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Perfluorinated substances, risk factors for multiple sclerosis and cellular immune activation

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A B S T R A C T

Perfluorinated alkylated substances (PFASs) have immunomodulatory effects but the impact on multiple sclerosis (MS) and cellular immune functions is only sparsely described. In the present study, we found lower concentrations of the long chain PFAS perfluorooctane sulfonic acid (PFOS) in MS than in healthy controls (HC). In HC, we did not detect associations between PFOS concentrations and immune phenotypes. Analyzing the impact of known MS risk factors on cellular immune functions, we found that smoking and Epstein–Barr nuclear antigen 1 antibodies were associated with distinct circulating immune cell changes. In summary, current background PFAS exposure is not an important risk factor for MS.

1. Introduction

Perfluorinated alkylated substances (PFASs) are synthetic chemical compounds produced since the 1950s. PFASs are substances with long carbon chains with fluorine atoms replacing the hydrogen atoms. Carbon-fluorine bindings are very stable, and biodegradation of PFASs is extremely slow (Li et al., 2018). Much focus has been on the "long-chain" PFASs as the toxicity and accumulation in the environment seems to increase with the length of the carbon chains. PFASs have been used as surfactants in a wide range of consumer products due to their combined hydrophilic and hydrophobic properties and has for the last two decades been investigated for their potential harmful effects in humans (Buck et al., 2011). Human exposure to PFASs stem from inhaled particles and from contaminated food products and drinking water due to a lack of environmental degradation (ATSDR, 2018).

In humans, PFASs are only slowly eliminated, and serum half-lives of up to 5.4 years are reported (Li et al., 2018, Stahl et al., 2011). PFASs bind to serum proteins and accumulate in kidneys, liver and blood (Jian et al., 2018). Mostly shorter chain PFASs are found to be excreted in urine whereas long-chain PFASs are suggested to be excreted through breast milk and blood (Fromme et al., 2009; Lorber et al., 2015; Wong et al., 2014). Reported health effects from PFASs includes several studies suggesting an immunosuppressive or immunotoxic effect of PFASs (DeWitt et al., 2019; Grandjean et al., 2017a; Stein et al., 2016).

Multiple sclerosis (MS) is an immune-mediated disease in the central nervous system (CNS). The disease is driven by autoimmune cells recruited from the periphery causing disease upon entry to the CNS (Compston and Coles, 2008; Sospedra and Martin, 2016). Genetic and environmental factors are believed to be main contributors to the etiology of MS. Some of the strongest risk factors reported are female sex, smoking, low vitamin D, Epstein-Barr virus (EBV) infection, and carrying the *HLA-DRB1*15:01* risk gene (Belbasis et al., 2015; Duan et al., 2014; Handel et al., 2011; The International Multiple Sclerosis Genetics and The Wellcome Trust Case Control, 2011).

One study has reported a slightly increased occurrence of MS at highly elevated exposures to perfluorooctanoic acid (PFOA), but the association between serum-PFOA concentrations and MS was not statistically significant (Steenland et al., 2013). However, the serum concentration obtained may not have reflected the pathogenic exposure. To our knowledge, effects of PFASs on MS risk factors and circulating immune cell characteristics are poorly known.

Accordingly, we aimed to investigate if serum-PFAS concentrations are associated with MS and to further examine the impact of PFASs and known MS risk factors on the relative distribution and activation of circulating immune cells.

2. Methods

2.1. Study population

Serum concentrations of PFASs were measured in 86 out of 100 healthy controls (HC) from whom we had collected blood samples and

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obtained data on immune cell frequencies and activation in a previous study (Ammitzboll et al., 2017) and 76 patients of whom 67 were diagnosed with relapsing-remitting MS (RRMS), and 9 with a clinically isolated syndrome (CIS). All patients were untreated at the time of blood sampling. From the RRMS patient cohort, 51 patients were newly diagnosed and untreated and 16 patients had not received immunomodulatory or immunosuppressive treatment for at least two months. All patients were diagnosed according to the 2010 McDonald criteria (Polman et al., 2011). All samples were collected within a 2-year period and we found no significant correlation with PFAS concentrations and sample date in neither HC nor patients with MS (Supplementary Fig. 1). Information of number of children, duration of menstruation and breastfeeding were obtained by questionnaires.

