Oxidative stress and immune aberrancies in attention-deficit/hyperactivity disorder (ADHD) : a casecontrol comparison

Oxidative stress and immune aberrancies in attention-deficit/hyperactivity disorder (ADHD): a case-control comparison

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Abstract

Objective: The objective of this study is to compare oxidative stress and immune biomarkers between attention-deficit/hyperactivity disorder (ADHD) patients and controls without ADHD.

Methods: A case-control comparison between 57 paediatric (6-12 years) untreated ADHD patients from the Antwerp University Hospital and 69 controls without ADHD from random schools in Flanders, Belgium, was conducted. Erythrocyte glutathione (GSH), plasma lipid soluble antioxidants (retinol, α-tocopherol, γ-tocopherol, retinyl palmitate, β-carotene, and co-enzyme Q10) were determined by HPLC with electrochemical detection, plasma malondialdehyde (MDA) by HPLC with fluorescence detection, plasma cytokines (interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, tumour necrosis factor (TNF), and interferon (INF)-γ) and immunoglobulins (IgE, IgG, and IgM) by flow cytometry and urinary 8-hydroxy-2'deoxyguanosine (8-OHdG) levels by ELISA assay. Dietary habits were determined by a food frequency questionnaire.

Results: Plasma MDA levels were on average 0.031 μM higher in patients than in controls ($p < 0.05$), and a trend for higher urinary 8-OHdG was observed. Erythrocyte GSH and plasma retinyl palmitate levels, as well as IgG and IgE levels were higher in patients than in controls as well (on average 93.707 μg/ml, 0.006 μg/ml, 301.555 μg/ml and 125.004 μg/ml, resp., $p < 0.05$). Finally, a trend for lower plasma IL-5 levels was observed. After Bonferroni correction for multiple

testing, the difference in GSH levels remained statistically significant (nominally significant for retinyl palmitate), while significance was lost for MDA, IgG and IgE levels. Dietary habits do not appear to cause the observed differences. Conclusion: These results point at the potential involvement of slight oxidative stress and immune disturbances in ADHD.

Keywords: ADHD, oxidative stress, antioxidants, immunity, diet

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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a disorder characterized by developmentally inappropriate levels of impulsive behaviour, hyperactivity and/or inattention [1, 2]. It is one of the most prevalent chronic paediatric neurodevelopmental conditions, with a worldwide pooled prevalence in children and youth of 5.29% [3, 4].

ADHD is a multifactorial disorder, influenced by genetic, environmental, biochemical and psychological factors, including common genetic variants underlying other neurodevelopmental and psychiatric disorders like schizophrenia and depression, as well as health risk behaviors such as smoking and obesity. This indicates a shared genetic basis across these traits, but no straightforward indication can yet be given about the exact pathophysiology of ADHD [5-7]. Dopaminergic and noradrenergic dysfunction are involved [5], but ADHD is also associated with immune and oxidative imbalances as reviewed recently [8]. Various studies demonstrate increased levels of oxidative damage markers and decreased activity of antioxidant enzymes in ADHD [9-12]. In addition, ADHD has a high comorbidity with both T_H1- and T_H2-mediated disorders [13-18]. Moreover, sub-cortical volumetric reductions have been found in ADHD, which, based on gene expression profiles, can be partly explained by increased vulnerability of these regions to apoptosis, oxidative stress and autophagy [19]. Oxidative and immune mechanisms may contribute to ADHD via neuronal damage and abnormal neurotransmitter regulation, but decisive evidence on their exact contributions to this disorder is yet to be published [18, 20, 21]. Results are often inconsistent and immune markers other than antibodies, like cellular activation and cytokine levels, have not been systematically studied in ADHD [10-12, 22-24]. A thorough investigation of oxidative and immune aberrancies and their interconnection in ADHD is thus needed. In this study, biomarkers of antioxidant status (erythrocyte glutathione (GSH) and plasma retinol, α-tocopherol, γ-tocopherol, retinyl palmitate, β-carotene, and co-enzyme Q10 (coQ10)), oxidative damage (plasma malondialdehyde (MDA) and urinary 8-hydroxy-2'deoxyguanosine (8-OHdG)) and immune status (plasma cytokines (interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, tumour necrosis factor (TNF), and interferon (INF)-γ) and immunoglobulins (IgA, IgE, IgG, and IgM)) were compared between ADHD patients and controls without ADHD. It is hypothesized that ADHD patients have more oxidative damage and a disturbed immune balance.

