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CANNABIS USE BY INDIVIDUALS WITH MULTIPLE SCLEROSIS: EFFECTS ON SPECIFIC IMMUNE PARAMETERS

Michelle Sexton, ND[1,2], **Eiron Cudaback, PhD**[3], **Rehab A. Abdullah, PhD**[4], **John Finnell, ND, MPH**[5], **Laurie K Mischley, ND, MPH**[2], **Mary Rozga, MS**[6], **Aron H. Lichtman, PhD**[4], and **Nephi Stella, PhD**[7,8]

[1]Center for the Study of Cannabis and Social Policy, Seattle, WA 98028

[2]Bastyr University Research Institute, Seattle, WA 98028

[3]Department of Pathology, Neuropathology Division, University of Washington, Seattle, WA 98104

[4]Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298

[5]AOMA Graduate School of Integrative Medicine, Austin, TX 78745

[6]Michigan State University, Seattle, WA 98185

[7]Department of Pharmacology, University of Washington, Seattle, WA 98185

[8]Department of Psychiatry and Behavioral Medicine, University of Washington, Seattle, WA 98185

Abstract

Cannabinoids affect immune responses in ways that may be beneficial for autoimmune diseases. We sought to determine whether chronic *Cannabis* use differentially modulates a select number of immune parameters in healthy controls and individuals with multiple sclerosis (MS cases).

Subjects were enrolled and consented to a single blood draw, matched for age and BMI. We measured monocyte migration isolated from each subject, as well as plasma levels of endocannabinoids and cytokines. Cases met definition of MS by international diagnostic criteria.

Monocyte cell migration measured in control subjects and individuals with MS were similarly inhibited by a set ratio of phytocannabinoids. The plasma levels of CCL2 and IL17 were reduced in non-naïve cannabis users irrespective of the cohorts. We detected a significant increase in the endocannabinoid arachidonoylethanolamine (AEA) in serum from individuals with MS compared to control subjects, and no significant difference in levels of other endocannabinoids and signaling lipids irrespective of *Cannabis* use. Chronic *Cannabis* use may affect the immune response to similar extent in individuals with MS and control subjects through the ability of phytocannabinoids to reduce both monocyte migration and cytokine levels in serum. From a panel of signaling lipids, only the levels of AEA are increased in individuals with MS, irrespective from

Correspondence: Michelle Sexton: msextonnd@cannabisandsocialpolicy.org; phone: 425-318-9307.

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Cannabis use or not. Our results suggest that both MS cases and controls respond similarly to chronic *Cannabis* use with respect to the immune parameters measured in this study.

Keywords

multiple sclerosis; endocannabinoids; inflammation; monocytes; cytokines; *Cannabis*; case control study

Introduction

The plant *Cannabis (CB)*, known since antiquity for its medicinal qualities, synthesizes over 400 metabolites that collectively participate in producing its' biological and therapeutic effects (ElSohly 2007, Russo 2007). The most abundant phytocannabinoid (pCB) is ^{9}-tetrahydrocannabinol (THC), known for the psychoactive, analgesic and anti-inflammatory effects that it produces. The studies of the molecular mechanisms underlying effects produced by THC led to the discovery of the cannabinoid CB_1 and CB_2 G protein-coupled receptors, endocannabinoid (eCBs) ligands and their modulatory function in many cell types (Pertwee 1997, Felder and Glass 1998, Stella 2010, Tanasescu and Constantinescu 2010, Downer 2011, Pini, Mannaioni et al. 2012). CB_1 receptors are expressed at a high level by neural cells while CB_2 receptors are predominately expressed by hematopoietic cells. Many other protein targets and receptors are modulated by pCBs and eCBs. The extent and action by which these non-CB_1/CB_2 targets mediate some of the biological and therapeutic properties carried by *Cannabis* have been described in detail elsewhere (Howlett, Barth et al. 2002, Mackie and Stella 2006, Pertwee, Howlett et al. 2010, Zhao and Abood 2012, Abood 2013).

It is well known that regimented use of pCB may induce prolonged immunosuppressive and anti-inflammatory effects (Klein 2003, Klein 2005). Clinical studies show that heavy use of smoked *Cannabis* decreased lymphocyte proliferation and suppressed cell-mediated immunity (Gaoni 1964, Nahas, Suciu-Foca et al. 1974, Nahas, Morishima et al. 1977). Peripheral blood mononuclear cells from chronic smokers have altered basal levels of the ratio of CB_1:CB_2 cannabinoid receptors compared to controls (Nong, Newton et al. 2002). Furthermore, THC acting on T cells induces a shift toward a T_H2 phenotype in animal models of infection (Newton, Klein et al. 1994, Massi, Fuzio et al. 2000, Smith, Terminelli et al. 2000). Accordingly, long-term *Cannabis* use decreases T cell proliferation and IL-2 levels (a T_H1 cytokine), and increases IL-10 levels (a T_H2 cytokine) in human blood (Pacifici, Zuccaro et al. 2003, Pacifici, Zuccaro et al. 2007). Thus, chronic use of *Cannabis* might bias immune effector mechanisms thereby affecting autoimmune reactions such as in MS.

The phenotype of circulating monocytes is relatively 'plastic', evidenced by cell-surface protein expression profiles, migratory potentials and differential cytokine secretion in response to specific environmental cues (Murray and Wynn 2011). Accordingly, phenotypic variability in circulating monocytes will influence the phenotype of the ensuing tissue macrophages. Evidence shows that pCB are likely to modulate the phenotype of circulating monocytes. A single low-dose of THC in mice induced recruitment of bone marrow

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monocytes (myeloid-derived suppressor cells, MDSC) into blood and alter cytokine release (also using cannabidiol (CBD, the second most abundant pCB) (Hegde, Nagarkatti et al. 2011). This direct modulation of the phenotype of MDSCs induced a four-fold increase in CD11bGR1^{+} cells (Hegde, Nagarkatti et al. 2011). MDSC are known to inhibit T-cell proliferation in humans after viral illness (Simmons 2001, McCoy, Tsunoda et al. 2006, De Santo, Salio et al. 2008, Hegde, Nagarkatti et al. 2010, Mameli, Poddighe et al. 2012). Together, these studies provide a mechanistic framework indicating that regimented use of pCB might affect the phenotype of circulating monocytes.

