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Clinical Investigation of French Maritime Pine Bark Extract on Attention-Deficit Hyperactivity Disorder as compared to Methylphenidate and Placebo: Part 2: Oxidative Stress and Immunological Modulation

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ABSTRACT

Objectives: To evaluate the effect of French Maritime Pine Bark Extract (PBE; Pycnogenol®) on immune, oxidative stress and neurochemical biomarkers in paediatric Attention-Deficit Hyperactivity Disorder (ADHD) as compared to methylphenidate (MPH) and placebo.

Results: Paediatric ADHD patients (n = 88, 70 % male, mean age 10.1 years) were randomised (placebo (n = 30), PBE (n = 32) and MPH (n = 26)) receiving 20 mg/day if < 30 kg or 40 mg/day if ≥ 30 kg PBE, or 20 mg/day if < 30 kg or 30 mg/day if ≥ 30 kg MPH for 10 weeks. In the oxidative stress pathway, catalase (CAT) activity was nominally significant different in the PBE group with a p-value of 0.025 whereas the immunity biomarkers IgA and IgG_2 were nominally significant different after MPH treatment with a p-value of 0.028 and 0.017

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AAS, Atomic Absorption Spectroscopy; ADHD, Attention-Deficit Hyperactivity Disorder; ANOVA, Analysis Of Variance; ApoJ, Apolipoprotein J; CAT, Catalase; CoQ10, Co-enzyme Q10; Ct, Cycles to Treshold; DMSO, Dimethylsulfoxide; DSM, Diagnostic and Statistical Manual of Mental Disorders; ELISA, Enzyme-Linked ImmunoSorbent Assay; FFQ, Food Frequency Questionnaire; GPX, Glutathione Peroxidase; GSH, reduced glutathione; HPLC, High Pressure Liquid Chromatography; IFN, Interferon; IL, Interleukin; LMM, Linear Mixed Model; MDA, Malondialdehyde; MAO, Monoamine oxidase; MPH, Methylphenidate; Nfr2, Nuclear factor erythroid 2 (NFE-2)-related factor 2; PBMC, Peripheral Blood Mononuclear Cells; PBE, French Maritime Pine Bark Extract; RNA, Ribonucleic acid; RT-qPCR, Real-Time quantitative Polymerase Chain Reaction; SOD, Superoxide dismutase; TNF, Tumour Necrosis Factor; UZ Ghent, University Hospital Ghent; USP, United States Pharmacopeia; UZA, University Hospital Antwerp; XO, Xanthine oxidase; ZNA, Hospital Network Antwerp.

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respectively, compared to baseline. Serum Neuropeptide Y (NPY) levels and weight were significantly lower after 10-weeks MPH.
Conclusions: Loss of appetite and weight loss was observed for MPH, whereas no differences in NPY concentrations and a significant weight gain, which is to be an expected physiological process in this age group, was noticed for PBE. Firm evidence that PBE increases antioxidant levels, reduces oxidative damage and improves immune status in general as compared to placebo or MPH could not be obtained.

1. Introduction

Attention-Deficit Hyperactivity Disorder (ADHD) is the most common paediatric neurocognitive behavioral disorder (Biederman & Faraone, 2005; Luo et al., 2019; Pelham et al., 2007; Polanczyk et al., 2014). Despite the worldwide use of psychostimulants such as methylphenidate (MPH) in the treatment of ADHD, concerns about side effects, long-term use and efficacy remain. Alternative therapeutic options are therefore urgently needed (Antshel et al., 2011; Schachter et al., 2001; Steer, 2005; Storebø et al., 2015; Yang & Li, 2019).

ADHD is a complex and multifactorial disorder, influenced by genetic, environmental, biochemical and psychological factors. Still, its exact pathophysiology remains unclear. Next to catecholaminergic dysfunction, ADHD is also associated with immune and oxidant-antioxidant imbalances (Ceylan et al., 2010, 2012; Güngör et al., 2013; Karim et al., 2011; Kawatani et al., 2011; Minter et al., 2001; Pelsser et al., 2009; Ross et al., 2013; Schmitt et al., 2010; Tsai, 2006; Verlaet et al., 2018; Verlaet et al., 2019). Indeed, several studies demonstrate increased levels of oxidative damage markers like plasma malondialdehyde (MDA) and decreased activity of antioxidant enzymes (Ceylan et al., 2010; Karim et al., 2011; Kawatani et al., 2011; Ross et al., 2013). Moreover, ADHD has comorbidity with T_H1- and T_H2-mediated disorders and increased levels of adenosine deaminase (a marker of cellular immunity) have been reported (Ceylan et al., 2012; Güngör et al., 2013; Minter et al., 2001; Pelsser et al., 2009; Schmitt et al., 2010; Tsai, 2006). These observations indicate the involvement of oxidative changes and cellular immunity, which may contribute to ADHD via neuronal damage and abnormal neurotransmitter regulation and offer potential for specific supplementation in ADHD therapy (Bulut et al., 2007; Ceylan et al., 2010; Kerschensteiner et al., 2009; Verlaet et al., 2014; Verlaet et al., 2018; Verlaet et al., 2019). The first line ADHD therapeutic MPH is in itself also associated with an increased oxidative stress, potentially deteriorating the existing imbalance even more, although conflicting results have been reported (Andreazza et al., 2007; Comim et al., 2014; Corona, 2020; Martins et al., 2006).

Additionally, loss of appetite is among the most common reported side effects for stimulant medication like MPH (Schachter et al., 2001). Neuropeptide Y, a potent orexigenic hormone released from the posterior pituitary and important in appetite regulation, was therefore investigated as well (Lu et al., 2015).

French Maritime Pine Bark Extract (PBE; *Pinus Pinaster,* Pycnogenol®, Horphag Research), a patented, proprietary polyphenol-rich herbal extract from French maritime pine (*Pinus pinaster* Aiton) bark, standardised to contain 70 ± 5 % procyanidins, has antioxidant and immune modulating properties and was therefore selected as a food supplement for this trial (D'Andrea, 2010; Dvoráková et al., 2006; Hsu et al., 2021; Robertson et al., 2020; Weyns et al., 2022; Wilson et al., 2010). Due to its strong antioxidative and anti-inflammatory capacity, PBE may be beneficial in the prevention and treatment of various chronic diseases including asthma and cardiovascular diseases (Nattagh-Eshtivani et al., 2022). Standardisation and characterisation of herbal extracts is critical when these nutraceuticals are administered in a clinical context. PBE used for this study complied with the USP requirement regarding procyanidin content (United States Pharmacopeial Convention, 2014). Quality control of herbal supplements is essential to conduct reliable research and to be able to compare results of various studies.

Therapeutic potential in ADHD was already suggested before but a more extensive analysis of the underlying mechanisms is needed. This double-blind, randomised controlled trial aims to make a contribution to this by investigating the antioxidant and immune modulating effects of PBE in ADHD therapy through analysis of an extensive series of biomarkers, as compared to MPH and placebo treatment (Dvoráková et al., 2006; Weyns et al., 2022). Results of this study, therefore, will increase insight into ADHD aetiology and (dietary) treatment options, which is highly desired by medical staff, parents and patients. It was hypothesized that, as compared to placebo and MPH, PBE increases antioxidant levels, reduces oxidative damage and improves immune status.

2. Materials and methods

The trial's methods are described briefly below. The study protocol is published in detail in Verlaet et al. (2017).

2.1. Ethics and registration

Ethical approval was acquired in the University Hospitals of Antwerp (UZA) (EC 15/35/365) and Ghent (UZ Ghent) (2016/0969) and Hospital Network Antwerp (ZNA) (EC approval 4656). The trial was registered at Clinicaltrials.gov (NCT02700685, registered 18 January 2016) and EudraCT (2016-000215-32, registered 4 October 2016) (Verlaet et al., 2017).

2.2. Quality control

The quality of PBE used for this clinical trial was assessed using the spectrophotometric method as described in the United States Pharmacopeia (USP) (United States Pharmacopeial Convention, 2014). Complementary analysis of phenolic compounds was achieved by HPLC with diode array detection (DAD) (see Supplementary information). PBE met all requirements as stated in the USP with an average procyanidin content of 78.300 % ± 3.000 %, and contents of selected polyphenols being 1.276 % ± 0.031 % catechin, 1.375 % ± 0.012 % taxifolin, 0.287 % ± 0.005 % caffeic acid and 0.274 % ± 0.003 % ferulic acid (Table 4 Supplementary Information).