Methods

Participants

A comparison was conducted between untreated ADHD patients (cases) from the paediatric outpatient department of Child Neurology of the Antwerp University Hospital (UZA) and controls without ADHD from 10 random schools in

Flanders (Belgium). Patients were diagnosed with ADHD according to DSM-4 or DSM-5 criteria by experienced staff [1, 2]. All participants were 6-12 years old, had no diagnosis of autism-spectrum disorder or chronic systemic disorder (e.g. diabetes or epilepsy), and no severe mental conditions, IQ < 70, or pervasive developmental disorder. Subjects were excluded if they had used medication or nutritional supplements for more than one week during the previous three months. Participants were recruited between June 2013 and September 2017 via a letter in schools and UZA and a poster in UZA waiting rooms. All participants and their legally accepted representative agreed with and signed the written informed consent. Upon inclusion, participants received a randomly assigned participation code.

Sample size calculation was based on SD values in previous studies [12, 18]. It was determined that 63 children per group were required to demonstrate group differences of 0.5 SD regarding the proposed biomarkers (power 0.80, significance level 0.05).

This study was approved by the UZA Ethical Committee (approved study number: 13/18/209; Belgian registration number: B300201317799) and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Sample collection

Blood was collected in EDTA tubes (BD, USA) and placed on ice immediately. After centrifugation (2000g, 4°C, 10 min), erythrocytes and plasma were frozen in -70°C and stored until analysis. Urine was collected in sterile urine containers, placed on ice immediately and stored at -70°C until analysis.

Questionnaires

Parents were asked to fill out several questionnaires. The Social-Emotional Questionnaire (SEQ) focusses on ADHD and on social behavioural problems, anxiety and autism, with items covering core symptoms according to the DSM-4 or DSM-5 [25]. Items are rated on a five-point scale, concerning the past six months: never, less than once a month, every month, every week, or (almost) every day. Dietary habits of participants were assessed by an unpublished food frequency questionnaire (FFQ) concerning 7 food groups: fruits, vegetables, cereal and potato products, dairy, meat and fish, drinks, and miscellaneous. Items were rated on a five-point scale: never or less than once a month, once to three times a month, once a week, twice to five times a week, or (almost) every day. This FFQ was based upon a FFQ assessing infant dietary habits in an unpublished pilot study [26]. The original FFQ consisted of questions on meal frequency (breakfast, lunch and dinner) and food frequency (60 questions on the intake of seven food groups, including portion sizes), which were rated on a six-point scale (never or less than once a month, once to three times a month, once a week, twice to four times a week, five to six times a week or every day). Fifteen additional questions assessed more specific information, e.g. bread

type. Comparison of this FFQ to a three-day food diary, the gold standard to investigate dietary habits, revealed acceptable validity, but potential overestimation of vegetable and potato product intake. For the present FFQ, 35 questions were extracted from the original FFQ as well as additional questions on e.g. type of bread, without questions on portion size. The rating scale was slightly adapted as well. Researchers involved in digitalizing questionnaire answers, calculating scores and statistics were blind for the participant groups.

Biological analyses

Technicians blinded for the participant groups performed biological analyses for patients and controls concurrently.

Erythrocyte GSH content, the most important intracellular antioxidant, was analysed after sample haemolysis and purification by a validated HPLC method [27, 28]. The lipid soluble antioxidants α- and γ-tocopherol, β-carotene, retinol, retinyl palmitate and coQ10 are non-enzymatic defence mechanisms against oxidants [29-32]. Therefore, plasma levels of these antioxidants were analysed by a validated HPLC method after extraction with hexane [33]. GSH and the lipid soluble antioxidants 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).