Prevalence of medicinal *Cannabis* use by individuals with MS is estimated at 14% in Nova Scotia, 17% in Spain and 22% in the UK (Clark, Ware et al. 2004, Ware, Adams et al. 2005, Martinez-Rodriguez, Munteis et al. 2008). Given the critical role of circulating immune cells in MS and the immuno-modulatory actions of pCB on these cells, we sought to determine if *Cannabis* use differentially modulates three parameters: *ex-vivo* migration of circulating monocytes (Sexton 2012), quantity of cytokines and eCB released in serum.

Methods and Materials

Subjects and Study Design

Subjects were recruited from 2009–2011 from the MS Center at the University of Washington (UW), the MS Center at Swedish Hospital in Seattle, and the MS Society of Greater Seattle and from medical, nursing and research staff at UW with approval from the UW Human Subjects Committee. Subjects were screened by telephone for inclusion/exclusion criteria prior to enrollment. Written informed consent was obtained prior to fasting, ante-cubital venipuncture using an approved protocol. Individuals with stable MS either were or were not currently using Disease-modifying therapy (DMT) and an we controlled for an equal number in each arm as a control for potential effects on our outcome measures. In addition, all subjects were asked not to use ibuprofen on the day prior to their blood draw, as part of the protocol. Subjects were stratified based on current *Cannabis* exposure.

All subjects were 21 and 50. MS Diagnostic criteria: positive diagnosis in the last 10 years of relapsing-remitting MS (made by a neurologist, according to international guidelines); an EDSS score between 3–5; not currently in exacerbation and no exacerbation less than 30 prior to the blood draw. Exclusion criteria: BMI 19 and 27; diagnosis of diabetes; diagnosis of other chronic inflammatory or autoimmune disease; current pregnancy; previous DSM IV diagnosis; tobacco use; or currently a performance athlete. These criteria were to minimize variables influencing the eCS or immune function. Subjects were stratified and matched by Cannabis exposure: non-naïve is current *Cannabis* use at least 2–3 times per week; and naïve, is ‘never used’ or no history of use in the last three years.

Monocyte Isolation and Migration

The buffy coat was collected from centrifuged whole blood using BD Vacutainer® CPT Cell Preparation Tubes with sodium citrate (VWR Scientific, San Francisco, CA). The buffy

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coat was washed with PBS (30 ml, centrifuged at 3000 rpm × 15 min). The resulting pellet was re-suspended in buffer and monocytes isolated using an *ex-vivo* deletion using the Midi Macs™ cocktail kit (indirect cell labeling by negative selection). The cells retained on a magnetized column are positive for CD3, CD7, CD16, CD19, CD56, CD123 and CD235a; unlabeled cells passing through the magnet are $CD14^+$. Serum was flash frozen at −80C for cytokine and eCB analysis.

The modified Boyden chamber cell migration assay was previously described (Miller and Stella 2009, Sexton 2012). Briefly, filters (pore diameter = 5 μm) were coated with human fibronectin (10 μg/ml in PBS for 30 min). Isolated $CD14^+$ monocytes were fluorescently labeled with DRAQ-5 (700 nM, 10 min at 37°C in RPMI 1640 supplemented with 0.1% BSA). Cells were rinsed in RPMI (0.1% BSA) and resuspended for a final density of 10^4 cells *per* upper well (390 μl). Lower wells were loaded with media (82 μl) containing vehicle (0.1% DMSO for basal migration) or the chemoattractant. Detection was by Odyssey® Imaging system (Li-COR Biosciences, Lincoln, NE). pCB 'mix' was a set ratio of 5.2 : consisting of 1μM THC /100 nM CBN / 300nM CBD (Broseus, Anglada et al. 2010).

Cytokine Analysis

Levels of 10 human cytokines/chemokines (CCL2, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17 and TNF-α) from human plasma were determined using a custom multiplex micro-bead based immunoassay kit (Millipore, Billercia, MA, USA). Undiluted human plasma samples were assayed according to the manufacturer's protocol and analyzed using Luminex (Liquichip, Qiagen, Valencia, CA). T_H1 cytokines were: IFN-γ, IL-2, IL-10; T_H2 cytokines were: IL-4, IL-6 and IL-13.

Endocannabinoid analysis

Serum samples were analyzed by liquid chromatography mass spectrometry (LC-MS). Two milliliters of serum was collected within 30 min after centrifugation of whole blood (at room temperature). Proteins were precipitated using 2 volumes of ice-cold methanol and centrifuged. Samples were completely dried down under a stream of nitrogen and flash frozen until the time of analysis. Plasma samples were reconstituted in 1ml chloroform:methanol (2:1 containing 34.8 mg PMSF/ml) from which 200 ul aliquots were mixed with 50 ul of each of deuterated internal standards (Cayman Chemicals, USA) and mixed with 2.8 ml chloroform: methanol (2:1 containing 34.8 mg PMSF/ml). Samples were vortexed and 0.6 ml of 0.73 % w/v NaCl was added to each sample, vortexed again and then centrifuged for 10 min at 3220*g 4°C. The aqueous phase plus debris were collected and extracted again twice with 1.6 ml chloroform. The organic phases from the three extractions were pooled and evaporated under nitrogen gas. The dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml cold acetone. The mixtures were then centrifuged for 5 min at 1811*g and 4 °C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in auto sample vials for analysis.