2.3. Design of the study

This was a phase III, randomised, double-blind, placebo and active product controlled, multicentre clinical trial with three parallel treatment arms (PBE, MPH and placebo). A total of 88 paediatric participants with ADHD, both diagnosed *de novo* and formerly treated, were recruited at UZA, and UZ Ghent and ZNA, as well as via general practitioners, paediatricians, speech therapists and physiotherapists. Patients were included between September 2017 and November 2020. Diagnosis was confirmed by a child neurologist or psychiatrist and written consent of the participants' legal representative was obtained before inclusion. An additional information brochure with information on specific details of the trial was given to the primary caregiver.

Following screening by medical staff at the outpatient departments of Child Neurology UZA and UZ Ghent and the University Child and Adolescent Psychiatry department of Hospital Network Antwerp (inclusion and exclusion criteria were determined by the medical staff in consultation with the NatuRA research group and can be found in

Table 1 Supplementary information) and baseline assessments, patients were randomised to one of the treatments for 10 weeks and received a dose based on their body weight: (Verlaet et al., 2017).

- MPH (Medikinet® Retard, Medice GmbH, MPH modified release): 20 mg/day if < 30 kg, 30 mg/day if ≥ 30 kg. Treatment started with 10 mg/day, increasing 10 mg per week.
- PBE (Pycnogenol®, Horphag): 20 mg/day if < 30 kg, 40 mg/day if ≥ 30 kg (20 mg/day during the first two weeks).
- Placebo: excipients (microcrystalline cellulose and magnesium stearate) only.

All treatments (1 or 2 oral capsules at breakfast) had an identical shape and appearance, and all were encapsulated, packaged and labelled by the same company (Qualiphar NV, Belgium).

2.4. Dietary habits

Baseline daily consumption of fruit and/or vegetables (and thus polyphenol intake) was assessed using a Food Frequency Questionnaire (FFQ) (Weyns et al., 2022).

2.5. Sample collection

Biological samples were collected at baseline and after 10 weeks. Blood was collected in EDTA and serum tubes (BD, USA). Per patient, 1 EDTA tube (12 mL) was placed on ice immediately while another EDTA tube was kept at room temperature. After centrifugation (2000*g*, 4 °C, 12 min) of cold EDTA blood, erythrocytes in phosphate buffer and plasma were aliquoted and stored at −80 °C until analysis. After centrifugation (200*g*, room temperature, 20 min) of the room temperature EDTA tubes, plasma was frozen at −80 °C, while peripheral blood mononuclear cells (PBMCs) were isolated and stored in a 20 % DMSO solution for long-term storage in liquid nitrogen (−196 °C) (PBMC isolation protocol can be found in Supplementary Information). After 30 min at room temperature to clot, serum samples were centrifugated (2000*g*, room temperature, 12 min) and stored at −80 °C until analysis. Urine was collected in sterile urine containers, placed on ice immediately and stored at −80 °C until analysis.

2.6. Biological analyses

To investigate the effect of PBE as compared to MPH and placebo with regard to antioxidant levels and oxidative damage (the oxidative stress pathway) and the immune status, various biomarkers were analysed at the start and the end of the study. Also the neurochemical parameter NPY was assessed. Researchers performing biological and statistical analyses were blind for the treatment groups.

2.6.1. Oxidative stress pathway: Antioxidants and gene expression profiles of antioxidant enzymes

After hemolysis and purification, erythrocyte GSH level was analysed by a validated HPLC method (Magielse et al., 2014; Pastore et al., 2003). Plasma levels of the lipid soluble antioxidants α- and γ-tocopherol, β-carotene, retinol and coQ10 were analysed by a validated HPLC method after extraction with hexane (Hermans et al., 2005). Both were analysed on an Agilent 1260 HPLC system (Agilent Technologies, Belgium) with an ESA-5600A CoulArray 8-channel electrochemical detector (ESA, Thermo Fisher Scientific, USA). Plasma CAT, SOD and GPX activities were analysed using the General Catalase Assay Kit (MBS8243260), the Glutathione Peroxidase Assay Kit (MBS841725) and the Superoxide Dismutase Colorimetric Assay Kit (WST-1 method, E-BC-K020-M), resp. for CAT and GPX activities from MyBioSource and for SOD activities from Elabscience, USA.

Gene expression profiles from PBMCs were analysed and quantified by real-time PCR (RT-qPCR) focusing on specific genes involved in pathways counteracting oxidative stress (GPX, CAT, SOD, XO) and the stress-related protein ApoJ. The QIAamp RNA Blood Mini kit (52304, Qiagen, Germany) was used to extract total RNA from PBMCs according to the manufacturer's protocol. A Qubit RNA Broad-Range Assay Kit (10174653, Thermo Fischer Scientific, USA), in combination with a Qubit Fluorometer, was used for the quantification of the isolated RNA. Total RNA (1 μg) extracted from each sample was converted into cDNA using SuperScript® II Reverse Transcriptase kit (M1705, Promega Benelux) according to the manufacturer's protocol.

Next, qPCR analyses were carried out using the GoTaq qPCR master mix (A6001, Promega Benelux) according to the manufacturer's instructions (See Supplementary Information). Differential expression of each gene was done by comparing the normalised Ct values (ΔCt) of all the biological replicates to the average ΔCt of all baseline values from each gene of interest. By comparing the normalised expressions it is possible to calculate the fold change of the expression of the miRNA (−ΔΔCt) of each gene of interest (Goni et al., 2009).

Serum zinc level was analysed by Atomic Absorption Spectroscopy (AAS; Flame AAS, Perkin-Elmer, Analyst 400) (Whitehouse et al., 1982).

2.6.2. Oxidative stress pathway: Oxidative damage

Oxidatively damaged fatty acids can degrade to reactive aldehydes like MDA. Plasma MDA was determined by competitive enzyme-linked immunosorbent assay (ELISA) (Human Malondialdehyde ELISA kit, MyBioSource, USA). Urinary 8-OHdG, indicating oxidative DNA damage, was analysed by a competitive ELISA kit (NWK-8OHDG01, NWLSS^{TM} Urinary 8OHdG ELISA, Northwest Life Science Specialties, USA) according to the manufacturer's protocol (Aruoma, 1998; Wu et al., 2004; Yano et al., 2009). To express urinary 8-OHdG concentration as ng/mg creatinine, urinary creatinine levels were analysed by the Creatinine Microplate Assay (CR01, Oxford Biomedical Research, USA) according to the manufacturer's protocol.

2.6.3. Immune status

Plasma cytokine levels (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ) were analysed by the MSD® V-PLEX Viral Panel 3 Human Kit according to the manufacturer's protocol (K15347D-1, Meso Scale Discovery, USA). Plasma antibody (IgA, $IgG_{1\text{-}4}$, IgE) levels were determined by the General Medical Laboratory (AML) in Antwerp.

2.6.4. The orexigenic peptide NPY and weight assessment

Serum NPY levels were measured using a Human NPY ELISA kit (EZHNPY-25K, Merck Millipore, USA).

2.7. Statistics

SPSS 27.0 (IBM) and R version 4.1.1 (R core team) were used for statistical analyses (R Foundation for Statistical Computing, 2017). Data were checked for outliers and a normal distribution (Shapiro-Wilk test and QQ-plot) and presented as mean ± standard deviation (SD). The three groups were compared regarding baseline characteristics by one-way ANOVA, Chi-square test or Cochran-Armitage trend test. For each biomarker of interest, the effect of treatment was investigated using linear mixed models (LMM) on the follow-up and baseline outcomes. Only for IL-1β and IL-4 a Tobit regression model was used due to the presence of many observations below the detection limit (Twisk & Rijmen, 2009). The biomarker was entered as dependent variable. Time, treatment and their interaction were entered as fixed effects. Sex, the processing time of the biological samples and time until analyses were entered as co-variates. Participant ID was entered as a random intercept to account for the dependence between observations from the same individual. The interaction between time and treatment and the corresponding p-values were used to test for a different effect between the treatment arms. In case of a significant fixed effect, post-hoc analyses were performed to check for pairwise differences in effect between the treatments. Moreover, paired-samples t-tests were conducted to

specifically evaluate the impact of MPH on the different biomarkers and to assess the effect of an active treatment on NPY and weight at baseline and after 10 weeks. Since multiple hypotheses were tested, thereby increasing the possibility of a type 1 error, a multiple testing correction needs to be carried out. The Bonferroni correction would be too strict here, since this correction assumes independent hypothesis tests, which is not the case when testing biomarkers within the same pathway. Therefore, we evaluated the significance of the p-values within each of the two pathways using false discovery rate (FDR) analysis (Hochberg, 1995). This analysis compares the observed distribution of the p-values to the uniform distribution between 0 and 1, which is expected in case all null hypotheses are true – that is, if none of the biomarkers differ between the three treatments. A clear difference between observed and expected p-value distribution, with an enrichment of low p-values, suggests that at least some of the significant p-values represent genuine associations. To further quantify the enrichment in low p-values in both pathways, we calculated q-values as implemented in the fdrtool package (Bernd Klaus & Strimmer Maintainer Korbinian Strimmer, 2021). In brief, observed p-values are sorted by significance. The q-value then indicates the fraction of false associations in case the p-value is declared significant. For the neurochemical parameter NPY, however, a Bonferroni correction was applied since NPY is an individual biomarker not involved in another pathway.

