: ~ 17 (SEM: 4) pmol/mL	Mean: ~ 1.6 (SEM: 0.2) pmol/mL	
		SPMS (8)	Mean: ~ 20 (SEM: 3) pmol/mL	Mean: ~ 1.6 (SEM: 0.3) pmol/mL	
		PPMS (6)	Mean: ~ 31 (SEM: 16) pmol/mL	Mean: ~ 1.4 (SEM: 0.2) pmol/mL	
Lötsch et al. 2018 [51]	Serum	Ctrl (301)	Median: ~ 25.6 (IQR: 19.8–38.3) pmol/mL	Median: ~ 5.5 (IQR: 4.6–6.6) pmol/mL	LC-ESI-MS/MS
		MS (102)	Median: ~ 26.9 (IQR: 17.2–46.8) pmol/mL	Median: ~ 3.5 (IQR: 2.3–4.3) pmol/mL	
Gustavsen et al. 2023 [31]	Plasma	Ctrl (46)	Median: 25.7 (IQR: 16.2–40.2) pmol/mL	Median: 5.9 (IQR: 4.9–6.8) pmol/mL	UHPLC-MS/MS
		MS (66)	Median: 22.7 (IQR: 17.1–35.4) pmol/mL	Median: 5.2 (IQR: 4.5–6.3) pmol/mL	
In this study	Serum	Ctrl (80)	Median: 8.1 (IQR: 5.7–15.9) pmol/mL	Median: 0.49 (IQR: 0.38–0.59) pmol/mL	LC-ESI-MS/MS
		MS (74)	Median: 7.9 (IQR: 5.3–13.6) pmol/mL	Median: 0.49 (IQR: 0.41–0.65) pmol/mL	

Ctrl: Control, HPLC: High pressure liquid chromatography, IQR: Interquartile range (25th to 75th percentile), LC-ESI-MS/MS: Liquid chromatography-electrospray ionization-tandem mass spectrometry, MS: Multiple sclerosis, PPMS: Primary-progressive MS, RRMS: Relapsing-remitting MS, SD: Standard deviation, SEM: Standard error of mean, SPMS: Secondary-progressive MS, UHPLC-MS/MS: Ultra high-performance liquid chromatography-tandem mass spectrometry

eCBs in a general comparison of MS and control patients, we found an intriguing sex specific increase of 2-AG in the CSF of male MS patients in comparison to female MS and control patients. Even though male and female MS patients have been included in the two earlier described studies by Centonze et al. and Di Filippo et al. [13, 18], sex specific alterations of eCBs in CSF of MS patients have not been reported, which may be due to the low number of included patients. In earlier clinical studies with high patient numbers sex associated differences in MS patients were identified and revealed a higher incidence in females, but also a potentially faster disease progression and higher severity in males [80, 88]. A study by Berek and colleagues recently reported sex-specific associations in the elevated cytokine patterns in CSF and serum of MS patients, indicating potential differences in the inflammatory response of males and females [8]. The finding of elevated 2-AG in the CSF of male MS patients in our study may therefore be associated with a higher disease severity and/or different inflammatory response in the males compared to the female patients. However, we did not find sex-specific differences of the available EDSS.

Besides sex we further identified age as impacting factor on eCB levels especially in the CSF, where 2-AG and AEA levels were positively correlating with the age of the control patients. A similar age correlation was found for 2-AG but not AEA in the serum of the controls. In contrast, no age correlation could be observed for the eCBs in CSF and serum of MS patients. We next focused on RRMS patients with an age and sex matched cohort of the control patients and split both cohorts by the median age (39 years). This revealed increased levels of the eCBs 2-AG and AEA in the CSF of young RRMS patients compared to the sex and age matched controls. These already elevated eCB levels in young MS patients are comparable to the levels found in older patients with and without MS and is accounting for the MS-specific loss of the positive correlation of the eCBs with age found in the control patients. The increased eCB levels in young MS patients may be beneficial to reduce neuroinflammation and disease severity as observed in pre-clinical models inhibiting MAGL or the eCB re-uptake [30, 72]. Furthermore, AA, the downstream metabolite of 2-AG and AEA, was found to be stronger positively associated with age in the CSF of controls compared to MS patients. A multiple linear regression analysis revealed that not only age and sex but potentially also MS impacts the AA levels in the CSF. Moreover, the CSF AA levels showed a tendency to be higher in young RRMS patients compared to matched controls, however not reaching significance ($p=0.0575$, Mann-Whitney test). Even though we could not observe significant changes of eCB levels in serum between young RRMS and control patients, we found a moderate positive correlation between 2-AG and AEA only in the serum of young RRMS patients in comparison to old RRMS and control patients. This might indicate potential changes in ECS metabolism in the periphery and circulation of young RRMS patients. The observed increased levels of eCBs in young RRMS patients may be associated with a potential acceleration of biological aging in MS patients identified in earlier clinical studies by analysing age-related biomarkers including telomere length, DNA methylation and marker of senescence and immunosenescence [48, 84, 96]. Since the observed elevated concentrations of both eCBs in the CSF of young RRMS patients were not reflected in serum eCB levels, we conclude that the analysis of serum eCB concentrations is not suitable for an approximation of MS-associated eCB changes in the CSF. Consistently, a correlation analysis between CSF and serum samples collected from the same patients revealed no associations of AEA in RRMS and control patients, while 2-AG only showed a weak positive correlation in RRMS patients. Since the impact of MS on serum and plasma eCB levels reported earlier are overall inconsistent [31, 39, 51], the analysis of eCBs in blood matrices like plasma and serum may not be suitable as a reliable readout for MS-associated changes in the CSF. However, two earlier articles reported increased eCB levels in lymphocytes of MS patients, which may indicate specific cell compartmental changes in the circulation [13, 78]. These cell-specific changes in the blood could be associated with the observed changes in the CSF and should be considered in more focused investigations in the future.