Oxidative damage to fatty acids leads to the generation of reactive aldehydes like MDA. Plasma MDA was determined by a validated HPLC method on an Agilent 1260 HPLC system (Agilent Technologies, Diegem, Belgium) with a Jasco FP-1520 fluorescence detector (Jasco, The Netherlands) [33]. Urinary 8-OHdG has been validated as a biomarker of oxidative DNA damage, though produced from both DNA guanine bases and nucleotides in cellular pools by nucleotide excision repair and was analysed by a competitive enzyme-linked immunosorbent assay (ELISA) kit (NWLSS™ Urinary 8OHdG ELISA, Northwest Life Science Specialties, USA) according to the manufacturer's protocol [34-36]. Urinary 8-OHdG concentration was expressed as ng/mg creatinine. Urinary creatinine levels were analysed by the Creatinine Microplate Assay (Oxford Biomedical Research, USA) according to the manufacturer's protocol.

Plasma cytokine levels (IL-1β, IL-5, IL-6, IL-8, IL-10, TNF, and IFN-γ) were determined by the Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit, and plasma IgA, IgM and IgG levels by the CBA Human Ig Flex Set (BD, USA) by flow cytometry (FACSCanto™ II, BD, USA,) according to the manufacturer's protocol. Total plasma IgE levels were determined by ImmnoCAP 250 analysis (Thermo Fisher Phadia, Uppsala, Sweden).

Statistical analyses

SPSS version 23.0.0.0 was used for statistical analyses. Data were checked for outliers and normality and presented as mean ± standard deviation (SD) or median ± interquartile range (IQR). Differences between patients and controls were

tested for significance by independent samples t-test, Mann-Whitney U, Chi-Square or Cochran-Armitage trend tests, or by multiple regression analysis. Bonferroni correction for multiple testing was performed. For all regression models, residuals versus the predicted values were plotted to check the assumptions of linearity and homoskedasticity. Using QQ-plots, the normality of the residuals was checked. A p-value < 0.05 is considered significant.

Results

In total 126 participants were included in this comparison: 57 ADHD patients and 69 controls. No significant differences were found between patients and controls regarding the demographic variables age, height and weight (independent samples t-tests, Table 1) and also gender ratio was not significantly different (Chi-Square test). Participants were mainly Caucasian (84% in the patient group, 86% in the control group).

Questionnaires

The median scores on all SEQ conditions and subconditions (Table 2) were significantly higher in the ADHD group than in controls ($p < 0.05$), except for social anxiety (Mann-Whitney U tests).

A trend was noticed for more physician's diagnosed allergies as reported by parents in patients than in controls (23.1% vs. 11.8%, $\chi(1)=2.715$, $p = 0.099$). Suspicion of undiagnosed allergy by parents, the use of antibiotics and the prevalence of ear infections were not significantly different between both groups ($p > 0.05$, Chi-Square tests, data not shown).

Regarding most items, dietary habits of patients and controls appeared very similar, except for consumption of sweet milk drinks and fish, which appeared less in patients ($p < 0.05$, Cochran-Armitage trend tests, data not shown). No significant difference was observed in percentages of participants meeting recommendations for daily fruit (55.6% in ADHD vs. 48.5% in controls) and vegetable intake (63.0% vs 70.6%).

Biological analyses

Mean levels and SD of each analysed oxidative damage and antioxidant biomarkers are presented per group in Table 3, those of each analysed immune biomarker in Table 4. A comparison of these levels between groups must account for different processing times as a potential confounder (processing time of each sample type can be found per group in Table 5), as processing time could influence biomarker levels. Therefore, a linear regression model was fitted with the biomarker as outcome variable, disease status as predicting variable and processing time as covariate. The adjusted differences between both groups can be found in Tables 3 and 4 as well. For example, assuming an equal processing time, erythrocyte

GSH level was on average 93.7 µg/ml higher in patients than in controls ($p < 0.05$). Also plasma retinyl palmitate levels were significantly higher in patients than in controls. Regarding oxidative damage biomarkers, plasma MDA levels were significantly higher in patients, while a trend for higher urinary 8-OHdG levels was observed. Regarding immune biomarkers, plasma IgG and IgE levels were significantly higher in patients, with a trend for lower plasma IL-5 levels ($p < 0.05$, linear regression). Antioxidant levels were not predicted by the amount of oxidative damage (data not shown, linear regression).

As for ADHD patients versus controls, a comparison was made between participants with and without a physician's diagnosed allergy as reported by parents. A linear regression model was fitted with the biomarker as outcome variable, allergy status as predicting variable and processing time as covariate. When accounting for processing time, none of the antioxidant, oxidative damage or immune biomarkers was significantly different between participants with and without allergies. ($p > 0.05$, data not shown)

A positive correlation was found between plasma MDA level accounted for processing time and SEQ impulsivity score ($p < 0.05$; Table 6).