3. Results

3.1. Basic characteristics

Eighty-eight ADHD patients (70 % male, 89 % Caucasian, mean age 10.1 years) were randomised to placebo (n = 30), PBE (n = 32) or MPH (n = 26) treatment; 76 finished their 10-week study period (Weyns et al., 2022). The 10-week biological sample could not be obtained from the 12 dropouts (14 %; n = 5 placebo, n = 5 PBE, n = 2 MPH) (Fig. 1). Due to lack of biological samples (e.g. no serum or urine at the moment of the study visit) needed for each specific analysis, not every biomarker was analysed for each participant. . No significant differences were found between the treatment groups regarding the demographic variables age, height and weight (one-way ANOVA, Table 1). Also, no significant differences in mean baseline concentrations for each biomarker were found between the groups (one-way ANOVA, Table 1). Sex ratio, compliance (an intake of > 90 % ingestion as scheduled, determined based on accountability of the treatment medication and self-reported adherence), general dietary habits and parents' highest educational achievement as proxy for socioeconomic status (data not shown) was not different between treatment arms (Chi-Square test, Table 1). The proportion of dropouts was not significantly different between groups (X^2 (1, 0.7068) = 0.401 > 0.05, Chi-Square test). Also dietary habits did not change significantly within treatment groups during the 10-week study period (Cochran-Armitage trend test, data not shown).

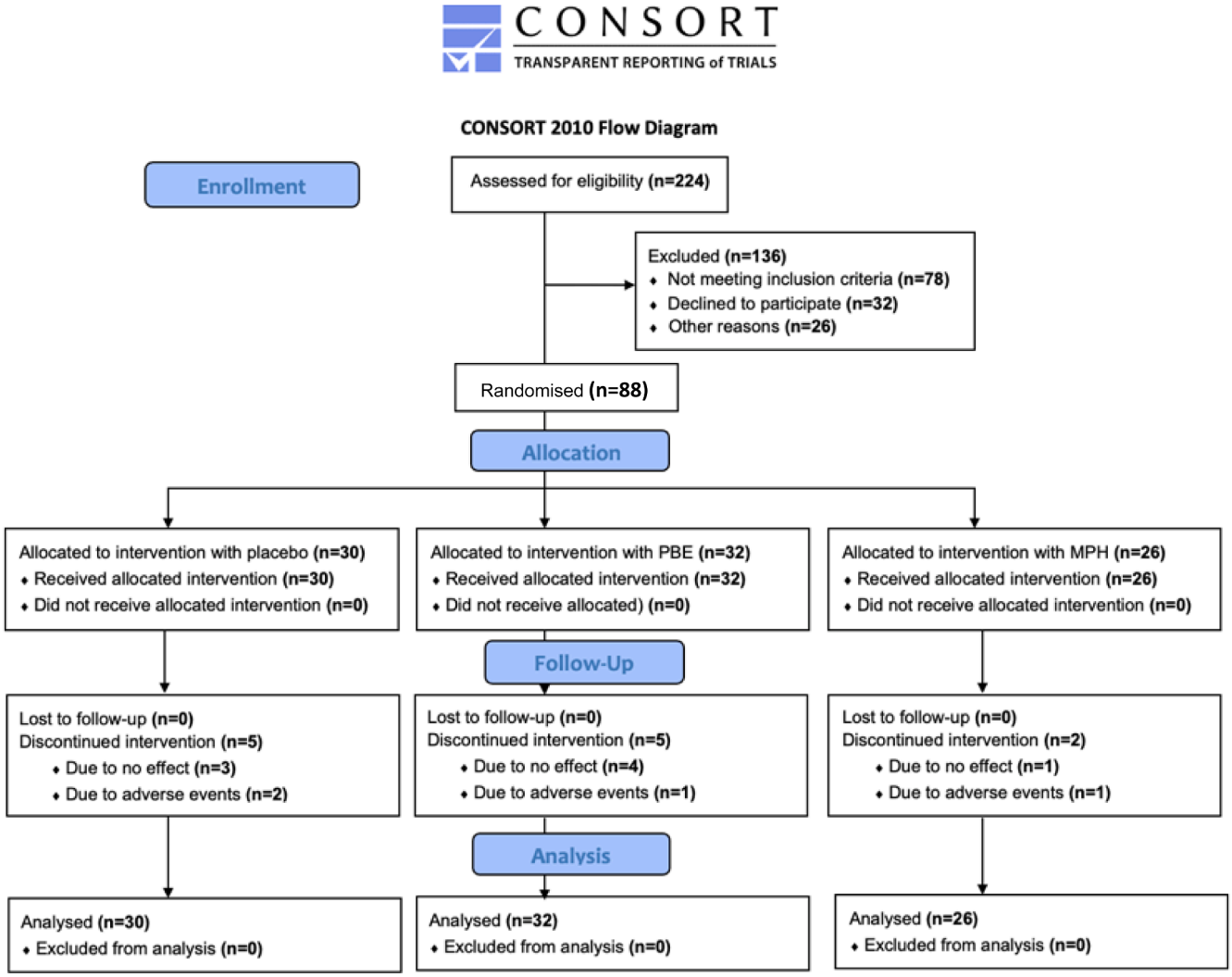

Fig. 1. Flow chart of included patients and dropouts. PBE: French Maritime Pine Bark Extract; MPH: methylphenidate hydrochloride.

Table 1
Baseline characteristics (mean (SD)) per treatment group.