All participants provided informed consent to study participation. Ethical approval was obtained from the local scientific ethics committee.

2.2. Methods

2.2.1. PFAS mass spectrometry

Analysis of the per fluorinated alkylated substances (PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFDA: perfluorodecanoic acid, PFHxS: Perfluorohexane sulfonic acid, PFHpS: perfluoroheptane sulfonic acid and PFOS: perfluorooctane sulfonic acid) were performed in serum using on-line solid phase extraction followed by Liquid Chromatography and tandem mass spectrometry (LC-MS/MS) as previously described (Grandjean et al., 2012; Haug et al., 2009). The extraction was performed on a Thermo Scientific EQuan MAX system, consisting of two Accela HPLC pump (Thermo Scientific, San José, CA) and a PAL autosampler module (CTC analysis AG, Zwingen, Switzerland). The tandem mass spectrometer was a TSQ Quantum Ultra Triple Stage Quadropole with heated electrospray ionization (Thermo Scientific, San Jose, CA). Calibration and quality control samples were included in each accomplished batch of samples. The imprecision within the batch were < 6% for all compounds. The limit of quantitation (LOQ) was 0.03 ng/mL for all PFASs.

2.2.2. ELISA and electrochemiluminescence protein binding assay

Anti-EBNA-1 antibodies and cotinine concentrations were measured by ELISA assays (Diasorin, Italy and Calbiotech, USA) as previously described in detail (Ammitzboll et al., 2017). 25-hydroxyvitamin D concentrations were analyzed at the Department of Clinical Biochemistry, Rigshospitalet, University hospital of Copenhagen, using electrochemiluminescence protein binding assays on a COBAS 8000 analyzer.

2.2.3. HLA-DRB1*15:01 genotyping

DNA extracted from buffy coats was genotyped by PCR assays using TaqMan® allelic discrimination according to manufacturer (Life Technologies, Denmark). SNP rs9271366 was used to tag *HLA-DRB1*15:01* as previously described (Ammitzboll et al., 2017).

2.2.4. Flow cytometry on circulating cells

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn blood samples using Lymphoprep (Axis-Shield, Norway) and density gradient centrifugation. PBMCs were washed and added FcR Blocking Reagent (Miltenyi Biotec, Germany). PBMCs were then stained with anti- CD14, CD1c, BDCA2, CD19, CD40, CD80, CD83, CD86, CD27, CD38, DC-Sign, ICOSL, CD3, CD4, CD8, CD45RA, CD62L, CCR6, CXCR3, CXCR5, ICOS, IL23R, CD26, CD161, CD25, CD127, CD31 and CD39. Data were acquired on a FACS Canto II (BD Biosciences, USA) and FlowJo (Tree star, USA) and FACS Diva software (BD Biosciences, USA) were used for data analyses as previously described in details (Ammitzboll et al., 2017).

2.2.5. T cell activation assays

Freshly isolated PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Denmark) and cultured 2×10^6 cells per well in 48 wells flat bottom culture plates (CellStar Greiner bio-one, Germany). Antigens, myelin basic protein (MBP; 30 μg/mL, HyTest, Finland) or myelin oligodendrocyte glycoprotein (MOG; 10 μg/mL, BioNordika, Denmark), EBV lysate (2 μg/mL, EastCoast Bio, USA) or no antigen as a negative control were added to the cells together with RPMI-1640/5% human AB-serum/Penicillin (50 units/mL) and streptomycin (50 μg/mL) (all Invitrogen, USA). After seven days, cells were stained with anti- CD3, CD4, CCR6, CXCR3, CXCR5 and a live/dead stain and analyzed by flow cytometry.