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The LC-MS method has been previously described (Ramesh, Ross et al. 2011). Briefly, LC-MS/MS was used to quantify anandamide (AEA), 2-Arachidonyl Glycerol (2-AG) palmitoyethanolamide (PEA), oleoylethanolamide (OEA) and arachidonic acid (AA). The mobile phase consisted of (10:90) water/methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 2.1×150 cm, 3 micron (Supelco, PA). A calibration curve was constructed based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.039 to 40 pmol for AEA, from 0.0625 to 64 nmol for 2-AG, from 0.039 nm to 1.25 nm for PEA and OEA and from 1 nm to 32 nm for AA. (Deuterated compound concentrations: 2 pmole AEA −d8, 1 nmole 2-AG-d^8, 3.3 nmole PEA-d4, 3 nmole OEA-d4 and 1 nmole AA-d8).

Materials

Miltenyi LD Midi Macs® separation unit and monocyte isolation kit II (Miltenyi Biotech Inc., Auburn, CA USA). Gibco® RPMI1640 (Invitrogen, Carlsbad, CA). DRAQ-5™ (Axxora, San Diego, CA). Multiplex microbead-based immunoassay kit (Millipore, Billercia, MA, USA). Luminex instrument (Liquichip, Qiagen, Valencia, CA). AEA, 2-AG, PEA, OEA and AA (Cayman Chemical, Ann Arbor, MI USA).

Study Design

This case control, cross-sectional study was designed to take a ‘snapshot’ of potential immunological effects of chronic *Cannabis* use. To control for immunomodulating effects of DMT’s, consistent numbers of DMT-naïve (n=4) and non-naïve (n=3) MS cases were using interferon beta-1a (Avonex® or Rebif®). Subjects were not administered any *Cannabis*, but were self-accessing this botanical medicine under a law passed in Washington State in 1998 allowing for its’ medical use. Healthy controls were recreational *Cannabis* users. Here we compare outcome measures between individuals with MS to healthy subjects assessing whether *Cannabis* exposure is an effect modifier of quantitative measurements in defined immune parameters.

Statistical Analysis

Data for monocyte migration and cytokine analysis were analyzed with Prism® 4.0 software (Graphpad, San Diego, CA) using 2-way ANOVA followed by Bonferroni’s post-test. Data for eCB were analyzed with and STATA IC 11.0 (StataCorp, College Station, TX) using two-tailed, paired t-tests. Results are expressed as mean +/− SEM. Point estimates for the odds ratio and 95% confidence interval (CI) were calculated using STATA IC (StataCorp, College Station, TX).

RESULTS

Eleven healthy subjects and ten individuals with MS were enrolled (Table 1). On the day of blood draw, subjects were matched by age, BMI and cannabinoid use (i.e. naïve *versus* non-naive). Isolated monocytes were tested to evaluate whether pCBs regulate the migration of these cells (Miller and Stella 2009, Sexton 2012). *In vitro* monocyte migration was insensitive to acute treatment with a set ratio of three pCBs when cells were isolated from either healthy subjects or from individuals with MS also naïve to cannabis (Figure 1). By

contrast, acute treatment with pCBs significantly inhibited the migration of monocytes isolated from both healthy subjects non-naïve and individuals with MS non-naïve to cannabis.

There were no significant differences in serum levels of CCL2 across cohorts (Figure 2a). Serum levels of IL17 were significantly reduced in non-naïve subjects, whether cases or controls. (Figure 2b) Cases and controls with *Cannabis* exposure had reduced levels of both T_H1 and T_H2 cytokines compared to naïve subjects (Figure 2c,d : T_H1 cytokines were below the limit of detection of the assay for the non-naïve subjects).

There was a statistically significant increase in levels of AEA (p=0.04) in cases compared to controls (Figure 3a). Serum levels of 2AG (p=0.14), PEA (p=0.99) OEA (p=0.08) and AA (p=0.09), were not significantly different between healthy controls and MS cases. (Figure 3b,c,d,e)

Discussion

Current strategies for MS attempt to reduce or stop the migration of T cells towards the brain. Despite a wealth of evidence pointing to the role of the eCB system in the pathogenesis of MS, there is little data on the migratory potential of monocytes isolated from human subjects and individuals with MS. (Pertwee 2002) Since brain macrophages and microglia accumulate in MS lesions, changes in circulating monocytes are likely to influence overall disease progression. (Kouwenhoven, Teleshova et al. 2001, Shechter and Schwartz 2012) Thus, specifically targeting circulating monocytes might influence brain repair by modifying the phenotype of these cells as they become brain macrophages.

Since *Cannabis* therapy may be for symptom palliation by individuals with MS (Boven, Van Meurs et al. 2006, Mikita, Dubourdieu-Cassagno et al. 2011), we addressed the question: “Does chronic Cannabis use induce a phenotypic change in circulating monocytes?” Monocyte cell migration was significantly inhibited by a mixture of pCBs in non-naïve subjects irrespective of whether these individuals are cases or controls. We previously reported that subjects naïve to Cannabis have an increased migratory response to CCL2. (Sexton 2012) CCL2 (monocyte chemo-attractant protein 1, MCP1) provides a strong chemotactic cue for monocytes, guiding these cells to inflammatory lesion sites throughout the body, including the brain. (Conductier, Blondeau et al. 2010) Our results suggest that while cannabis use affects the cell migration properties of circulating monocytes, it does not affect the serum levels of CCL2, a major chemotaxic cue for these cells (Fig 2A).