Discussion

To the best of our knowledge, this is the first comparison of erythrocyte GSH and plasma CoQ10, retinyl palmitate, IL-5, IL-8 and IgM between ADHD patients and controls. Significantly increased plasma MDA levels were found in ADHD patients as compared to controls ($p < 0.05$), as well as a trend for higher urinary 8-OHdG levels. In addition, significantly increased erythrocyte GSH as well as plasma retinyl palmitate were observed in ADHD patients as compared to controls, as well as significantly increased total IgE and IgG levels, along with a trend for more diagnosed allergies. Finally, a trend for lower plasma IL-5 levels was observed. After Bonferroni correction for multiple testing however, only a borderline significant difference was found for retinyl palmitate, while significance was lost for MDA, IgG and IgE levels. Nevertheless, these data are consistent with a potential role of oxidative stress that affects the immune system in children with ADHD as compared to controls. As a result, paediatric ADHD patients might develop more IgE and non-IgE-mediated allergies [15]. **Questionnaires**

No differences were found in demographic variables between patients and controls. The expected higher scores on various SEQ (sub)conditions in the ADHD group compared to controls, even after Bonferroni correction to take into account false-positive significant differences due to multiple testing (in this case: 13 conditions and subconditions, so significant if $p < 0.05/13$), is a confirmation of more comorbidity in ADHD, though high comorbidity rates could also be due to

selection of the patient group via a university hospital. However, these comorbidities could influence the results of this study.

Dietary habits of patients and controls appeared very similar, as the only significant differences found concern less consumption of sweet milk drinks and fish by ADHD patients ($p < 0.05$). Again, Bonferroni correction for multiple testing should be taken into account (35 questioned items, so significant if $p < 0.05/35$). Therefore, these differences can be neglected. Though dietary habits can influence oxidative and inflammatory status [37, 38], they probably do not explain differences observed regarding the biomarkers analysed. In addition, similar dietary habits reflect a similar socioeconomic status of both groups, despite different recruitment sources [39].

Oxidative damage

Significantly higher adjusted plasma MDA levels in ADHD patients were observed, while MDA correlated weakly with the SEQ impulsivity score. Though after Bonferroni correction for multiple testing significance was lost for the adjusted difference and correlation, these results still indicate potential involvement of slight oxidative damage in ADHD. Another study, without Bonferroni correction, also reported more plasma MDA in paediatric ADHD, but no correlation with ADHD subtype was found [10]. Other studies, however, found lower plasma or serum MDA levels [23, 24]. Still, increased lipid peroxidation in ADHD is also evidenced by raised urinary acrolein-lysine levels as well as by exhaled ethane levels, a marker of omega-3 fatty acid oxidation [11, 12]. Omega-3 fatty acids are essential in brain development and function [40-42]. Multiple reviews and meta-analyses report small, beneficial effects of n-3 PUFAs (sometimes in combination with n-6 PUFAs) on inattention, hyperactivity, cognition and/or ADHD overall [43-50], but others do not support a therapeutic effect [41, 46, 51-54]. Especially the duration and composition of supplementation appear of influence on the behavioural effect, with EPA doses of at least 500 mg/day or the combination of n-3 with n-6 PUFAs (γ-linolenic acid (GLA) and EPA, possibly with antioxidants like vitamin C or E) generating the most promising results [5, 42, 44, 49, 55, 56].

A trend for higher adjusted urinary 8-OHdG levels was observed in ADHD. Literature on oxidative DNA damage in ADHD is contradictory, as increased levels of total DNA damage were found, determined by 8-oxoG in lymphocytes [22], as well as reduced 8-OHdG levels in serum [24].

Considering the strength of the correlations between SEQ scores and biomarkers, especially impulsivity appeared related to higher levels of oxidative lipid damage. This could imply that the ADHD spectrum not only varies in terms of behavioural manifestations, but also regarding underlying mechanisms and thus potentially also appropriate treatment.