Intracellular cytokine staining was done by re-stimulating the PBMCs with phorbol 12-myristate 13 acetate (PMA; 10 ng/mL, Sigma-Aldrich, USA) and ionomycin (0.5 μg/mL, Sigma-Aldrich, USA) for 30 min and then incubating the cells for 4 h with Brefeldin A (5 μg/mL, Sigma-Aldrich, USA). Cells were then stained with anti- CD3 and CD8, and a live/dead stain and fixed and permeabilized according to manufacturer's protocol (Fixation Buffer and Permeabilization Buffer, Biolegend, USA). PBMCs were then stained with anti- IL − 17A, IFNγ and GM-CSF and analyzed by flow cytometry as previously described (Ammitzboll et al., 2017).

2.2.6. Statistical analyses

Statistics on PFOS concentrations were performed using parametric analyses of logarithmically transformed values. We used Pearson's correlation analyses, analyses of covariance (ANCOVA) and multiple linear regression analyses using the “forced entry” method. Analyzing cell frequencies was done using non-parametric methods since many of the cell frequencies were not suitable for parametric analyses even in a transformed form. Mann-Whitney's *U* test and Spearman's rank correlation analyses were performed. We used a significance level of $P < .005$ and did not correct for multiple testing (Benjamin et al., 2018).

3. Results

One hundred HC and 76 patients with RRMS or CIS were included in the study. Demographics are shown in Table 1. From 86 HC and from all 76 patients, we obtained serum concentrations of six long-chain PFASs: the per fluorinated alkylated sulfonic acids perfluorohexane sulfonic acid (PFHxS) (C6), perfluoroheptane sulfonic acid (PFHpS) (C7) and perfluorooctane sulfonic acid (PFOS) (C8); and the perfluoroalkyl carboxylic acids perfluorooctanoic acid (PFOA) (C_7), perfluoro nonanoic acid (PFNA) (C_8) and perfluorodecanoic acid (PFDA) (C_9).

To identify demographic factors related to PFASs in the blood, we initially studied correlations of age, years of menstruation, number of children, total months of breastfeeding and sex with PFASs in the HC cohort (Supplementary Table 1). We found that concentrations of PFHxS, PFHpS, and PFOS all increased significantly ($P < .005$) with age. PFHxS, PFHpS, PFOS and PFOA decreased significantly with both years of menstruation and number of children. PFHxS, PFHpS, PFOS, PFOA, and PFNA concentrations decreased with months of breastfeeding and PFHxS, PFHpS, PFOS and PFOA concentrations were higher in men than in women after correcting for age by ANCOVA analyses (Supplementary Table 1). The difference between serum-PFAS concentrations in men and women was non-significant when adjusting for age, breastfeeding, years of menstruation and number of children (data not shown). All serum-PFAS concentrations were closely correlated ($P < .0001$).

To determine if PFAS exposure is related to MS, we performed multiple regression analyses. We found that only PFOS concentrations differed significantly between HC and patients with MS or CIS (Table 2). These analyses were adjusted for sex, age and breastfeeding. Age was more closely associated with PFOS concentrations than years of menstruation, and the latter was therefore not included as covariate. Likewise, number of children showed collinearity with duration of

Table 1
Participant demographics and PFAS levels. Participants, MS type and HLA-genotypes are summarized in counts. Age, breastfeeding, cotinine, vitamin d, anti-EBNA-1 and PFAS concentrations are given as mean and median values with inter quartile ranges (IQR) in brackets. Abbreviations: Relapsing remitting multiple sclerosis = RRMS; Clinically isolated syndrome = CIS.