	Placebo	PBE	MPH	P-value
No. male/female (% male)	24/6 (80)	21/11 (66)	17/9 (65)	0.369
Age (years)	9.96 (1.90)	10.31 (1.37)	10.0 (1.73)	0.660
Weight (kg)	36.21 (11.68)	35.47 (9.82)	34.32 (8.79)	0.788
Height (m)	1.42 (0.14)	1.42 (0.11)	1.39 (0.10)	0.664
Dose (mg/kg)	–	0.88 (0.03)	0.78 (0.02)	–
Compliance (%)	0.94 (0.24)	0.99 (0.38)	0.89 (0.15)	–
GSH (μg/mL)	849.98 (164.89)	823.15 (185.10)	864.38 (128.96)	0.625
Retinol (μg/mL)	0.27 (0.07)	0.28 (0.07)	0.26 (0.05)	0.417
α -tocopherol (μg/mL)	8.96 (2.43)	8.95 (1.55)	9.51 (2.31)	0.547
γ -tocopherol (μg/mL)	0.51 (0.23)	0.50 (0.19)	0.49 (0.18)	0.964
β-carotene (μg/mL)	0.35 (0.25)	0.46 (0.33)	0.40 (0.26)	0.319
coQ10 (μg/mL)	0.47 (0.16)	0.51 (0.17)	0.54 (0.19)	0.402
GPX (mU/mL)	131.88 (54.13)	167.26 (82.64)	171.88 (92.38)	0.104
CAT (U/mL)	173.61 (87.10)	173.36 (85.85)	170.94 (81.41)	0.992
SOD (U/mL)	56.71 (8.34)	53.01 (8.80)	56.15 (9.87)	0.233
GPx gene (normalised value)	1.13 (0.62)	1.20 (0.55)	1.37 (1.39)	0.628
CAT gene (normalised value)	1.16 (0.47)	1.14 (0.58)	1.03 (0.46)	0.645
SOD gene (normalised value)	1.32 (0.78)	1.22 (0.87)	1.32 (0.79)	0.861
XO gene (normalised value)	1.94 (2.44)	1.43 (1.38)	2.07 (3.78)	0.624
ApoJ gene (normalised value)	1.41 (0.98)	1.38 (1.24)	1.38 (0.98)	0.994
Zinc (μg/dL)	117.84 (27.01)	112.22 (24.66)	126.10 (44.14)	0.301
MDA (μM)	5.46 (2.76)	5.11 (2.33)	3.92 (1.95)	0.054
8-OHdG (ng/mg creatinine)	7.63 (3.20)	9.27 (4.46)	7.96 (3.44)	0.208
IL-1β (pg/mL)	0.04 (0.02)	0.06 (0.09)	0.04 (0.04)	0.646
IL-4 (pg/mL)	0.02 (0.01)	0.02 (0.03)	0.02 (0.01)	0.790
IL-5 (pg/mL)	0.42 (0.42)	0.38 (0.69)	0.39 (0.50)	0.972
IL-6 (pg/mL)	0.38 (0.43)	0.34 (0.27)	0.31 (0.28)	0.729
IL-8 (pg/mL)	2.90 (1.06)	2.87 (1.18)	2.69 (1.37)	0.794
IL-10 (pg/mL)	0.33 (0.27)	0.26 (0.13)	0,49 (1.04)	0.336
IL-12p70 (pg/mL)	0.12 (0.10)	0.18 (0.37)	0.17 (0.17)	0.683
TNF-α (pg/mL)	1.07 (0.34)	0.91 (0.25)	1.06 (0.55)	0.226
IFN-γ (pg/mL)	8.74 (18.59)	5.27 (5.83)	11.26 (25.44)	0.445
IgA (g/L)	1.21 (0.53)	1.00 (0.37)	0.91 (0.48)	0.058
IgE (kU/L)	415.68 (730.53)	243.66 (417.92)	244.61 (489.35)	0.420
IgG1 (g/L)	4.94 (1.15)	4.91 (0.87)	4.65 (1.47)	0.622
IgG_2 (g/L)	1.67 (0.54)	1.62 (0.71)	1.55 (0.51)	0.765
IgG_3 (g/L)	0.56 (0.25)	0.58 (0.19)	0.56 (0.26)	0.940
IgG_4 (g/L)	0.36 (0.27)	0.35 (0.31)	0.38 (0.25)	0.949
NPY (pg/mL)	10.03 (6.86)	9.78 (7.67)	11.07 (7.26)	0.792

GSH: glutathione; coQ10: co-enzyme Q10; GPX: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; IL-1β: interleukin-1 beta; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; IL-12p70: interleukin-12; NPY: Neuropeptide Y; TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon gamma; IgA: immunoglobuline A; IgE: immunoglobuline E; IgG_1: immunoglobuline G_1; IgG_2: immunoglobuline G_2; IgG_3: immunoglobuline G_3; IgG_4: immunoglobuline G_4; MPH: methylphenidate hydrochloride; NPY: Neuropeptide Y; PBE: French Maritime Pine Bark Extract. GSH, lipid soluble antioxidants, GPX and CAT analyses were performed on respectively n = 30, n = 32 and n = 25; SOD analysis was performed on n = 30, n = 31 and n = 24. Gene expression analysis was carried out on n = 28, n = 31 and n = 25. Zinc analysis was performed on n = 29, n = 29 and n = 24. MDA concentrations were measured of n = 28, n = 33 and n = 25. 8-OHdG analysis was carried out on n = 29, n = 32 and n = 24. Cytokines levels were analysed in n = 28, n = 32 and n = 25. Antibodies were measured in n = 29, n = 31 and n = 25.

3.2. Biological analyses

Mean concentrations of the analysed biomarkers are listed per treatment group at baseline and follow-up in Table 2. Test statistics and p-values of the interaction between treatment and time are also shown in Table 2. In case of a significant interaction term, false discovery rate analysis was applied (Table 3) to account for the multitude of hypotheses tested. For the oxidative stress parameters, the two qq-plots show that the observed p-values more or less follow the expected distribution under the null hypothesis of no association. For the immunological markers, there is a slight increase in significance regarding the expected null distribution (Fig. 2). Results of the paired samples t-test for NPY and weight assessement in the active treatment groups are listed in Table 4.

3.2.1. Oxidative stress pathway: Antioxidants

Regarding effects on GSH levels, no significant differences in mean GSH concentrations between the treatments were found. Also no significant differences in mean plasma lipid soluble antioxidant concentrations (retinol, α- and γ-tocopherol, β-carotene and coQ10) were found between the three groups after 10 weeks (Table 2). LMM analyses of the measured plasma antioxidant enzymes did show a nominally significant difference in CAT activity between the groups after 10 weeks ($p = 0.025$) (Table 2). However, since multiple hypotheses are tested within one particular pathway, a multiple testing correction needs to be performed. The calculated q-value ($q = 0.425$) now indicates that this association is likely to be false positive, 42.5 % of this significance will be a false positive result (Table 3). This result is consistent with the qqplot (Fig. 2), which showed that the observed p-values are following the expected distribution under the null hypothesis of no association. Gene expression of the different target genes (GPX, CAT, SOD, XO and ApoJ) did not reveal any significant differences between the three groups after the study period (Table 2). Lastly, the LMM analysis did not reveal any significant differences in serum zinc concentrations (Table 2). In order to specifically evaluate the effect of MPH on oxidative stress biomarkers, paired-samples t-tests (data not shown) were performed. No significant differences could be demonstrated for this oxidative stress pathway at the end of the 10-week period compared to the start.

3.2.2. Oxidative stress pathway: Oxidative damage

LMM did not show any significant difference on neither MDA nor on 8-OHdG concentration between the treatment groups after 10 weeks (Table 2).

3.2.3. Immunity

In most of the analysed plasma samples, IL-1β and IL-4 levels were lower than the limit of detection of the method used. For statistical analysis of these two cytokines, a more suitable regression method, Tobit regression, was used (Twisk & Rijmen, 2009). Both Tobit regression as well as the LMM analyses used for the other cytokines failed to show any significant differences in cytokine levels between the groups over time (Table 2). LMM analyses did, however, reveal a significant difference in IgA and IgG_2 plasma concentrations between the three treatment arms over time ($p = 0.028$ and $p = 0.017$ resp.). FDR analyses of these immunity biomarkers showed a slight enrichment of lower p-values, with the two most significant markers, IgA and IgG_2 having a q-value of 0.098 (Table 3). Therefore, IgA and IgG_2 have been shown, with a 90 % probability, to significantly differ between the treatment arms. It could thus be assumed that after 10 weeks, increased IgA and IgG_2 concentrations in the MPH group as compared to those in the PBE group could be found. Statistical analyses of the other antibodies (IgG_1, IgG_3, IgG_4 and IgE) did not show any significant differences between the three groups after 10 weeks (Table 2). In particular for the MPH group, post-hoc analyses after the paired-samples t-test, to compare baseline to 10 weeks, demonstrated a q-value of 0.023 for TNF-α, IgA and IgG_2 and 0.100 for IL-5. Assuming TNF-α, IgA and IgG_2, and IL-5 are considered significant, respectively only 2 % and 10 % would represent false

Table 2
Baseline and follow-up concentrations (SD) of evaluated biomarkers per treatment group.