Antioxidants

When correcting for processing time, ADHD patients had significantly higher erythrocyte GSH levels than controls, even after Bonferroni correction, though GSH levels did not correlate with any of the SEQ ADHD scores ($p > 0.05$). Higher antioxidant levels do not necessarily imply less oxidative stress, as these could be a compensation mechanism for increased oxidative stress [10]. In fact, levels of GSH S-transferase and GSH peroxidase, both necessary for GSH activities, were found to be lower in ADHD before [10, 18]. These reduced levels, leading to low GSH consumption and thus lack of an efficient antioxidant defence, could also explain higher GSH levels. In a randomized double-blind placebo-controlled trial, administration of a procyanidin rich extract (1 mg/kg body weight/day) significantly increased whole blood GSH level in paediatric ADHD patients from 0.103 ± 0.019 mM to 0.130 ± 0.008 mM, but no GSH levels were determined for healthy controls [57]. The striking difference in concentration level between this and the present study (2-3 mM) is probably caused by methodological differences, especially sample type used (whole blood vs. erythrocytes).

Vitamin A, including retinol and retinyl palmitate, is required for various biological events, including cell differentiation and survival. However, excessive vitamin A intake can have negative effects, including redox impairment, mitochondrial dysfunction and neurotoxicity. For instance, *in vitro* as well as *in vivo*, vitamin A possesses antioxidant abilities [58-61], though depending on the dose and other factors (incl. nutritional status and pathology), it may also exert pro-oxidative effects, leading to cell death and inflammation [59, 62]. This prooxidant inversion also accounts for other well-known antioxidants, including β-carotene, a vitamin A precursor [58]. In the present study however, physiological levels have been measured, which can be assumed to exert antioxidant rather than prooxidant effects. As retinyl palmitate is the storage form of vitamin A, and low levels were found in plasma, it is questionable whether the borderline significant difference after Bonferroni correction is biologically relevant. No difference was found for adjusted plasma retinol levels as well as for its precursor β-carotene, as confirmed by Spahis *et al.* [23] No difference regarding retinol is not surprising as retinol concentrations in plasma are strictly regulated due to toxicity at high levels [63].Therefore, the authors doubt that vitamin A is involved in ADHD, irrespective of pro- or antioxidant effects.

Spahis *et al.* found significantly higher plasma α- and γ-tocopherol levels in ADHD patients [23], but another study, like the present study when accounting for processing time, found no difference regarding serum tocopherol levels [64].

Redox imbalance

Research generally points towards more oxidative damage in ADHD. Although non-enzymatic antioxidant levels generally do not appear reduced in ADHD, despite conflicting results, levels or activities of antioxidant enzymes were found to be lower in various studies [10, 18, 22, 65-70]. Therefore, oxidative damage biomarkers appear more reflective

of the actual oxidative stress situation compared to antioxidant levels, since even relatively high antioxidant levels can still be too low to balance high oxidant levels [71].

Immune biomarkers

In patients as compared to controls and when accounting for processing time, no significantly different plasma cytokine levels were found, despite a trend for lower IL-5 levels (without Bonferroni correction). In autism, IL-6 and IFN-γ levels correlated negatively with full-scale IQ, verbal comprehension index and working memory index [72], but these cognitive abilities were not assessed in the present study. Nevertheless, significantly higher adjusted plasma IgG and IgE levels in ADHD were observed (indicating an immune imbalance, though significance was lost after Bonferroni correction), without correlation with any of the SEQ ADHD scores.

Oxidative and inflammatory processes are closely related [18]. An oxidant/antioxidant imbalance is responsible for changes in the nervous and immune system [18, 68, 73-78]. Moreover, immune cells are an important source of both oxidant and proinflammatory compounds, like reactive oxygen and nitrogen species (ROS and RNS) and inflammatory cytokines, which stimulate NF-κB activation, leading to production of more oxidants and inflammatory compounds, and thereby establishing a vicious circle [18, 78]. Antioxidants could exert beneficial effects through an improvement of oxidative stress and immune cell functions, and might therefore have potential in ADHD therapy [8].

ADHD-associated increased oxidative stress might, in case of a chronic state, lead to immune dysfunction resulting in elevated T_H2 induction and thereby increased IgE levels. Although IgE is found in every individual, and levels increase with age until reaching a stable level at adulthood, elevated concentrations of total IgE reflect a predisposition to develop IgE-mediated allergic diseases, despite not being directly related to allergic status [79, 80]. Oxidative stress might thus facilitate the development of allergic conditions in ADHD patients [8, 75]. Indeed, the observed higher IgE levels were supported by a trend for more diagnosed allergies in patients than in controls.