Our results suggest that chronic CB use may lead to global reduction of cytokines (Fig 2B,C) in both healthy controls and individuals with MS. Previous clinical reports have measured either no change in levels of IFNγ, IL-10 or IL-12 with THC administration, or saw promotion of pro-inflammatory cytokines with THC or nabiximols (Sativex**®,** G W Pharmaceuticals) administration (Killestein, Hoogervorst et al. 2003, Katona, Kaminski et al. 2005). Differences between this study and other trials include the fact that these were prospective studies where a protocol included known and standardized doses of cannabinoids, administered regularly. Here we had an observational approach to probe

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patients were self-titrating at an unknown dose and rate. Additionally, differences in administration, such as oral vs. inhaled, and administration of products having various cannabinoid ratios may yield different results. A balance in T_H1 and T_H2 cytokines is important in controlling the progression of both EAE and MS pathogenesis. Specifically, EAE and MS are T_H1-driven and there is compelling data that a T_H2 shift would be beneficial for achieving a desired anti-inflammatory response by the immune system. (Kennedy and Karpus 1999, Oreja-Guevara, Ramos-Cejudo et al. 2012). However, our results extend previous studies showing that *Cannabis* use may globally suppress cytokine production. IL17-producing T-cells (T_H17) are important players in a number of autoimmune processes and IL17 is considered to be a key player in the cytokine milieu in patients with MS, a putative biomarker for disease activity, and predictor of drug response in MS (Axtell, de Jong et al. 2010, Hecker, Paap et al. 2011, Li, Wang et al. 2011, Chen, Wang et al. 2012) (Frisullo, Nociti et al. 2008, Kallaur, Oliveira et al. 2013, Kozela, Juknat et al. 2013). Notable in this study is the global suppression of IL17 in subjects who are exposed to Cannabis, as cannabinoids were shown elsewhere to decrease the Th17-associated autoimmune phenotype (Kozela, Juknat et al. 2013). Our cytokine results suggest general immunosuppression instead of immune biasing, an environment likely to regulate the phenotype of circulating monocytes. These innate effector cells might be targets for immunotherapeutic strategies that guide the innate immune reaction. (Downer 2011) Further studies are required to extend our results.

eCB levels in human serum change in disease states such as obesity, Alzheimer's disease, schizophrenia, depression and liver cirrhosis. (De Marchi, De Petrocellis et al. 2003, Hill, Miller et al. 2009, Koppel, Bradshaw et al. 2009, Caraceni, Viola et al. 2010, Matias, Gatta-Cherifi et al. 2012) Little is known about eCBs in MS and previously published data on serum eCBs levels in individuals with MS were inconclusive. (Jean-Gilles, Feng et al. 2009) Our results suggest that AEA levels may be increased in individuals with MS. A change in eCB signaling is not apparently sufficient to affect disease process in MS, but may participate in MS progression. (Pryce 2012) Specifically, since AEA has been suggested to be neuro-protective and have an anti-inflammatory profile in CNS parenchyma (Correa, Mestre et al. 2009) (Eljaschewitsch, Witting et al. 2006), eCB changes likely influence both immune response and resulting cell damage in brain tissue.

Drugs that boost eCB signaling system are thought to induce both palliative and neuro-protective responses by tempering the immune response, promoting oligodendrocyte survival, reducing demyelinated lesions and attenuating neuronal loss. These results were obtained by studying the EAE mouse model, a T cell-mediated autoimmune disease often used to study MS. (Jackson, Pryce et al. 2005, Pryce and Baker 2007, Rog, Nurmikko et al. 2007, Kuerten 2011, Notcutt, Langford et al. 2012) With regard to individuals with MS, an oral-mucosal spray that delivers a standardized 1:1 ratio of THC:CBD has been shown to be effective for treating refractory spasticity in MS with few side-effects and low potential for addiction. (Wade, Collin et al. 2010, Sastre-Garriga, Vila et al. 2011)

The odds ratio (OR) that *Cannabis* use is more likely in cases *versus* controls is 0.36 (95% CI: 0.40 to 2.89, Fisher's exact p=0.39). The interpretation of the OR is that the odds for exposure to Cannabis are 64% (non-significant) lower in MS cases than in controls in our

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study population. We also acknowledge the small sample size may not be adequate for firm conclusions as our original enrollment powered for eCB measurement was 18 subjects for each cohort.

In summary, our results suggest a model wherein pCB (in a whole plant context), are affecting monocyte phenotype, cytokine production and eCB signaling molecules. (Palazuelos, Davoust et al. 2008) The combined effects of eCB modulation are: a) skewing the phenotype of select immune cells toward a potentially anti-inflammatory phenotype; and b) contributing to global immune suppression by a decrease in cytokine production. Our results provide the foundation for conducting prospective human trials to investigate the effects of supplementing individuals with MS with regimented intake of pCB and measuring the long-term effects on inflammatory response and disease outcome in MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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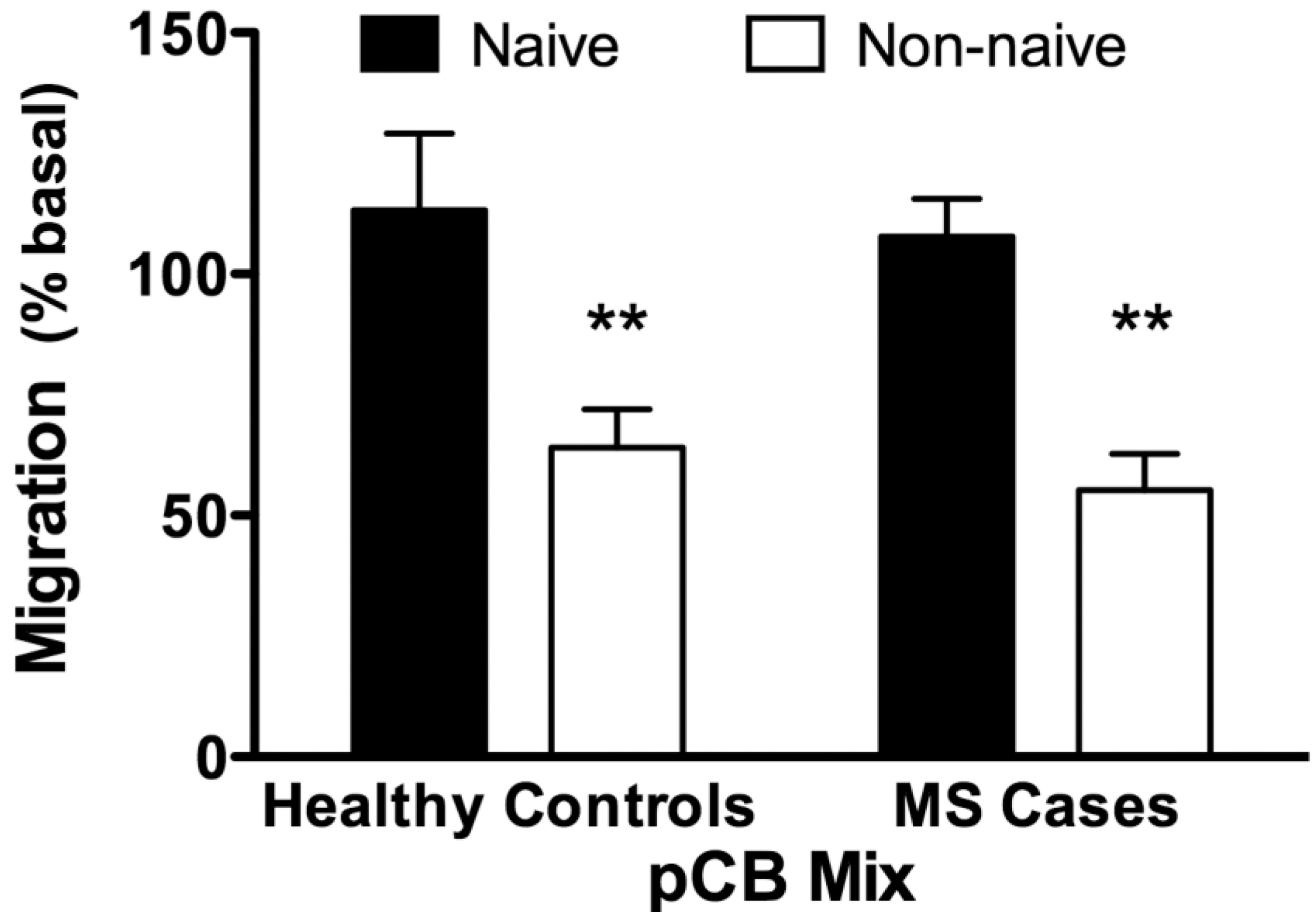