Biomarker	Time point	Placebo	PBE	MPH	Test value	P-value
GSH (µg/mL)	Baseline	849.98 (164.89)	823.15 (185.10)	864.38 (128.96)	F(2, 153) = 0.438	0.646
	10 weeks	862.99 (134.12)	777.70 (175.88)	845.64 (156.85)		
Retinol (µg/mL)	Baseline	0.27 (0.07)	0.28 (0.07)	0.26 (0.05)	F(2, 70.160) = 0.041	0.960
	10 weeks	0.27 (0.06)	0.29 (0.07)	0.26 (0.07)		
α -tocopherol (µg/mL)	Baseline	8.96 (2.43)	8.95 (1.55)	9.51 (2.31)	F(2, 68.330) = 0.199	0.820
	10 weeks	8.48 (2.09)	8.77 (1.71)	9.68 (2.28)		
γ -tocopherol (µg/mL)	Baseline	0.51 (0.23)	0.50 (0.19)	0.49 (0.18)	F(2, 73.144) = 0.353	0.704
	10 weeks	0.58 (0.25)	0.58 (0.23)	0.63 (0.23)		
β-carotene (µg/mL)	Baseline	0.35 (0.25)	0.46 (0.33)	0.40 (0.26)	F(2, 69.146) = 2.468	0.092
	10 weeks	0.37 (0.34)	0.44 (0.37)	0.30 (0.13)		
CoQ10 (µg/mL)	Baseline	0.47 (0.16)	0.51 (0.17)	0.54 (0.19)	F(2, 69.051) = 0.086	0.918
	10 weeks	0.43 (0.13)	0.46 (0.14)	0.50 (0.13)		
GPX (mU/mL)	Baseline	131.88 (54.13)	167.26 (82.64)	171.88 (92.38)	F(2, 69.940) = 0.915	0.405
	10 weeks	158.12 (47.55)	184.79 (79.64)	195.75 (96.12)		
CAT (U/mL)	Baseline	173.61 (87.10)	173.36 (85.85)	170.94 (81.41)	F(2, 66.568) = 3.922	0.025*
	10 weeks	186.58 (72.80)	132.16 (59.39)	180.48 (72.49)		
SOD (U/mL)	Baseline	56.71 (8.34)	53.01 (8.80)	56.15 (9.87)	F(2, 68.428) = 1.039	0.359
	10 weeks	57.33 (6.93)	58.20 (8.67)	58.85 (8.01)		
GPX gene (normalised value)	Baseline	1.13 (0.62)	1.20 (0.55)	1.37 (1.39)	F(2, 77.623) = 0.266	0.767
	10 weeks	1.09 (0.66)	1.35 (0.47)	1.40 (0.85)		
CAT gene (normalised value)	Baseline	1.16 (0.47)	1.14 (0.58)	1.03 (0.46)	F(2, 74.546) = 0.828	0.441
	10 weeks	1.20 (0.40)	1.40 (0.62)	1.33 (0.74)		
SOD gene (normalised value)	Baseline	1.32 (0.78)	1.22 (0.87)	1.32 (0.79)	F(2, 68.777) = 0.841	0.436
	10 weeks	1.38 (0.85)	1.12 (0.69)	1.28 (0.81)		
XO gene (normalised value)	Baseline	1.94 (2.44)	1.43 (1.38)	2.07 (3.78)	F(2, 39.932) = 1.605	0.214
	10 weeks	1.14 (1.06)	1.91 (1.73)	1.37 (1.04)		
ApoJ (normalised value)	Baseline	1.41 (0.98)	1.38 (1.24)	1.38 (0.98)	F(2, 69.694) = 0.327	0.722
	10 weeks	1.55 (1.03)	1.34 (0.60)	1.29 (0.62)		
Zinc (µg/dL)	Baseline	117.84 (27.01)	112.22 (24.6)	126.10 (44.14)	F(2, 77.541) = 0.274	0.761
	10 weeks	104.00 (29.73)	113.87 (31.69)	116.62 (30.41)		
MDA (µM)	Baseline	5.46 (2.76)	5.11 (2.33)	3.92 (1.95)	F(2, 65.511) = 1.053	0.355
	10 weeks	4.52 (2.48)	4.95 (1.98)	3.94 (2.16)		
8-OHdG (ng/mg creatinine)	Baseline	7.63 (3.20)	9.27 (4.46)	7.96 (3.44)	F(2, 75.755) = 1.351	0.265
	10 weeks	9.75 (3.51)	9.05 (2.88)	8.48 (2.60)		
IL-1β (pg/mL)	Baseline	0.04 (0.02)	0.06 (0.09)	0.04 (0.04)	Z = 0.600	0.539
	10 weeks	0.05 (0.02)	0.05 (0.04)	0.06 (0.07)		
IL-4 (pg/mL)	Baseline	0.02 (0.01)	0.02 (0.03)	0.02 (0.01)	Z = 0.187	0.900
	10 weeks	0.02 (0.01)	0.02 (0.03)	0.02 (0.01)		
IL-5 (pg/mL)	Baseline	0.42 (0.42)	0.38 (0.69)	0.39 (0.50)	F(2, 72.116) = 0.209	0.812
	10 weeks	0.29 (0.41)	0.31 (0.28)	0.25 (0.28)		
IL-6 (pg/mL)	Baseline	0.38 (0.43)	0.34 (0.27)	0.31 (0.28)	F(2, 145) = 1.003	0.369
	10 weeks	0.28 (0.14)	0.37 (0.37)	0.35 (0.34)		
IL-8 (pg/mL)	Baseline	2.90 (1.06)	2.87 (1.18)	2.69 (1.37)	F(2, 77.227) = 0.963	0.386
	10 weeks	3.14 (1.21)	2.86 (1.02)	3.21 (2.15)		
IL-10 (pg/mL)	Baseline	0.33 (0.27)	0.26 (0.13)	0.49 (1.04)	F(2, 81.891) = 1.893	0.157
	10 weeks	0.55 (0.58)	0.36 (0.50)	0.30 (0.18)		
IL-12p70 (pg/mL)	Baseline	0.12 (0.10)	0.18 (0.37)	0.17 (0.17)	F(2, 60.998) = 0.525	0.594
	10 weeks	0.12 (0.11)	0.19 (0.37)	0.14 (0.14)		
TNF-α (pg/mL)	Baseline	1.07 (0.34)	0.91 (0.25)	1.06 (0.55)	F(2, 74.046) = 2.163	0.122
	10 weeks	0.91 (0.22)	0.88 (0.23)	0.87 (0.28)		
IFN-γ (pg/mL)	Baseline	8.74 (18.59)	5.27 (5.83)	11.26 (25.44)	F(2, 146) = 1.458	0.236
	10 weeks	7.09 (5.73)	8.48 (16.95)	3.97 (2.55)		
IgA (g/L)	Baseline	1.21 (0.53)	1.00 (0.37)	0.91 (0.48)	F(2, 67.847) = 3.781	0.028*
	10 weeks	1.24 (0.55)	1.00 (0.37)	0.99 (0.53)		
IgE (kU/L)	Baseline	415.68 (730.53)	243.66 (417.92)	244.61 (489.35)	F(2, 66.179) = 0.868	0.425
	10 weeks	441.04 (892.65)	289.23 (676.60)	227.91 (430.15)		
IgG_1 (g/L)	Baseline	4.94 (1.15)	4.91 (0.87)	4.65 (1.47)	F(2, 68.689) = 1.833	0.168
	10 weeks	5.06 (1.27)	4.75 (0.82)	4.76 (1.39)		
IgG_2 (g/L)	Baseline	1.67 (0.54)	1.62 (0.71)	1.55 (0.51)	F(2, 67.492) = 4.302	0.017*
	10 weeks	1.74 (0.51)	1.62 (0.69)	1.64 (0.59)		
IgG_3 (g/L)	Baseline	0.56 (0.25)	0.58 (0.19)	0.56 (0.26)	F(2, 67.921) = 1.189	0.311
	10 weeks	0.59 (0.25)	0.58 (0.22)	0.58 (0.30)		
IgG_4 (g/L)	Baseline	0.36 (0.27)	0.35 (0.31)	0.38 (0.25)	F(2, 67.026) = 1.272	0.287
	10 weeks	0.33 (0.27)	0.29 (0.22)	0.37 (0.26)		
NPY (pg/mL)	Baseline	10.03 (6.86)	9.78 (7.67)	11.07 (7.26)	F(2, 73.690) = 1.304	0.278
	10 weeks	9.61 (6.79)	11.59 (6.29)	8.77 (4.80)		

Test value and p-value of the interaction between time and treatment between all treatment groups, 10 weeks versus baseline with sex, processing time and time until analysis as covariates (LMM and Tobit regression for respectively IL-1β and IL-4), *: p-value < 0.05 if interaction term is significant. GSH: glutathione; coQ10: coenzyme Q10; GPX: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; IL-1β: interleukin-1 beta; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; IL-12p70: interleukin-12; TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon gamma; IgA: immunoglobuline A; IgE: immunoglobuline E; IgG_1: immunoglobuline G_1; IgG_2: immunoglobuline G_2; IgG_3: immunoglobuline G_3; IgG_4: immunoglobuline G_4; MPH: methylphenidate hydrochloride; NPY: Neuropeptide Y; PBE: French Maritime Pine Bark Extract. Follow-up analyses of GSH, lipid soluble antioxidants, GPX and CAT were performed on respectively n = 23, n = 27 and n = 23. SOD activity levels were measured in n = 23, n = 26 and n = 22. Gene expression analysis was performed on n = 22, n = 26 and n = 23. Zinc concentrations were measured in n = 23, n = 27 and n = 24

respectively. MDA concentrations were analysed in n = 21, n = 27 and n = 22. 8-OHdG analysis was carried out on n = 22, n = 27 and n = 22. Cytokines analyses were performed on n = 21, n = 27 and n = 23. Antibodies were measured in n = 23, n = 27 and n = 24.