It should be noted that the use of medication was an exclusion criterion of this study, so that patients with intense allergy symptoms were likely to be excluded. It is therefore no surprise that none of the antioxidant, oxidative damage or immune biomarkers was significantly different between participants with and without allergies or that no significantly aberrant systemic cytokine levels were found, which would indicate active inflammation. In addition, cytokine concentrations in mucosal secretions are predominantly the result of local cytokine production, causing a potential lack of correlation between low plasma IL-5 levels and, presumably, high concentrations in mucosal secretions reflecting local T_H2 expansion [81]. In addition, as the analysed cytokine levels in both groups were very low, it is questionable whether any observed difference would be biologically relevant. Though elevated oxidative stress can cause higher basal (without

antigen stimulation) levels of inflammatory cytokines [82], oxidative stress in ADHD might be too limited for clear effects on plasma cytokine levels.

The shifted immune balance due to oxidative stress results in a modified humoral immune response. Though IgE levels are more strongly T-cell regulated and have a much shorter half-life than IgG, under chronic conditions also IgG levels will be changed [83]. The observed higher IgG levels in ADHD support involvement of inflammation [84].

Due to the use of various methodologies in literature, it is hard to compare the results of different studies. For example, despite the increased prevalence of atopies in ADHD in literature [15, 85], their association appears based on a non-IgE-mediated mechanism [86-89]. In addition, a role for IgG in ADHD pathophysiology was countered before as well [86]. Moreover, indications of more T_H1 cytokines have been found in previous research, e.g. higher detection rates for IFN-γ and TNF-β in cerebrospinal fluid [90]. Another study however reports no significant difference regarding serum TNF-α, IL-1β, IL-6 and IL-10, but did find higher levels of IFN-γ in ADHD patients than in controls [91]. Further research with consistent methodology is thus required to draw a final conclusion. Nevertheless, cytokines could be important in ADHD as they can pass the blood-brain barrier and affect synaptic plasticity and neurogenesis and can even cause T-cell mediated neuroinflammation [91-93].

Strengths and limitations

This case-control comparison has limitations. For example, the nature of this study does not address the investigation of causality. It is therefore unknown whether increased oxidative stress or immune dysbalance are causative factors in the pathophysiology of ADHD, or a consequence of the disorder [18]. For instance, increased oxidative stress in ADHD could be the result of the restlessness related with ADHD [69]. Still, case-control studies are a good start, though not providing solid proof, to unravel underlying mechanisms of action.

Furthermore, though all included patients in this study were diagnosed by a child neurologist as having ADHD according to DSM criteria (DSM-4 or DSM-5), several patients would not be classified as such based solely on the SEQ scores of their parents. In addition, several controls had a positive SEQ ADHD score, without ADHD diagnosis or parental complaints. This underscores the subjectivity of questionnaires and might explain the lack of correlations between biomarkers and SEQ (sub)scores.

Another limitation is that the FFQ questioned consumption frequency but not portion size, which makes it impossible to draw conclusions on actual intake. In addition, the probability of recall bias and overestimation of vegetable and potato product intake should be taken into account [26], but both are not expected to differ between patients and controls.

An important strength of this study is the use of regression analysis correcting for differences in processing time due to practical issues (e.g. sample transport to the analytical facility from the neighbouring UZA was faster than from schools). Systematically recording processing time and correcting for this potential confounder was found to be crucial to obtain valid results and should thus be implemented in future research. In addition, Bonferroni correction for multiple testing is essential, but often not performed in literature.

Finally, the comparison of erythrocyte GSH and plasma CoQ10, retinyl palmitate, IL-5, IL-8 and IgM between ADHD patients and controls has not been published before.