Fig. 1.

Freshly isolated monocytes respond differentially to pCB stimulation. Cell migration of freshly isolated human monocytes was significantly reduced by addition of phytocannabinoids (pCB Mix) to the lower wells of the migration chamber. There was a 50% reduction in monocyte migration in Cannabis users, both in healthy controls and MS cases. (2-way ANOVA followed by Bonferroni's post-test).

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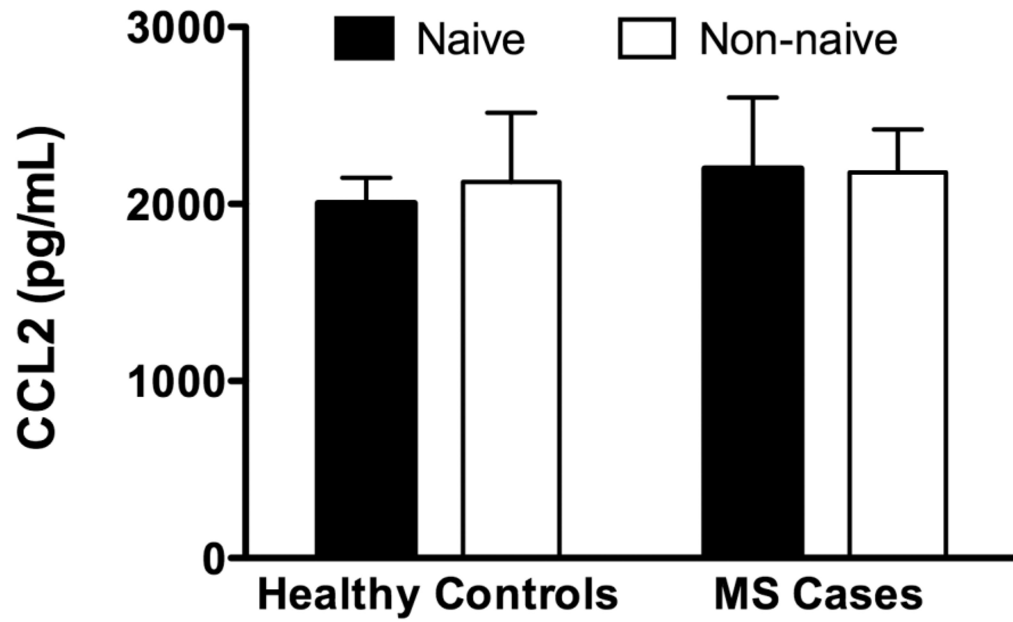