Since serum levels of 2-AG, AEA and AA were 50- to 330-fold higher compared to CSF levels, the positive age correlations found for these analytes in the CSF of control patients could be associated with an enhanced impairment of the blood-CSF barrier, which has been described to occur also in healthy people with increasing age [23, 25, 87]. A loss of integrity and function of the brain barriers is a typical inflammation associated phenomenon of MS [62, 99]. An impairment of the blood-CSF barrier may enable a higher influx of eCBs and AA from the circulation into the CSF, which could potentially explain the higher CSF levels found in young RRMS patients. The CSF is produced from plasma by the epithelial cells of the choroid plexus [22]. Although the lipophilic eCBs are generally associated to membranes and binding proteins, it is not fully understood how the diffusion of eCBs from the plasma into the CSF is regulated and why eCB levels in CSF are significantly lower compared to the circulation. Since components of the ECS, including the eCB hydrolysing enzymes FAAH and MAGL, were identified in the cells of the choroid plexus in rodents, eCB hydrolysis during CSF generation could potentially regulate the diffusion of plasma eCBs into the CSF [20, 21, 82]. The

sex, age and MS associated differences of eCBs in the CSF identified in this study could therefore be mediated by eCB hydrolysing enzymes in the choroid plexus.

Poly-unsaturated fatty acids (PUFA) and their downstream lipid mediators are important regulators of inflammatory processes [28, 69, 81]. Because the PUFA AA is released through hydrolysis of AEA and 2-AG, and is thereby closely associated to the ECS (see Fig. 1) [54], we also quantified AA and the AA-derived prostaglandins PGE2 and PGD2 in the collected samples of MS and control patients. As mentioned earlier, a multiple linear regression model indicated that MS, age and sex are potential impacting factors for CSF AA levels. Furthermore, the levels of AA in the CSF were found to be generally higher in male compared to female patients. In rodent models, 2-AG has been shown to serve as source of AA in various tissues, especially in the brain [27, 54, 63]. Interestingly, we observed a positive correlation of 2-AG with AA in serum as well as in CSF possibly indicating that in humans 2-AG serves as major supply of AA as presented in Fig. 1 and described in preclinical models [27, 54, 63]. In serum we found higher levels of the AA-derived prostaglandin PGE2 in MS compared to control patients. Moreover, PGD2 was significantly increased in older (age ≥ 39 years) RRMS patients compared to matched controls, with the same tendency for PGE2, potentially indicating a higher inflammatory state of the older RRMS patients. In agreement, PGE2 was earlier reported to be significantly increased in CSF of MS patients with an active disease state in comparison with MS patients with an inactive disease state [69]. In a mouse EAE model a selective antagonist of the PGE2 receptor EP4 was shown to reduce the accumulation of both TH1 and TH17 cells in regional lymph nodes and to suppress the disease progression [97]. This indicates a likely role of prostaglandins like PGE2 in the modulation of the inflammatory processes in MS with the associated demyelination and neuronal damage.