Conclusion

An evaluation of redox and immune status in ADHD was performed. Significantly higher plasma MDA levels were found in ADHD patients as compared to controls, and a trend for higher urinary 8-OHdG levels. Erythrocyte GSH and plasma retinyl palmitate as well as plasma IgG and IgE levels were significantly higher in patients than in controls. Finally, a trend for lower plasma IL-5 levels was observed. After Bonferroni correction for multiple testing, the result for GSH remained statistically significant, while only a borderline significant difference was found for retinyl palmitate. Significance was lost for MDA, IgG and IgE levels. Still, though only slightly elevated, an indication of more oxidative damage was thus found in ADHD. Antioxidant levels however did not differ or were even higher than in the control group. In addition, higher IgE levels were supported by a trend for more diagnosed allergies in patients than in controls and might point at an immune dysbalance in ADHD. However, due to the nature of this study, it is unknown whether oxidative stress and immune dysbalance have a causative role in ADHD. Dietary habits do not appear to explain the observed biomarker differences. Further confirmation of these results is required, as well as further investigation of potential differences between ADHD subtypes. Finally, systematically correcting for processing time is crucial to obtain valid results.

The authors declare that they have no conflict of interest.

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Tables

Table 1. Demographic variables per group.

	ADHD	Control	Test value	P-value (2 sided)
Age (years; mean ± SD)	8.98 ± 1.75	8.37 ± 1.69	T(121)=-1.959	0.052
Height (m; mean ± SD)	1.36 ± 0.11	1.36 ± 0.12	T(114)=-0.105	0.916
Weight (kg; mean ± SD)	32.54 ± 9.48	31.15 ± 7.64	T(114)=-0.878	0.382
Gender (n; male/female)	41/16	45/24	$\chi(1)=0.649$	0.420

SD: standard deviation

Table 2. Median scores and IQR per (sub)condition of SEQ.

	ADHD		Control		Test value	P-value (2 sided)
	Median	IQR	Median	IQR		
ADHD total	40.0	22.8	12.0	11.5	U=329.5	<0.001*
ADHD inattention	15.0	8.0	4.0	4.0	U=199.5	<0.001*
ADHD hyperactivity	16.0	12.5	5.0	6.0	U=664.0	<0.001*
ADHD impulsivity	9.0	8.5	4.0	4.8	U=649.0	<0.001*
Autism	6.0	6.0	2.0	2.3	U=827.5	<0.001*
Social problem behavior total	17.0	19.0	10.0	11.0	U=963.0	<0.001*
Oppositional Defiant Disorder	7.0	12.5	4.0	4.8	U=1168.5	<0.001*
Conduct Disorder – Aggression	1.0	3.8	0.0	2.0	U=1393.5	0.004*
Conduct Disorder – Antisocial	9.0	8.0	4.0	6.0	U=928.0	<0.001*
Anxiety total	16.5	16.0	7.0	10.0	U=982.5	<0.001*
General anxiety	6.0	6.0	3.0	4.0	U=902.5	<0.001*
Social anxiety	3.0	6.5	3.0	3.9	U=1621.0	0.114
Depression	5.0	5.0	2.0	4.0	U=1017.5	<0.001*

**Statistically significant if $p < 0.05$. IQR: interquartile range.*

Table 3. Mean levels and SD for oxidative damage and antioxidant biomarkers in ADHD patients and controls and adjusted mean difference, accounting for processing time, by linear regression.

	ADHD		Control		Adjusted		Test value	P-value
	Mean	**SD**	**Mean**	**SD**	**difference**	**95% CI**		**(2 sided)**
8-OHdG (ng/mg Crn)	13.749	5.168	12.229	4.259	1.851	-0.290 – 3.992	F(1,116)=2.932	0.089
MDA (µM)	0.374	0.078	0.348	0.056	0.031	0.004 – 0.058	F(1,118)=5.030	0.027*
α-tocopherol (µg/ml)	10.302	2.199	9.708	1.709	0.319	-0.470 – 1.109	F(1,117)=0.641	0.425
β-carotene (µg/ml)	0.590	0.229	0.657	0.267	-0.020	-0.118 – 0.079	F(1,118)=0.153	0.696
γ-tocopherol (µg/ml)	0.462	0.170	0.420	0.147	0.041	-0.024 – 0.106	F(1,117)=1.586	0.210
CoQ10 (µg/ml)	0.578	0.143	0.534	0.134	0.043	-0.013 – 0.099	F(1,119)=2.337	0.129
GSH (µg/ml)	805.662	129.761	670.742	112.641	93.707	45.684 – 141.729	F(1,111)=14.951	<0.001*
Retinol (µg/ml)	0.310	0.043	0.316	0.050	-0.003	-0.022 – 0.016	F(1,116)=0.084	0.772
Retinyl palmitate (µg/ml)	0.019	0.013	0.011	0.008	0.006	0.002 – 0.011	F(1,108)=7.780	0.006*

**Significantly different if p<0.05. CI: confidence interval; CoQ10: co-enzyme Q10; Crn: creatinine; GSH: reduced glutathione; SD: standard deviation.*

Table 4. Mean levels and SD for immune biomarkers in ADHD patients and controls and adjusted mean difference, accounting for processing time, by linear regression.