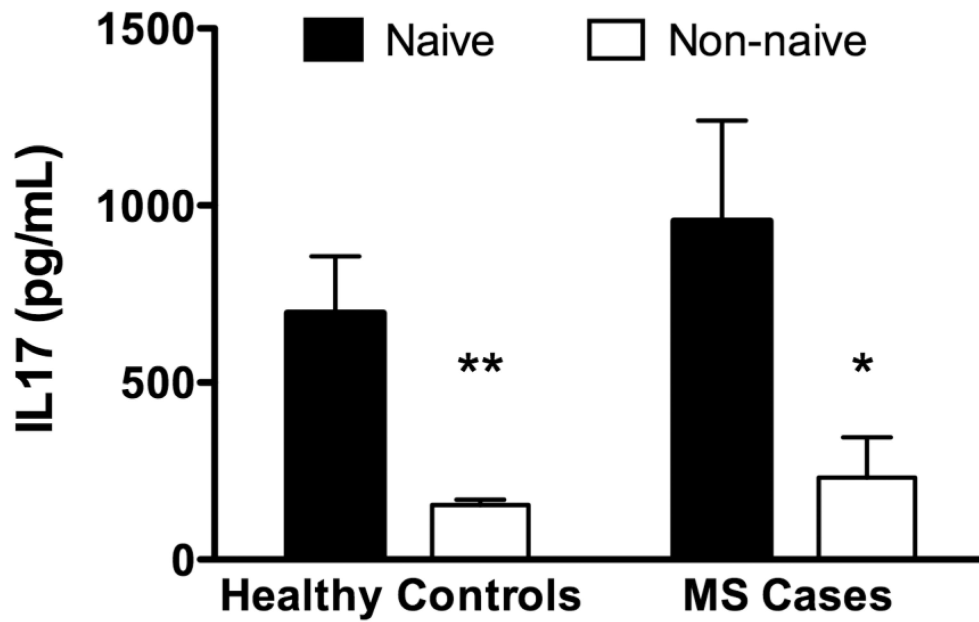

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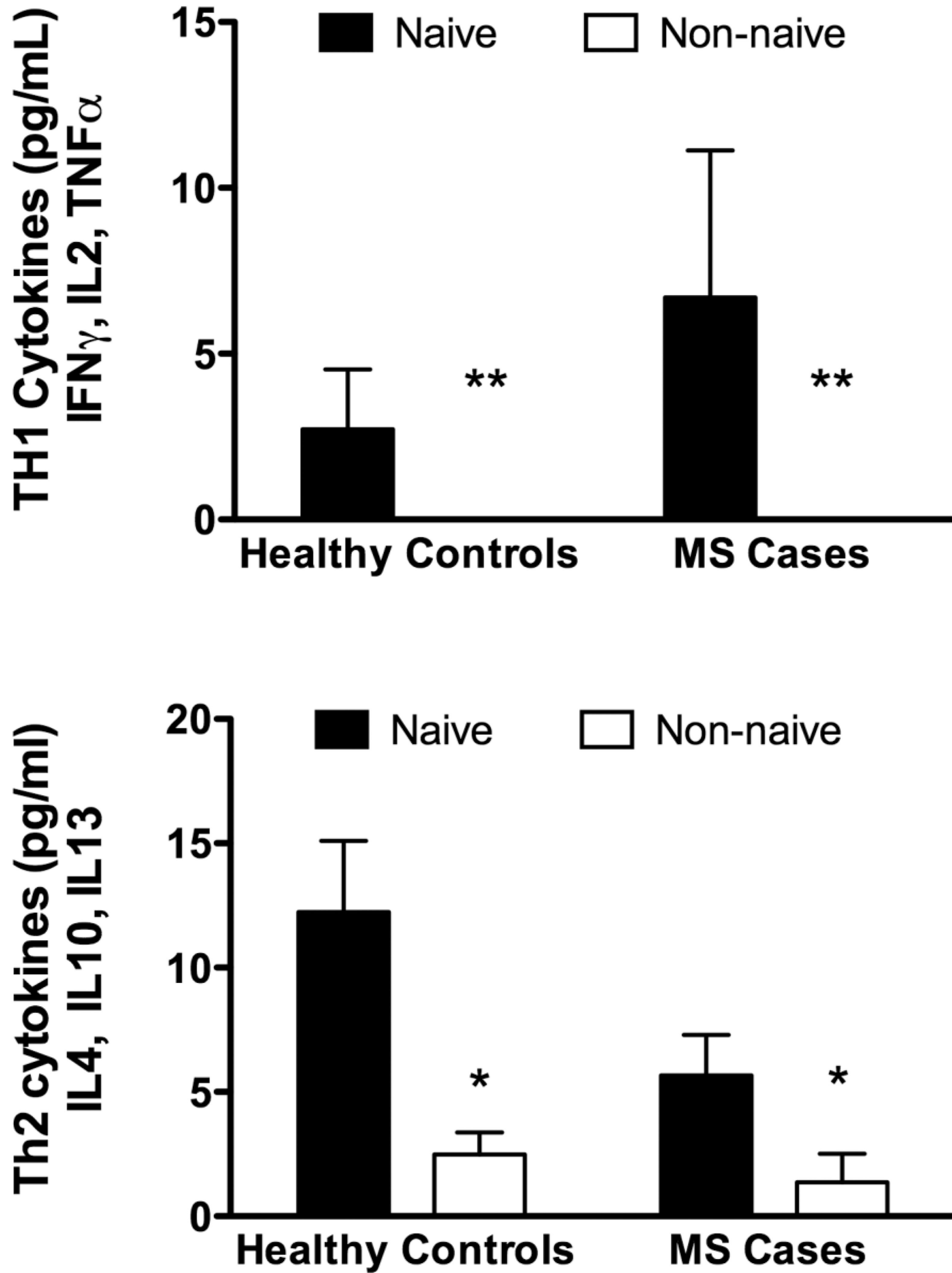

Fig. 2.

Serum Cytokine Levels: Using a bead array assay we measured CCL2, IL17 and panels of Th_1 and Th_2 cytokines. a) There was no significant difference in the level of CCL2 across cohorts. b) Levels of IL17 were significantly reduced in both cohorts who were current Cannabis users (non-naïve), compared to naïve subjects c,d) Levels of both Th_1 and Th_2 cytokines were significantly suppressed (undetectable in some cases) in current *Cannabis* users compared to naïve subjects (2-way ANOVA followed by Bonferroni's post-test).