Table 3
Q-values as calculated by the fdr tool package for each biomarker of the oxidative stress and immunological pathway.

	Biomarker	P-value	Q-value
Oxidative stress pathway	CAT (U/mL)	0.025	0.425
	β-carotene (μg/mL)	0.092	0.782
	XO gene (normalised value)	0.214	0.833
	8-OHdG (ng/mg creatinine)	0.265	0.833
	MDA (μM)	0.355	0.833
	SOD (U/mL)	0.359	0.833
	GPX (U/mL)	0.405	0.833
	SOD gene (normalised value)	0.436	0.833
	CAT gene (normalised value)	0.441	0.833
	GSH (μg/mL)	0.646	0.929
	γ -tocopherol (μg/mL)	0.704	0.929
	ApoJ gene (normalised value)	0.722	0.929
	Zinc (μg/dL)	0.761	0.929
	GPX (normalised value)	0.767	0.929
	α -tocopherol (μg/mL)	0.820	0.929
	CoQ10 (μg/mL)	0.918	0.960
	Retinol (μg/mL)	0.960	0.960
Immunity pathway	IgG_2 (g/L)	0.017	0.098
	IgA (g/L)	0.028	0.098
	TNF-α (pg/mL)	0.122	0.208
	IL-10 (pg/mL)	0.157	0.224
	IgG_1 (g/L)	0.168	0.228
	IFN-γ (pg/mL)	0.236	0.247
	IgG_4 (g/L)	0.287	0.257
	IgG_3 (g/L)	0.311	0.260
	IL-6 (pg/mL)	0.369	0.267
	IL-8 (pg/mL)	0.286	0.269
	IgE (kU/L)	0.425	0.272
	IL-1β (pg/mL)	0.539	0.321
	IL-12p70 (pg/mL)	0.594	0.343
	IL-5 (pg/mL)	0.812	0.416
	IL-4 (pg/mL)	0.900	0.442

The observed p-values of the biomarkers linked to the oxidative stress and the immune pathway generated with the LMMs are sorted by significance. The q-value then indicated the fraction of false associations, in case that p-value is declared significant. GSH: glutathione; coQ10: co-enzyme Q10; GPX: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; IL-1β: interleukin-1 beta; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; IL-12p70: interleukin-12; TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon gamma; IgA: immunoglobuline A; IgE: immunoglobuline E; IgG_1: immunoglobuline G_1; IgG_2: immunoglobuline G_2; IgG_3: immunoglobuline G_3; IgG_4: immunoglobuline G_4.

positives (data not shown).

3.2.4. The orexigenic peptide NPY and weight assessment

LMM analyses of the serum NPY levels did not show any significant differences between the treatment groups (Fig. 3). However, results of the paired-samples t-test did reveal a significant decrease in serum NPY concentrations (p = 0.021) after MPH treatment for 10 weeks (Table 4). Moreover, a significant weight loss (p < 0.001) was observed in the MPH group. No significance in serum NPY concentrations was observed for the participants receiving PBE, however, a significant increase in body weight was established (p < 0.001).

4. Discussion

Most research on nutritional supplements or medication in ADHD primarily assesses effects on behaviour (Konofal et al., 2008; Rucklidge et al., 2018). This trial, however, also takes into account a broad range of immune and oxidative biomarkers, and the neurochemical biomarker NPY, in order to potentially identify processes underlying behavioural improvement by PBE or MPH (Weyns et al., 2022).

This is the first report on the effects of PBE on *in vivo* lipid soluble antioxidant and zinc levels, CAT, SOD and GPX activity, and XO, CAT, SOD, GPX and ApoJ gene expression, as well as on levels of cytokines and antibodies in paediatric ADHD patients. Moreover, to our

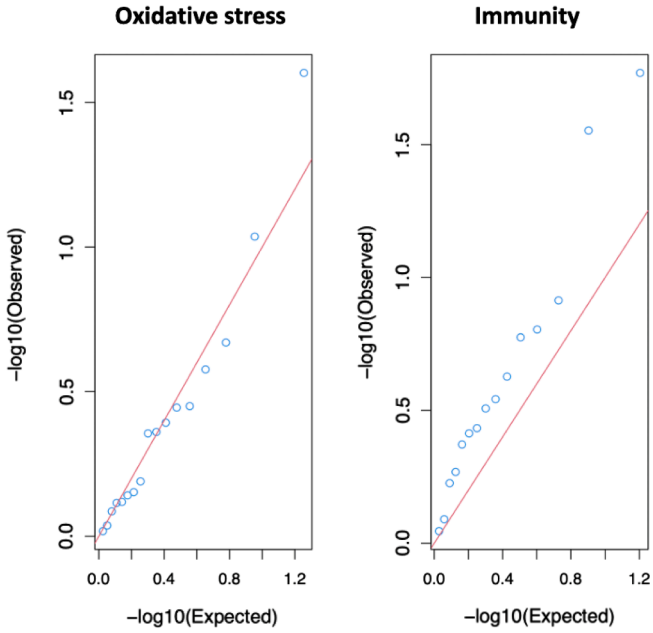

Fig. 2. QQ-plots of the oxidative stress pathway and the immunity pathway. Diagonal red line: expected distribution under the null hypothesis; open circles: observed p-values. Deviations from the distribution under the null hypothesis indicates an enrichment in low p-values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Baseline and follow-up concentrations (SD) of the neurochemical parameter NPY, height and weight for the active treatment groups.

Biomarker	Time point	PBE	Test value[a]	P-value[a]	MPH	Test value[b]	P-value[b]
NPY (pg/mL)	Baseline	10.65 (7.75)	t(23) = −0.646	0.525	11.07 (7.26)	t(23) = 2.471	0.021*
	10 weeks	11.80 (6.34)			8.77 (4.80)		
Height (m)	Baseline	1.42 (0.11)	t(25) = −5.305	<0.001***	1.39 (0.10)	t(23) = −6.699	<0.001***
	10 weeks	1.44 (0.11)			1.41 (0.10)		
Weight (kg)	Baseline	35.37 (10.02)	t(26) = −3.007	0.006**	34.47 (9.34)		
	10 weeks	36.04 (9.97)			33.36 (9.25)	t(22) = 6.743	<0.001***

[a] Test value and p-value of the mean difference for each parameter between the start and the end of the study for the PBE treatment group. [b]Test value and p-value of the mean difference for each biomarker between the start and the end of the study for the MPH treatment group *: if p-value < 0.05; ** p-value < 0.01 and if interaction term is significant. [b]Test value and p-value of post-hoc pairwise comparisons with Bonferroni correction. ***: p-value < 0.001; NPY: Neuropeptide Y; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract.

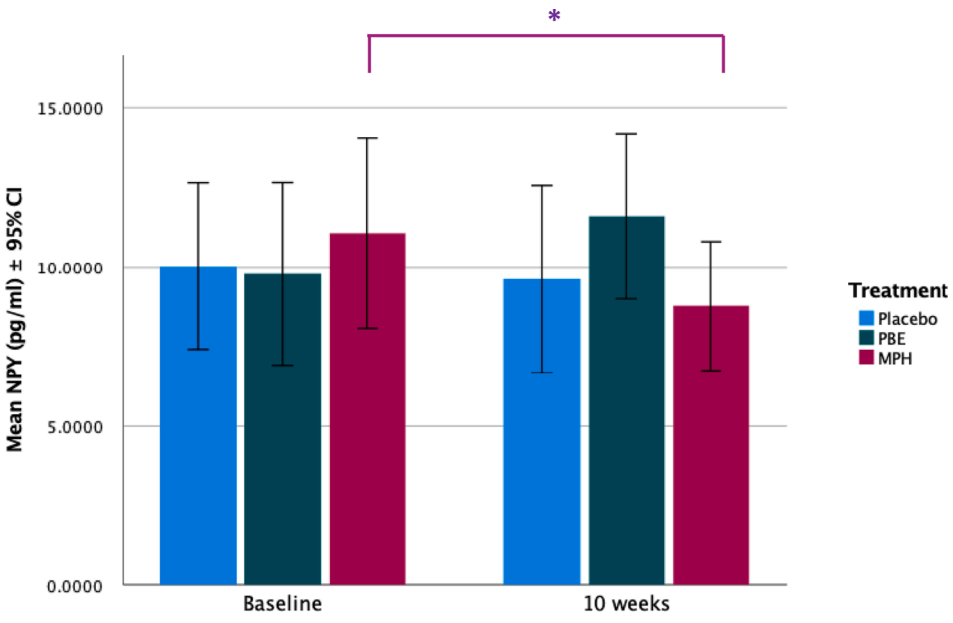

Fig. 3. Evaluation of serum NPY levels analysed at the start and end of the study for French Maritime Pine Bark Extract, methylphenidate and placebo groups. *: p-value < 0.05 for the difference between mean serum NPY concentrations at baseline and after 10 weeks in the MPH group. CI: confidence interval; NPY: Neuropeptide Y; MPH: methylphenidate hydrochloride; PBE: French Maritime Pine Bark Extract. NPY analysis was performed at baseline for n = 32, n = 30 and n = 23 and after 10-weeks for n = 28, n = 25 and n = 22 for respectively placebo, PBE and MPH.

knowledge, this is also the first study investigating the possible effects of MPH, considered one of the first-line treatment options in ADHD, on numerous peripheral immune and oxidative stress related biomarkers.