In addition to the well-established arachidonic acid-prostaglandin-axis, pre-clinical data strongly indicate a regulatory association between the ECS and the HPA-axis [57, 59]. Since a potential dysregulation of the HPA-axis and cortisol levels was already reported earlier in MS patients [24, 43, 49, 67, 79, 98], we included the glucocorticoids cortisol and corticosterone in our analysis of serum and CSF samples and identified increased cortisol levels in the CSF of MS patients compared to the controls. As observed for 2-AG, the sex-based differentiation of the patient groups revealed specifically increased levels of cortisol and corticosterone in the CSF of male MS patients compared to female MS and control patients. In accordance, a positive correlation between cortisol and 2-AG, as well as AA was identified in the CSF of RRMS patients but was absent in the controls. The HPA axis activity follows a circadian rhythm modulating the cortisol release from the adrenals into the circulation [15]. Thereby, the cortisol concentrations regularly increase very early in the morning and drop over the day [15]. Fittingly, we observed a negative correlation of cortisol levels with the sampling time. Importantly, the time of day of blood and CSF collection did not differ between MS and control patients, as well as between RRMS patients and age and sex matched controls. As already described in an earlier published study of Katayama et al., we also observed a negative correlation of cortisol with age, which was more pronounced in RRMS patients compared to the age and sex matched controls [42]. In addition, we observed a mild negative correlation between the serum cortisol of RRMS patients and the reported EDSS, as well as the time between the occurrence of the first symptoms and the sampling. These findings are in agreement with an earlier post-mortem study, where high CSF cortisol levels of 42 MS patients were associated with slow disease progression, whereas low CSF cortisol was associated with a fast disease progression, higher numbers of active lesions and less remyelinated plaques [55, 56]. In serum of control patients, we further observed a clear positive correlation of both glucocorticoids with AEA and related *N*-acylethanolamines. However, this was not the case for the RRMS patients potentially indicating a disturbed interplay between the HPA-axis and the ECS.

The analysis of ECS-associated lipids further revealed increased serum levels of SEA, a *N*-acylethanolamine which is structurally related to the eCB AEA [54]. Of note, SEA was positively correlating with the age of MS patients, as well as with the time between the occurrence of the first symptoms and sample collection and SEA was found to be especially increased in already treated MS patients. This may indicate that circulating SEA is increasing with the disease progression or during the treatment. In pre-clinical inflammation models, SEA was shown to have anti-inflammatory function, restricting the spread of peripheral inflammation to the brain and thereby reducing the progression of associated neuropathologies [7, 41, 53]. Together with our observations, this may imply an anti-inflammatory, CNS protecting function of SEA also in MS patients. Furthermore, 2-OG, which is structurally related to the eCB 2-AG, was observed to be increased in CSF of MS patients, especially in treated MS patients. The analysis of young and old RRMS patients with age and sex matched controls further revealed significantly elevated 2-OG concentrations in old RRMS patients. Like for SEA, the elevated 2-OG levels in CSF of MS patients might therefore be associated either with the disease progression or with the applied treatment. 2-OG is an agonist of the G protein-coupled receptor 119 (GPR119) [34, 83]. In a recent

preclinical study GPR119 was found to be expressed in spinal cord and other neuronal tissues of rats, where it is contributing to the processing of neuronal pain [100]. Therefore, 2-OG might also be involved in the pain perception of MS patients. Generally, the ECS-associated lipids are less studied compared to the eCBs 2-AG and AEA. The presented findings show the connectivity of the lipidome and may suggest that also ECS-associated lipids should be considered in future pre-clinical and clinical investigations.

Besides the eCBs and the ECS-associated lipids we were interested in peptide endocannabinoids (pepcans). RVD-hemopressin (pepcan-12) acts as a negative allosteric modulator of CB1R and as a positive allosteric modulator of CB2R, and is therefore hypothesised to be a potential modulator of inflammatory processes [6, 29, 68]. Fittingly, enhanced levels of RVD-hemopressin and its precursor peptide pepcan-23 were observed in inflammation inducing mouse models of endotoxemia and ischemia reperfusion injury [29, 68]. Therefore we hypothesised that pepcan levels would be increased in MS patients due to the clear inflammatory pathomechanism of the disease. Even though we could detect both pepcans (RVD-hemopressin/pepcan-12 and pepcan-23) in the CSF samples, there were no significant differences between MS and control patients. However, we found a positive correlation of pepcan-23 and RVD-hemopressin, which is in accordance with findings reported earlier by our group describing the function of pepcan-23 as precursor of RVD-hemopressin [29].