	Healthy controls (n = 100)			RRMS + CIS (n = 70)		
	Mean	Median	Count/IQR	Mean	Median	Count/IQR
Women/men			51/49			49/27
RRMS/CIS						67/9
HLA-DRB1 (non- carriers/carriers of *rs9271366G*)			73/26			
Age	37	35	(28–45)	39	39	(32–44)
Cotinine (ng/mL)	24.98	0	(0–43.11)			
Vitamin D (nmol/L)	63.16	57.71	(45.64–77.26)			
Anti-EBNA-1 (AU/mL)	203.3	168.4	(66.8–235.6)			
PFHxS (ng/mL)	0.74	0.68	(0.44–0.97)	0.71	0.62	(0.41–0.93)
PFHpS (ng/mL)	0.17	0.15	(0.09–0.22)	0.15	0.14	(0.87–0.20)
PFOS (ng/mL)	10.61	9.41	(6.41–13.05)	8.07	7.14	(5.76–9.93)
PFOA (ng/mL)	2.47	1.94	(1.38–3.01)	1.96	1.88	(1.34–2.32)
PFNA (ng/mL)	1.01	0.92	(0.69–1.16)	1.01	0.94	(0.72–1.18)
PFDA (ng/mL)	0.29	0.26	(0.21–0.34)	0.31	0.29	(0.19–0.37)
For women only	Healthy controls (n = 43)			RRMS + CIS (n = 49)		
Age	34	32	(26–42)	39	41	(31–44)
Breastfeeding (months)	6	0	(0 − 12)	7	0	(0–12)
Number of children	0.83	0	(0–2)	0.83	0	(0–2)
Years of menstruation	20.05	18	(13–26)	24.95	25.5	(17.5–31)

breastfeeding. Because women with and without MS had comparable number of children, the variable was therefore excluded from the analysis (Table 1). Overall, serum-PFOS concentrations were 17% lower in patients with MS than in controls (Table 2). This result was confirmed ($P < .05$) when data were analyzed separately for men (with adjustment for age) but was not statistically significant for women ($P = .093$) (adjusted for age and breastfeeding).

MS is considered an autoimmune disease influenced by age, sex, genes, lifestyle- and environmental factors. Smoking, *HLA-DRB1*15:01* risk genotype, low vitamin D concentrations and EBV are factors associated with an increased risk of developing MS (Belbasis et al., 2015, Handel et al., 2011). To study whether these MS risk factors were related to serum concentrations of PFOS in HC we assessed previously obtained serum concentrations of the nicotine metabolite cotinine, vitamin D and anti-EBNA-1 as well as *HLA-DRB1*15:01* genotype (Ammitzboll et al., 2017) but found no associations between any of the mentioned MS risk factors and PFOS (data not shown).

To get an impression of the cellular mechanisms affected by MS risk factors or PFOS, we performed exploratory correlation analyses of age, sex, *HLA-DRB1*15:01* genotype, cotinine, anti-EBNA-1 and vitamin D concentrations and a large data-set of different immune cell frequencies in the blood of the 100 HC. Moreover, we studied data of T cell activation, including cytokine production upon stimulation with EBV lysate and the MS autoantigens myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) (Supplementary Tables 2 and 3). Median frequencies of proliferating CD4 T cells were 0.16% with no antigen, 5.2% when stimulated with MBP, 3.6% with MOG and 6.2% with EBV (Supplementary Table 2).

We found that increasing age was associated with increased frequencies of CD39+ regulatory CD4+ T cells (T_{reg}) and CD8+ effector memory (EM) T cells, and lower frequencies of circulating naïve CD8+ T cells and MOG- and EBV-reactive CD4+ T cells secreting IFN-γ upon stimulation (Supplementary Table 2). Women had higher frequencies of circulating CD4+ T cells and men had higher frequencies of IL-21-secreting CD4+ T cells (Supplementary Table 2).

We did not find significant associations between PFOS concentrations and any of the circulating immune cell types, T cell activation or cytokine producing T cells, but we did see a tendency of circulating CD8+ T cells being skewed towards a memory phenotype (Supplementary Table 3). Cotinine concentrations, as a measure of smoking intensity, has previously been explored in a sub-group analysis of HC (Ammitzboll et al., 2017). We confirm our previous findings of lower frequencies of CD8+ T cells characterized by the expression of $CD26^{hi}CD161^{hi}$ in smokers, presumably defining mucosal associated invariant T (MAIT) cells (Ammitzboll et al., 2017; Sharma et al., 2015), and increased percentages of ICOSL+ plasmacytoid dendritic cells (pDC). Furthermore, cotinine was positively correlated with the percentage of CD86+ B cells (Supplementary Table 3).