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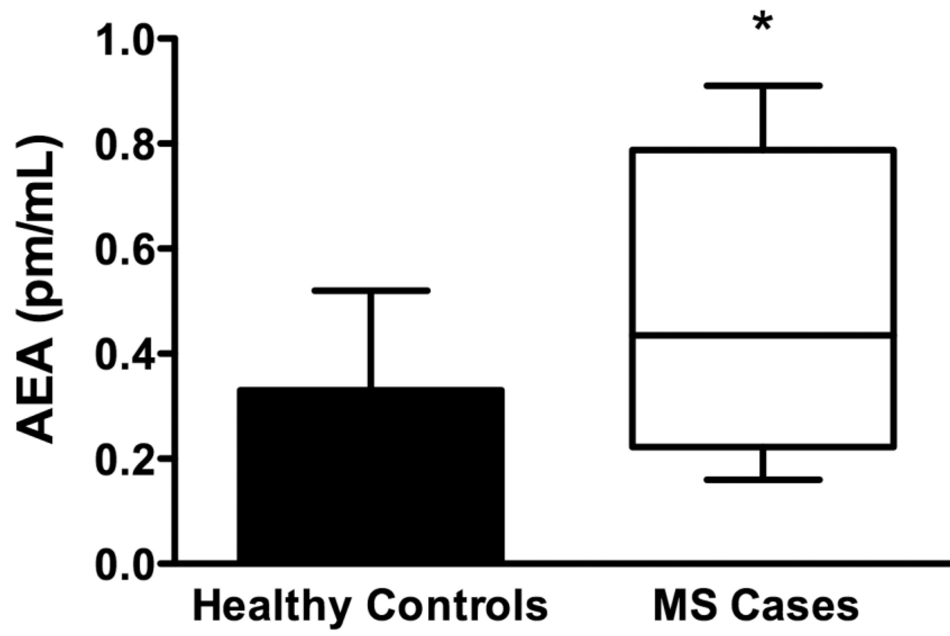

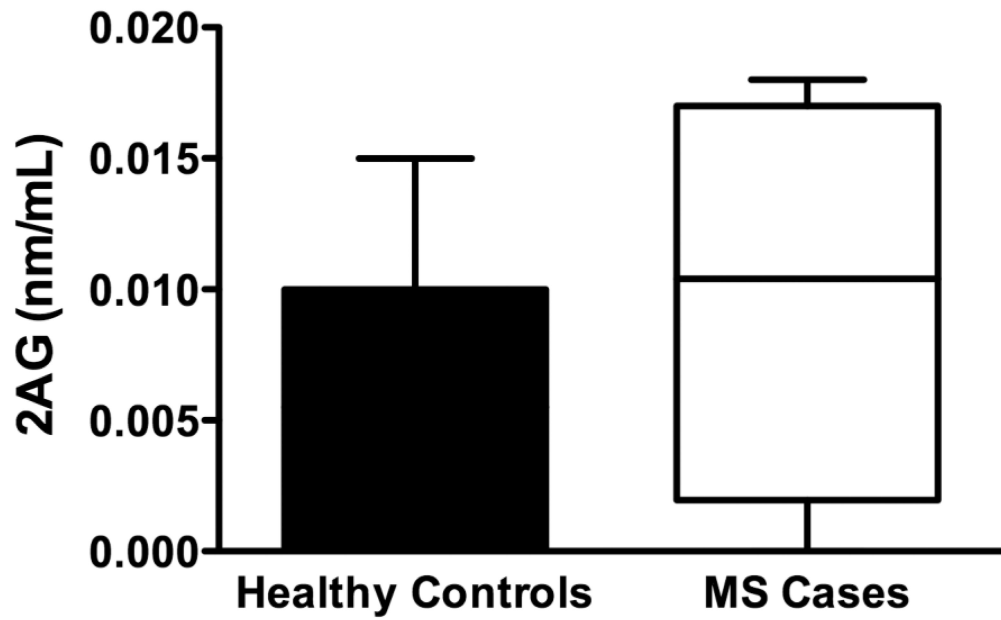

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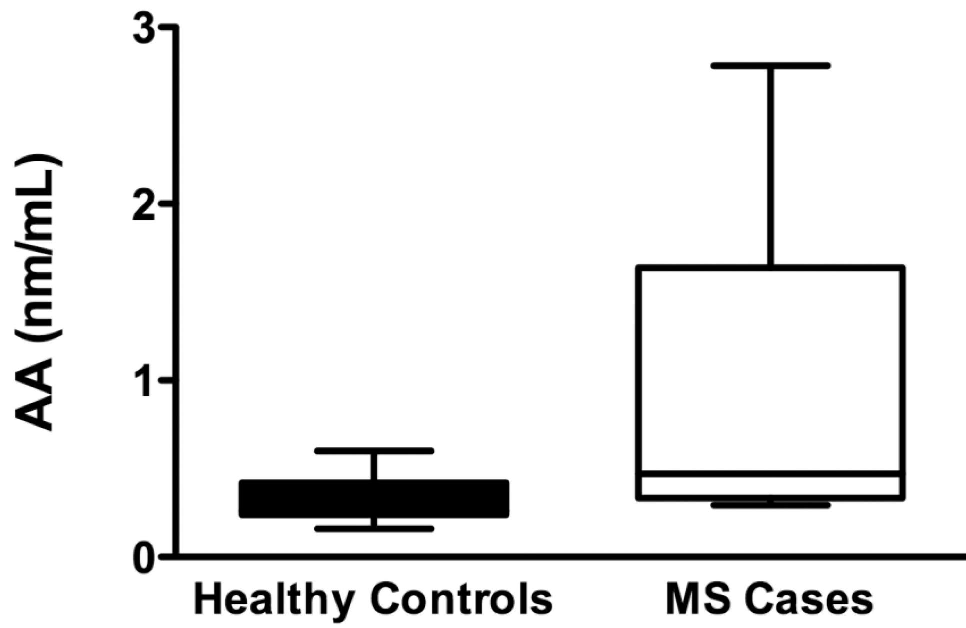