The presented study has several limitations, which should be considered for the interpretation, mostly due to the retrospective study design. We could not control for aditional patient parameters associated with circulating 2-AG and AEA levels, including the body mass and body fat percentage [50], physical activity [17] and food intake [58]. This might explain the variability of the 2-AG and AEA level measured in serum to a certain extend. Due to the low number of each specific treatment, we were unable to analyse the impact of a single treatment on the lipid profiles. In addition, we cannot exclude that a potential impact of other uncontrolled or unknown factors, as well as non-neuroinflammatory diseases of the control cohort may disguise a potential effect of MS on the analytes in serum or CSF in the general comparison. In an earlier study, increased levels of serum eCBs, especially 2-AG and the 1-AG/2-AG ratio, were observed with prolonged ex-vivo coagulation times (>30 min) indicating that the control of the blood incubation before centrifugation is a crucial pre-analytical factor for the analysis of serum eCBs [45]. For this study serum samples collected earlier and stored in the LBB were used. Thus, the coagulation time was not the same for all blood samples. However, the incubation of the blood was short (<20 min) for most of the samples and comparable between samples of MS and control patients. Additionally, we had no MRI data for the assessment of Gd-enhanced lesions and could not sort the RRMS patients according to the disease activity.

Overall, this study reports sex and age-specific changes of eCBs, AA and cortisol in CSF of MS patients compared to non-neuroinflammatory control patients. The observed impact of age and sex might in part explain the inconsistencies in the so far reported MS associated changes of CSF eCBs. The elevated CSF levels of both eCBs 2-AG and AEA in young, but not old RRMS patients indicate a modified ECS regulation in the CNS especially in younger RRMS patients. Changes of eCB levels in the CSF were not reflected in the serum and only 2-AG correlated weakly between CSF and serum highlighting that a quantification of serum eCBs are not sufficient to analyse MS associated changes in the CNS of MS patients. Our data further suggest that sex and age should be considered in future clinical studies and probably also in the development and clinical trials applying treatment strategies to increase eCB concentrations in MS patients.

Abbreviations

1/2-AG	1-arachidonoylglycerol and 2-arachidonoylglycerol
1/2-OG	1-oleoylglycerol and 2-oleoylglycerol
2-AG	2-arachidonoylglycerol
2-OG	2-oleoylglycerol
AA	Arachidonic acid
AEA	Arachidonoylethanolamide, N-arachidonoylethanolamine, anandamide
BSA	Bovine serum albumin
CBR1	Cannabinoid receptor 1
CBR2	Cannabinoid receptor 2
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
Ctrl	Control
DAGL	Diacylglycerol lipase
EAE	Experimental autoimmune encephalomyelitis
eCBs	Endocannabinoids
ECS	Endocannabinoid system
EDSS	Expanded disability status scale
EtNH2	Ethanolamine
FAAH	Fatty-acid amide hydrolase
GPR	G protein-coupled receptor
HPA-axis	Hypothalamic-pituitary-adrenal-axis
HPLC	High performance liquid chromatography
IQR	Interquartile range
LBB	Liquid Biobank Bern
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LEA	Linoleoylethanolamide
LOQ	Limit of quantification
MAGL	Monoacylglycerol lipase
MRM	Multiple-reaction monitoring
MS	Multiple sclerosis
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
NArPE	N-arachidonoyl phosphatidylethanolamine
n.d.	Not detectable
no.	Number
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
Pepcans	Peptide endocannabinoids

PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGH2	Prostaglandin H2
PMS	Progressive MS
PPT	Protein precipitation
PPMS	Primary progressive MS
PUFA	Poly-unsaturated fatty acids
RRMS	Relapse-remitting MS
SAG	1-Stearoyl-2-arachidonoyl-sn-glycerol
SEA	Stearoylethanolamide
SD	Standard deviation
SEM	Standard error of mean
SPE	Solid-phase extraction
SPMS	Secondary-progressive MS
UHPLC-MS/MS	Ultra high-performance liquid chromatography-tandem mass spectrometry

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

Sandra Glasmacher, Anke Salmen, Andrew Chan and Jürg Gertsch contributed to the study conception and design. The application for the ethical approval was written by Sandra Glasmacher and Jürg Gertsch. Sorting and selection of available patient samples was conducted by Sandra Glasmacher, Anke Salmen and Philip Meier. Material preparation and data collection were performed by Sandra Glasmacher and Philip Meier. Philip Meier analysed the data and generated the graphical data representations. Jürg Gertsch supervised the analytical measurements and data acquisition and analysis. The first draft of the manuscript was written by Philip Meier and Jürg Gertsch. All authors commented on previous versions of the manuscript and approved the final manuscript.

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Data availability

All analyte concentrations and patient information included in this study, as well as additional information for the presented correlation analyses are summarised in the spreadsheet in the Online Resource 2.

Declarations

Ethics approval

The study was performed in accordance with the declaration of Helsinki and was approved by the cantonal ethics committee of Bern (Project-ID: 2020–02164).

Consent to participate

All patients provided written informed consent for the collection, storage and usage of biological material and medical documentation for research purposes.

Competing interests

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