Vitamin D concentrations did not correlate with any of the circulating cell populations but were positively associated with a CCR6+ CD4+ T cell phenotype of resting, unstimulated cells. Also, MOG and EBV proliferating CCR6+ CD4+ T cells showed a tendency to correlate positively with vitamin D concentrations ($P < .05$) (Supplementary Table 3).

High anti-EBNA-1 concentrations were significantly associated with lower frequencies of circulating Tfh cells being ICOS positive and higher frequencies of central memory (CM) CD8+ T cells. Resting, unstimulated CD4+ T cells producing GM-CSF or IFN-γ were also increased with higher concentrations of EBNA-1 antibodies (Supplementary Table 3). Anti-EBNA-1 were not associated with CD4+ T cell proliferation.

Carriers of the MS risk allele *HLA-DRB1*15:01* among HC did not have increased frequencies of any of the studied circulating immune cell types or increased proliferating or cytokine producing T cells. We found a tendency ($P = .007$) to higher frequencies of circulating CD80+ B cells in HC who did not carry the risk allele (Supplementary Table 3).

4. Discussion

With the present study, we show novel data on the cross-sectional relationship between PFAS exposure and RRMS and conclude that RRMS is associated with lower serum concentrations of PFOS compared with HC, whereas we found no difference for the other PFASs studied after adjustment for relevant confounding factors.

Being one of the largest PFAS, PFOS is poorly eliminated from the human body. Consequently, the health problems associated with PFOS exposure, as compared with other PFASs, have attracted the most attention. Early-life exposure to PFOS was previously found to be associated with poor antibody responses in children upon vaccination, and a high MS prevalence was reported in an area of high exposure of PFOA, another long chain PFAS (Grandjean et al., 2012, Grandjean et al.,

Table 2
PFAS serum concentrations and relations with multiple sclerosis. Results from multiple regression analyses shown for all individuals and separately for women and for men. Results are adjusted for sex, age and breastfeeding (all individuals) age and breastfeeding (women) and age (men). Logarithmically transformed PFAS values were used and unstandardized coefficients (B) and 95% confidence intervals are given in percentages after anti-log transformation. In the analyses, HC and women were given values of 0 and RRMS + CIS and men were given values of 1. Statistical significant findings are shown with bolded *P*-values ($P < .005$). Results are not adjusted for multiple testing. Abbreviations: RRMS = Relapsing remitting multiple sclerosis, CIS = Clinically isolated syndrome, PFOA = Perfluorooctanoic acid, PFNA = Perfluorononanoic acid, PFDA = Perfluorodecanoic acid, PFHxS = Perfluorohexane sulfonic acid, PFHpS = Perfluoroheptane sulfonic acid, PFOS = Perfluorooctane sulfonic acid, CI = Confidence interval, HC = Healthy controls.

All individuals (n = 162)	PFHxS			PFHpS			PFOS			PFOA			PFNA			PFDA		
	B (% change/ unit)	95.0% CI (%)	P	B (% change/ unit)	95.0% CI (%)	P	B (% change/ unit)	95.0% CI (%)	P	B (% change/ unit)	95.0% CI (%)	P	B (% change/ unit)	95.0% CI (%)	P	B (% change/ unit)	95.0% CI (%)	P
HC/RRMS + CIS	2	(−9–15)	0.719	0	(−13–14)	0.971	–17	(−27 – −6)	**0.004**	−12	(−24–2)	0.099	0	(−13–14)	0.951	0	(−14–16)	0.979
	Adjusted R Square = 0.57			Adjusted R Square = 0.55			Adjusted R Square = 0.41			Adjusted R Square = 0.27			Adjusted R Square = 0.14			Adjusted R Square = 0.02		
Women (n = 92)																		
HC/RRMS + CIS	2	(−15–23)	0.807	2	(−16–25)	0.819	−14	(−28–3)	0.093	7	(−13 – 32)	0.526	10	(−11–35)	0.372	12	(−10–39)	0.320
	Adjusted R Square = 0.24			Adjusted R Square = 0.25			Adjusted R Square = 0.17			Adjusted R Square = 0.20			Adjusted R Square = 0.12			Adjusted R Square = 0.73		
Men (n = 70)																		
HC/RRMS + CIS	5	(−10 – 22)	0.522	−2	(−18–18)	0.862	−19	(−32 – −3)	0.023	−28	(−42 – −9)	0.006	−10	(−25–7)	0.232	−12	(−29–8)	0.207
	Adjusted R Square = 0.40			Adjusted R Square = 0.32			Adjusted R Square = 0.22			Adjusted R Square = 0.23			Adjusted R Square = 0.16			Adjusted R Square = 0.06		