Since the FFQ shows similar baseline dietary habits between the three groups, without differences in polyphenol-containing food intake, effects observed in the PBE group can be attributed to PBE's polyphenol content.

Forty-eight patients per treatment were to be included (n = 144 in total) according to power calculations (Verlaet et al., 2017). However, due to slow inclusion during the covid-19 pandemic and expiry of the study capsules, the trial was stopped after inclusion of 88 patients in total. Although the dropout ratio was lower than expected (14 % vs 20 %), power was too low to complete subgroup analyses. Specific differences between treatments (e.g. PBE and MPH) could therefore potentially remain unexplored. Nevertheless, statistical analyses revealed specific significant differences in clinical effect between the treatment groups (Weyns et al., 2022).

An important strength of this study is the use of LMM for the statistical analyses. LMM has the ability to accommodate missing data points often encountered in longitudinal datasets (i.e. dropouts or persons lost to follow-up with only baseline values) as compared to ANOVA, which is a complete case analysis. LMM will thus provide higher power as incomplete cases will also be included in the analyses (Krueger & Tian, 2004). Moreover, co-variates such as processing time and time until analysis (due to practical issues) were taken into account. Systematically recording processing time and time until analysis as well as correcting for these potential confounders was found to be crucial to obtain valid results (Verlaet et al., 2019).

In vitro, animal and/or human studies found PBE to have potent antioxidant and anti-inflammatory activity and to improve endothelial function, among various other effects (Grimm et al., 2004; Packer et al., 1999; Verlaet et al., 2019; Wei et al., 1997). Specifically, a previous study on ADHD patients aged 6–14 years found that treatment with 1 mg/kg/day PBE for one month improved ADHD behaviour and increased GSH levels and GSH/GSSG ratio, while there was no change in the placebo group (Dvoráková et al., 2006; Trebatická et al., 2006). However, oxidized glutathione GSSG was not measured in our present study as earlier work by our research group demonstrated that GSSG measurements were not accurate and prone to artefact GSH oxidation, resulting in GSH/GSSG ratios not reflecting real *in vivo* situation (Magielse et al., 2013). Moreover, previous work has shown that PBE reduced levels of 8-oxo-7,8-dihydroguanine (8-oxoG, a marker of oxidative DNA damage) compared with baseline and placebo. Also total antioxidant status (TAS) non-significantly increased following treatment with PBE, while the decrease in DNA damage and increase in TAS correlated with an improvement in inattention score (Chovanová et al., 2009).

In the present study, no clear evidence for an increase in antioxidant levels, reduced oxidative damage or improvement of immune status could be found after treatment with PBE as compared to the other treatment arms. In fact, in the oxidative stress pathway LMM only revealed a nominally significant reduced CAT activity after treatment with PBE as compared to placebo. This was unexpected, as multiple animal studies report upregulation of CAT activity after PBE supplementation. Nevertheless, these studies typically used high PBE concentrations, up to 100 mg/kg, as opposed to 1 mg/kg in the present trial, while the level of oxidative stress (and thus potential for improvement) in these animal models (e.g. type-2 diabetic rats) was possibly higher than in the ADHD patients participating in the present trial (Atta et al., 2020; Goel & Saxena, 2019; Lee et al., 2012; Parveen et al., 2010, 2013; Xiao et al., 2017). Moreover, lower antioxidant enzyme levels do not necessarily imply more oxidative stress. In fact, CAT can be upregulated in case of increased oxidative stress (and vice versa) in child and adolescent patients with ADHD (Ceylan et al., 2010). This has also been observed in *in vivo* models, where following administration of alcohol to rats inducing lipid peroxidation, plasma catalase levels increased. This increase could be prevented with a natural antioxidant like pineapple peel extract (Okafor et al., 2011). An observed reduction in CAT activity by PBE treatment might therefore be explained by PBE's antioxidant effects. However, in this case, this association needs to be interpreted with caution as it is possibly a false positive result demonstrated by its respective q-value (0.425). There is therefore about 43 % chance of getting a false positive result whereas the probability of obtaining a true significant result is only 57 %. A firm conclusion on lowered CAT activity after PBE treatment is therefore not possible.

Similarly, a slightly lower mean GSH concentration after PBE treatment can be noticed. Though this effect is not significant, this could be in line with polyphenols activating nuclear factor erythroid 2–related factor 2 (Nfr2) pathway as described before, increasing the expression of cytoprotective genes coding for i.e. glutathione-S-transferase (GST) (Ma, 2013; Martínez-Huélamo et al., 2017). Higher GST activity could lead to lower GSH concentrations by the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds.

Although increased lipid peroxidation has been reported for ADHD patients (Bulut et al., 2013), this could not be confirmed in this study. Indeed, no significant change in MDA levels, a lipid peroxidation biomarker, could be demonstrated between the groups. Therefore, no evidence was provided that increased lipid peroxidation in patients can be improved upon treatment with PBE or with MPH (Ceylan et al., 2010; Oztop et al., 2012). Although no significant differences in 8-OHdG concentration could be observed, in the placebo group a slightly increased (although not significantly) 8-OHdG concentration could be noted, which might suggest that untreated ADHD could lead to more oxidative DNA damage. Still, a wide variety of oxidative damage biomarkers exist, of which only two were analysed in the present study. Analysis of additional markers should thus be performed in order to draw firm conclusions. Nevertheless, reasons for discrepancies between our findings and those of previous research in paediatric ADHD are unclear (Trebatická et al., 2006).

Although no strong associations were found for the oxidative stress pathway, our results suggest that the p-values observed for the immunity biomarkers are more significant than could be expected in case none of the markers would differ between the active treatment groups. The concentrations of the two most significant biomarkers, IgA and IgG_2, were found to be increased in the MPH group as compared to the PBE group after 10 weeks. Although MPH potentially increases neuroinflammation whereas PBE is known for its anti-inflammatory effects, IgA and IgG_2 are no direct markers of a neuroinflammatory status which is therefore not a plausible explanation for the increased concentrations. However, increased IgE levels under treatment with MPH were demonstrated before as well, but were not found in the present study (Auci et al., 1997).

An important limitation lies in the fact that only immune parameters were assessed in plasma whereas the relevance of the immune dysfunction and the putative immunomodulatory activities of MPH and PBE could be restricted to local tissues. Nevertheless, MPH medication does not appear to affect mucosal immunity, while decreased systemic immunity, associated with increased risk on certain infections might be restored in ADHD patients (Oliva et al., 2020). Over the past years several studies demonstrated the role of cytokines in tryptophan and dopaminergic pathways in the brain, which are implicated in ADHD (Anand et al., 2017). Hence alterations in pro-inflammatory and anti-inflammatory cytokines might influence ADHD pathogenesis. However, the LMM analysis did not reveal any significant differences in effects on plasma cytokine levels between the groups. It is important to note that this study only includes ADHD patients. Differences in cytokine levels in literature are often demonstrated between ADHD and healthy controls (Donfrancesco et al., 2020). Since in our study however, no differences in baseline cytokine levels between the groups could be demonstrated, possible small modulations could remain undetected. Also, the use of medication was an exclusion criterion of this study. Participants with intense immunological symptoms were therefore likely to be excluded. Moreover, cytokine concentrations are generally low unless in case of an active inflammation in which they are locally and transiently produced as a response to stimuli, thereby modulating the functioning of individual cells (Foster, 2001). Effects might thus have been too subtle to be detected in this study due to limited power.