	ADHD		Control		Adjusted		Test value	P-value
	Mean	**SD**	**Mean**	**SD**	**difference**	**95% CI**		**(2 sided)**
IFN-γ (pg/ml)	5.802	0.228	5.815	0.291	-0.031	-0.139 – 0.078	F(1,116)=0.316	0.575
IL-1β (pg/ml)	5.463	0.253	5.480	0.286	-0.036	-0.149 – 0.076	F(1,114)=0.406	0.525
IL-5 (pg/ml)	5.442	0.312	5.603	0.410	-0.130	-0.284 – 0.023	F(1,112)=2.819	0.096
IL-6 (pg/ml)	6.669	0.368	6.669	0.481	-0.057	-0.236 – 0.122	F(1,110)=0.403	0.527
IL-8 (pg/ml)	7.321	1.131	7.291	1.193	0.009	-0.464 – 0.481	F(1,115)=0.001	0.972
IL-10 (pg/ml)	5.549	0.528	5.602	0.710	-0.061	-0.309 – 0.187	F(1,116)=0.237	0.627
TNF (pg/ml)	6.308	0.553	6.418	0.492	-0.102	-0.304 – 0.101	F(1,120)=0.993	0.321
IgE (µg/ml)	250.358	270.350	132.348	158.592	125.004	25.298 – 224.709	F(1,97)=0.779	0.015*
IgG (µg/ml)	2198.126	985.728	2100.781	116.924	301.555	143.382 – 746.491	F(1,111)=0.182	0.016*
IgM (µg/ml)	328.257	190.206	294.117	163.720	17.293	-54.126 – 88.712	F(1,114)=0.230	0.632

**Significantly different if p<0.05. CI: confidence interval; IFN: interferon; Ig: immunoglobulin; IL: interleukin; SD: standard deviation; TNF: tumor necrosis factor.*

Table 5. Mean levels and SD sample processing time for each biological sample in ADHD patients and controls.

	ADHD		**Control**	
	Mean (h)	**SD**	**Mean (h)**	**SD**
Plasma	4.05	3.08	6.82	2.49
Erythrocytes	4.20	2.76	6.80	2.46
Urine	3.93	3.34	6.37	2.93

Table 6. Correlations (Pearson's r) between oxidative stress biomarkers and ADHD SEQ scores.

	ADHD total	Inattention	Hyperactivity	Impulsivity
8-OHdG	0.011	0.001	0.034	0.019
MDA	0.111	0.069	0.079	0.208*
α-tocopherol	-0.114	-0.044	-0.154	-0.178
β-carotene	-0.008	-0.021	0.009	-0.033
γ-tocopherol	-0.043	-0.021	-0.036	-0.077
CoQ10	0.051	0.048	0.019	0.047
GSH	-0.037	-0.013	-0.044	-0.060
Retinol	0.004	0.042	0.040	-0.042
Retinyl palmitate	0.032	0.011	0.020	0.049
IFN-γ	-0.037	-0.057	-0.001	-0.072
IL-1β	0.000	-0.065	0.053	0.019
IL-5	-0.144	-0.157	-0.127	-0.132
IL-6	-0.063	-0.065	-0.057	-0.069
IL-8	-0.078	-0.111	-0.059	-0.054
IL-10	-0.121	-0.104	-0.096	-0.140
TNF	-0.043	-0.068	-0.003	-0.050
IgE	-0.007	-0.015	-0.011	-0.013
IgG	-0.109	-0.098	-0.101	-0.100
IgM	-0.100	-0.027	-0.091	-0.141

**Statistically significant if $p < 0.05$. CoQ10: co-enzyme Q10; GSH: reduced glutathione; IFN: interferon; Ig: immunoglobulin; IL: interleukin; MDA: malondialdehyde; TNF: tumor necrosis factor.*