2017b, Steenland et al., 2013). Based on this information, we speculated that PFAS have modulating effects on the immune system that influence the development of MS. Finding that serum concentrations of PFOS were negatively associated with MS was unexpected, but considering PFOS being immunosuppressive, an actual protective effect on MS development is conceivable. Still, serum-PFOS concentrations in MS patients included in the present study may not reflect the PFOS exposure during a vulnerable time window in the MS pathogenesis. On the other hand, PFASs have very long elimination half-lives in humans, and the concentrations measured indicate exposure at least during the most recent years before diagnosis.

We did not find serum-PFOS associated with known MS risk factors and we were not able to identify an effect of PFOS on circulating immune cells. However, we relied on blood samples from HC and we cannot rule out that an effect of PFOS on immune cells may occur in patients with MS. Pregnancy, breastfeeding and blood loss are some of the important elimination routes of PFASs. We have tried to adjust for confounding factors influencing serum concentrations of PFOS. However, for number of children we did not know if some children were adopted and we were not able to quantify the loss of blood due to menstruation. Moreover, we do not have information on blood donation or other significant sources of blood loss in the cohorts.

Investigations of the influence of MS risk factors on circulating and activated immune cells were explorative and we did not adjust for multiple testing. Accordingly, we used a significance level of $P < .005$, but the results should be validated in an independent cohort.

We found that women have more CD4+ T cells but less IL-21 secreting CD4+ T cells. Higher frequencies of circulating CD4+ T cells in women might be part of the reason why women are more susceptible to developing MS, as autoreactive CD4+ T cells are believed to be major players in MS pathogenesis (Sospedra and Martin, 2005). Higher frequencies of IL-21 producing CD4+ T cells in men are difficult to interpret. IL-21 has immunomodulatory roles and is most often considered to induce pro-inflammatory immune responses. However, the effect in MS is controversial (Ghalamfarsa et al., 2016).

Not surprisingly, age was associated with more antigen-experienced CD8 T cells. Increased frequencies of CD39+ T reg cells with age is previously described (Ruhnau et al., 2016). CD39+ T reg cells are described as having suppressor functions on effector T cells and, in MS, an impaired IL-17 suppression by CD39+ T reg cells has been found (Fletcher et al., 2009). The lower frequencies of IFN-γ producing antigen-reactive CD4+ T cells could possibly reflect a less aggressive circulating T cell pool with higher age. MS is primarily affecting younger adults (Filippi et al., 2018), but whether this is related to a protective effect of circulating CD39+ T reg cells with higher age and more easily activated CD4+ T cells with young age is highly speculative.

We confirm that smoking is associated with fewer circulating MAIT-like cells using cotinine as a measure of smoking intensity. The role of MAIT cells in MS is yet undetermined (Ammitzboll et al., 2017). MAIT cells are found in MS brains but frequencies in blood of patients with MS are reported with contradictory results (Annibali et al., 2011; Illes et al., 2004; Miyazaki et al., 2011; Willing et al., 2014). A recent study reports a more pro-inflammatory MAIT cell profile in patients with MS than HC (Willing et al., 2018). Accordingly, MAIT cells have been associated with both proinflammatory and protective functions in MS (Treiner and Liblau, 2015). The lower MAIT-like cell frequency we found in smokers might be suggestive of cells migrating to the lung mucosa of smokers, but how and if this worsens MS disease is highly speculative.