In a case-control study by Verlaet et al. (2019) in which oxidative stress and immune biomarkers between unmedicated paediatric ADHD patients and healthy controls were compared, only a significant difference in GSH levels was observed in the unmedicated paediatric ADHD group as compared to the control group. Moreover, no significance was observed for the other biomarkers, which may indicate the potential involvement of only marginal oxidative stress and immune disturbances in ADHD and might therefore be a possible explanation for our findings.

Furthermore, over the last years interest in preclinical studies investigating the role of MPH on neurological functioning is growing. Evidence from these studies suggest that (mis)use of MPH is associated with redox and energy metabolism changes in the CNS (Foschiera et al., 2022). According to a study of Gomes *et al.* it was observed that MPH alters SOD activity in different brain structures like the cerebellum, prefrontal cortex in an adult rat model emphasizing that the effect of MPH on redox homeostasis is dependent on specific brain regions (Comim et al., 2014). Moreover, in an animal model of ADHD it was demonstrated that MPH causes lipid peroxidation in the brain and altered antioxidant enzymatic activities, hereby confirming that MPH can trigger oxidative stress even in an ADHD model (Comim et al., 2014; Foschiera et al., 2022). In addition to alternating activities of antioxidant enzymes in the brain, another animal study demonstrated that intraperitoneal administration of MPH (20 mg/kg) resulted in reduced levels of GSH in isolated hippocampal mitochondria (Foschiera et al., 2022; Motaghinejad et al., 2016). Studies also pointed out that chronic treatment with MPH dose-dependently increases lipid peroxidation in the brain reflected by increasing MDA levels (Martins et al., 2006). Among the neurotoxic aspects induced by MPH, it is also necessary to highlight its inflammatory potential (Foschiera et al., 2022). A study by Schmitz *et al.* found that young animals after chronic treatment with MPH (2 mg/kg) have an increased production of cytokines TNF-α and IL-6 (Foschiera et al., 2022) while another one demonstrated that treatment with MPH at a dose of 20 mg/kg increased the levels of pro-inflammatory cytokines TNF-α and IL-1β in the cerebellum (Raoofi et al., 2020). Unlike MPH, evidence from *in vitro*, animal and human studies suggests that PBE has neuroprotective effects and even enhances mental performance (Verlaet et al., 2019). For instance, research work by Kim *et al.* (Kim et al., 2020) demonstrated that PBE supplementation exerts a neuroprotective role against ischemic stroke in a gerbil model.

Despite a few studies investigating the effects of MPH on (anti) oxidative and immunological biomarkers in animal or laboratory studies, possible consequences of long-term exposure to MPH in humans are not yet known. A study by Guney *et al.* (Guney et al., 2015) explores the possible effects of a 12-week MPH treatment on oxidative metabolism in children and adolescents with ADHD. Pre-treatment oxidative stress index (OSI) values and plasma total oxidative status (TOS) were statistically higher than those of healthy controls suggesting an oxidative imbalance in ADHD. Moreover, plasma levels of antioxidant enzymes (paraoxonase (PON), stimulated paraoxonase (SPON), arylesterase (ARES) and thiol) and total antioxidative status (TAS) increased after 12-week treatment with MPH whereas post-treatment OSI was significantly lower than the pre-treatment value. It could thus be demonstrated that MPH reduces oxidative stress by way of increasing the plasma antioxidant defence mechanisms in children and adolescents with ADHD. Nevertheless, there are many antioxidant enzyme activities in plasma whereas TAS only measures the cumulative effect of antioxidative molecules in a plasma sample (Guney et al., 2015). In our recent study however, various markers of oxidative stress status were investigated but none was reported to be significant.

From the cytokines and antibodies studied, TNF- α and IL-5 concentrations decreased while IgA and IgG_2 levels increased after 10-week MPH treatment. In the past years, some evidence has indicated that high-dose administration of MPH could play an active role in the increase of neuroinflammation factors and cytokines like IL-6, IL-1β, TNF-α in different brain regions (Raoofi et al., 2020). On the other hand, previous research work by Chuang *et al.* (Chuang et al., 2019) demonstrated that there were no significant differences in a series of serum cytokines levels between pre- and post-treatment with MPH in male ADHD patients aged between 6 and 12 years. To the best of our knowledge, the presented study is the first investigating the possible effects of MPH, considered one of the first-line treatment options in ADHD, on numerous peripheral oxidative stress and immunological biomarkers.

NPY is found to be one of the most appetite-stimulating neuropeptides in a large number of species and thus involved in the control of food intake (Kalra et al., 1991; Kuo et al., 2001). In the present study we could demonstrate a significant decrease in serum NPY levels for the MPH group, which can imply a loss of appetite at the end as compared to the

start of the trial. Moreover in the MPH group, a significant weight loss was also observed ($p < 0.001$) after 10 weeks treatment. This is in line with earlier research work demonstrating the effect of a two-month treatment with MPH on poor appetite and weight loss by investigating several biomolecules like ghrelin and adiponectin (Sahin et al., 2014). As far as we know, this study is the first to report the effect of MPH treatment on NPY. In addition, unlike the MPH group, patients receiving PBE underwent a moderate weight gain though no significant differences in serum NPY levels could be observed. Participants in both active treatment groups significantly increased in height, reflecting a normal growth curve for all patients, attributing the weight loss seen in the MPH group to a loss of appetite. Moreover, in this study up to five times more side effects were reported for MPH as for PBE (Weyns et al., 2022).

5. Conclusions

Based on this study, it is not possible to conclude that PBE increased antioxidant levels, reduced oxidative damage or improved immune status in general as compared to placebo or MPH. However, despite the limited number of significant results, our trial confirmed the impact of psychostimulants such as MPH on appetite and weight. Nevertheless, biochemical effects on the oxidative or immunological pathways resulting from PBE or MPH therapy are not clear yet and need to be further elucidated.

For this reason, insights regarding ADHD pathophysiology and possible treatment options, especially for young children, must be further explored. Nevertheless, our recent publication focusing on the behavioral effects of this clinical trial, does report beneficial effects of PBE on ADHD behavior, especially in the school environment (Weyns et al., 2022). Based upon the teacher-rated ADHD-RS questionnaires, both PBE and MPH significantly improved the total and hyperactivity/impulsivity scores after 10 weeks (Döpfner et al., 2006). PBE was thus proven to be a good alternative for MPH for those willing to wait a few weeks, especially in view of its almost complete lack of side effects (Weyns et al., 2022). No discriminating biochemical marker correlating with the significant effects of PBE on symptom scores were detected as oxidative stress/inflammatory markers were close to physiological status at baseline.

Ethical approval

Ethical approval for this clinical trial was acquired in the University Hospitals of Antwerp (UZA) (EC 15/35/365) and Ghent (UZ Ghent) (2016/0969) and Hospital Network Antwerp (ZNA) (EC approval 4656). The trial was registered at Clinicaltrials.gov (NCT02700685, registered 18 January 2016) and EudraCT (2016-000215-32, registered 4 October 2016).

Statement of human rights

The clinical trial was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Statement of informed consents

All participants and their legally accepted representatives agreed with and signed the written informed consent

CRediT authorship contribution statement

Anne-Sophie Weyns: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Annelies A.J. Verlaet:** Conceptualization, Methodology, Investigation, Funding acquisition, Data curation. **Maxim Van Herreweghe:** Investigation. **Annelies Breynaert:** Investigation. **Erik Fransen:** Methodology, Formal analysis. **Ingrid De Meester:** Investigation, Writing – review & editing. **Emilie Logie:** Investigation. **Wim Vanden Berghe:** Investigation. **Helene Verhelst:** Conceptualization, Methodology, Investigation, Data curation. **Dirk Van West:** Investigation, Supervision. **Ingrid Van Ingelghem:** Investigation. **An I. Jonckheere:** Investigation, Data curation. **Diane Beysen:** Investigation. **Sandra Kenis:** Investigation. **Els Moens:** Investigation. **Aalt P.J. van Roest:** Resources. **Huub F.J. Savelkoul:** Conceptualization, Methodology. **Tess De Bruyne:** Writing – review & editing. **Luc Pieters:** Funding acquisition, Resources. **Berten Ceulemans:** Conceptualization, Supervision. **Nina Hermans:** Conceptualization, Methodology, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105247.

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