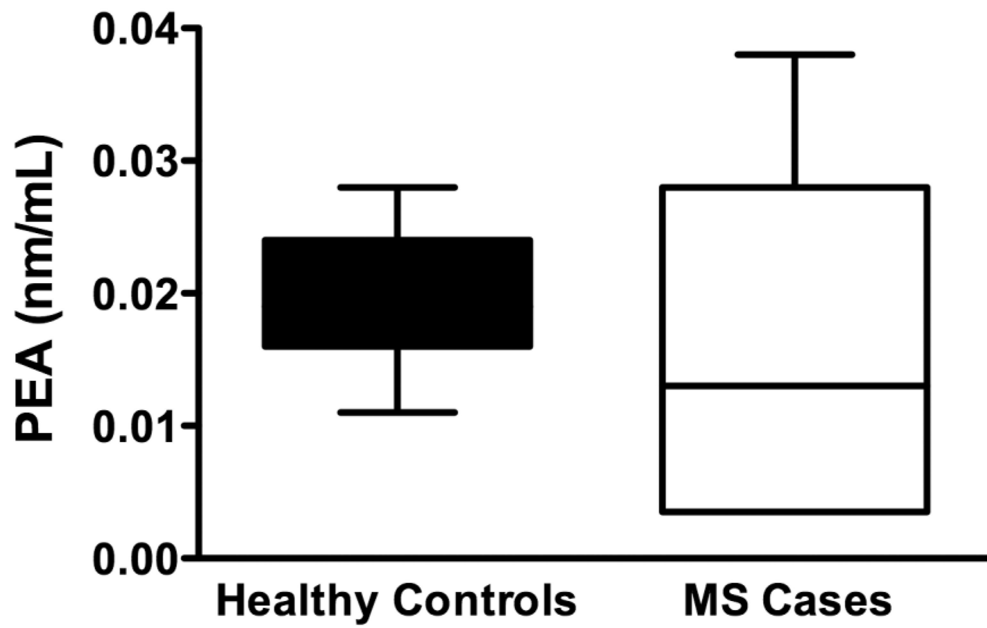

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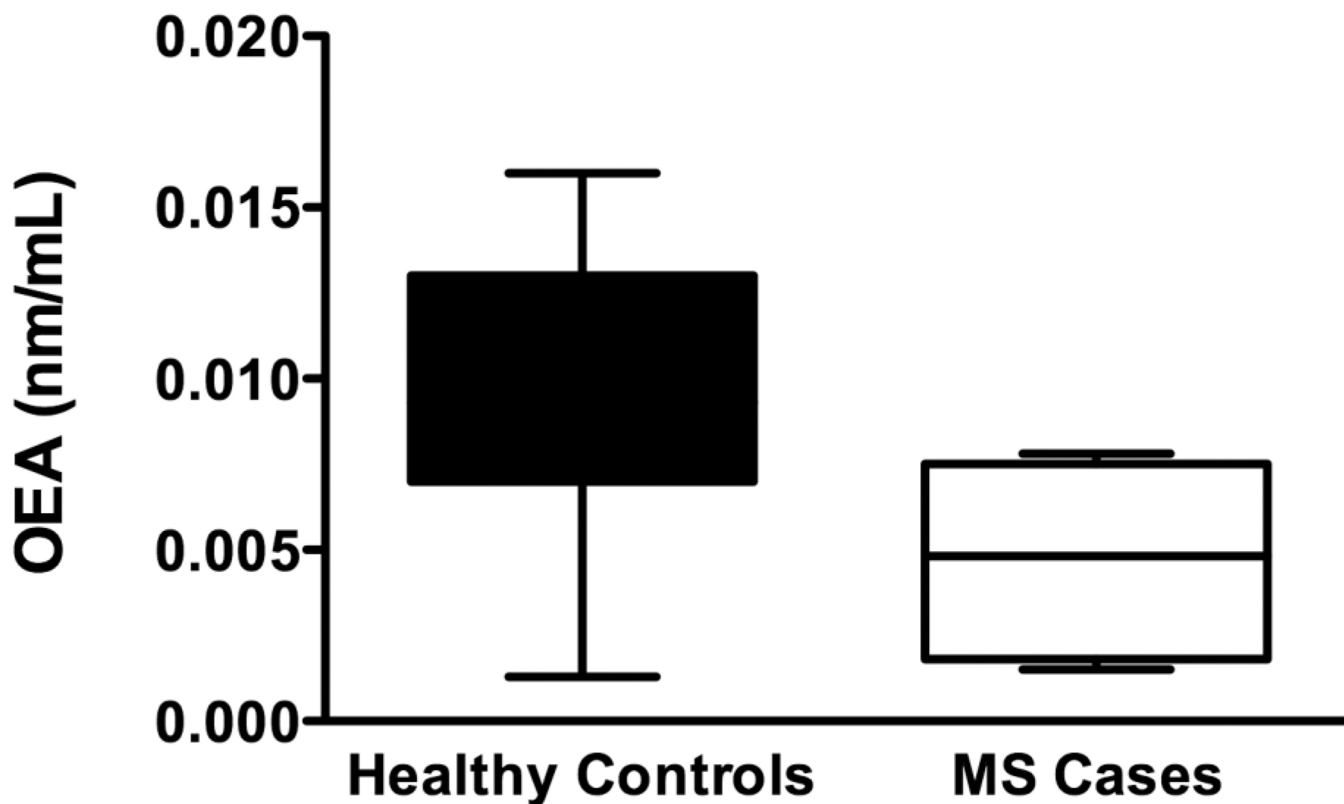

Fig. 3.
Serum levels of endocannabinoids (eCB): Using HPLC/MS/MS we measured serum levels of 5 eCBs. a) Serum AEA levels were elevated in patients with MS compared to controls. b–e) We found no significant differences in the levels of 2AG, AA, PEA or, OEA between healthy controls and MS cases. However there was a trend for elevated 2AG and AA in patients with MS compared to controls. These data are not presented as stratified for *Cannabis* exposure as there were no significant differences in that regard (two-tailed, paired t-tests).

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Table 1

Demographic and descriptive data of subjects

In patients with MS, two non-naïve subjects were on no disease-modifying therapy (DMT) Of the naïve, subjects, 3 were on DMTs (Rebif, Avonex.)

Subject	**Healthy Controls**	**MS Cases**
Male Female	5 6	3 7
Total	11	10
Average age (+/– s.d.)	37.6 (+/– 7.1)	32.8 (+/– 3.4)
Cannabis Non-naïve	4	5
Cannabis Naïve	7	5
BMI	23.8 (+/– 2.4)	23.6 (+/– 2.25)