We also found increased co-stimulatory molecules ICOSL and CD86 on antigen presenting cells, pDCs and B cells from smokers. Both molecules are essential in T cell activation and whereas ICOSL is specifically upregulated on mature pDCs, CD86 is universal on antigen presenting cells (Ito et al., 2007; Janke et al., 2006). ICOSL-ICOS interaction are furthermore important for the development of Tfh cells

(Fan et al., 2015). Our group has previously found increased frequencies of ICOS+ Tfh cells in patients with RRMS. Other research groups support this finding (Cunill et al., 2018; Romme Christensen et al., 2013; Shekhar and Yang, 2012). In this study, we did not find increased frequencies of ICOS+ Tfh cells in HC smokers. Overall, smoking seems to induce antigen presenting cells with higher T cell activation properties.

Vitamin D concentrations seemed generally associated with a CCR6+ CD4+ T cell phenotype in resting and activated T cells. In MS, vitamin D is generally agreed to have a protective effect (Duan et al., 2014). Since CCR6+ CD4+ T cells are suggested to be MS-pathogenic, we would have expected CCR6+ CD4+ T cells to be negatively correlated with vitamin D concentrations. This is further supported by findings in autoimmune diseases of vitamin D inhibiting *CCR6* gene expression in activated CD4+ T cells (Dankers et al., 2016). Our findings of higher vitamin D concentrations related to a CCR6+ phenotype on resting, non-proliferating CD4+ T cells could be explained by the fact that we studied HC and therefore reflect a healthy condition. This is supported by findings of vitamin D being essential for T cell activation (von Essen et al., 2010).

Anti-EBNA-1 concentrations were associated with IFN-γ and GM-CSF production in CD4+ T cells resting in culture for seven days. Anti-EBNA-1 also were found to be associated with lower ICOS+ Tfh and higher $CD8_{CM}$ T cell frequencies. Previous studies have shown that patients with MS have more EBNA-1 specific CD4+ T cells than HC and that these cells are predominantly of a Th1 cell type (Lunemann et al., 2008). This is consistent with our findings in HC as IFN-γ secretion defines Th1 cells. Frequencies of T cells secreting GM-CSF also have previously been found to be higher in patients with MS compared with HC and is associated with MS severity biomarkers (Hartmann et al., 2014; Rasouli et al., 2015). The lower frequencies of ICOS+ Tfh with increasing anti-EBNA-1 concentrations suggest an overall skewed Th1/Tfh ratio with increasing concentrations of anti-EBNA-1.

In conclusion, we were not able to support PFAS exposure as an environmental risk factor for MS. Conversely, our findings suggest a potential protective effect of serum-PFOS concentrations on MS which may be attributed to an immunosuppressive effect of PFOS. However, we were not able to characterize any such effect in our detailed immuno-phenotype study. From studying already known MS risk factors and their effect on immune cell mechanisms, we confirm the suppressing effect of smoking on circulating MAIT-like cells as well as the linkage between EBV infection and GM-CSF and IFN-γ producing CD4+ T cells. These findings add to the importance of studying the effect of MS risk factors in healthy controls and its capacity to provide mechanistic insights into the pathogenesis of complex immune-mediated diseases such as MS.

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Conflict-of-interest disclosure

C.A., L.B. H.B·S and P.G. report no financial conflicts of interest. E.R.P. has served on scientific advisory board for Teva and received support for congress participation from Roche. A.B.O. has served on scientific advisory boards for Biogen Idec, Novartis and Sanofi Genzyme; has received research support from Novartis and Biogen Idec; has received speaker honoraria from Biogen Idec, Novartis and TEVA; and has received support for congress participation from, Merck, TEVA, Biogen, Roche, Novartis and Sanofi Genzyme. F.S. has served on scientific advisory boards, been on the steering committees of clinical trials, served as a consultant, received support for congress participation, received speaker honoraria, or received research support for his laboratory from Biogen, EMD Serono, Merck, Novartis, Roche, and Sanofi Genzyme.

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