Proinflammatory mediators and their associations with medication and comorbid traits in children and adults with ADHD

Liu L. Yang\textsuperscript{a,b}, Miranda Stiernborg\textsuperscript{a,b}, Elin Skott\textsuperscript{a,b,c}, Åsa Söderström\textsuperscript{c}, MaiBritt Giacobini\textsuperscript{a,c}, Catharina Lavebratt\textsuperscript{a,b,*}

\textsuperscript{a}Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden
\textsuperscript{b}Center for Molecular Medicine, Karolinska University Hospital Solna, Stockholm, Sweden
\textsuperscript{c}PRIMA Child and Adult Psychiatry, Stockholm, Sweden

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Abstract
Peripheral immune activation can influence neurodevelopment and is increased in autism, but is less explored in attention deficit hyperactivity disorder (ADHD). Patients with ADHD often display comorbid autism traits and gastrointestinal (GI) symptoms. Plasma protein levels of two acute phase reactants, C-reactive protein (CRP) and serum amyloid A (SAA), and two endothelial adhesion molecules, soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1), which share important roles in inflammation, were analyzed in 154 patients with ADHD and 61 healthy controls. Their associations with ADHD diagnosis, severity, medication and comorbid autistic symptoms, emotion dysregulation and GI symptoms were explored. The ADHD patients had increased levels of sICAM-1 and sVCAM-1 compared to healthy controls ($p = 8.6e-05$, $p = 6.9e-07$, respectively). In children with ADHD, the sICAM-1 and sVCAM-1 levels were higher among those with ADHD medication than among children ($p = 0.0037$, $p = 0.0053$, respectively) and adults ($p = 3.5e-09$, $p = 1.9e-09$, respectively) without ADHD medication. Among the adult ADHD patients, higher sICAM-1 levels were associated with increased comorbid autistic symptoms in the domains attention to detail and imagination ($p = 0.0081$, $p = 0.00028$, respectively), and higher CRP levels were associated with more GI symptoms ($p = 0.014$). sICAM-1 and sVCAM-1 levels were highly correlated with each other, and so were CRP and SAA levels. To conclude, vascular inflammatory activity may be overrepresented in ADHD, with elevated sICAM-1 and sVCAM-1 levels and this may in chil-

\*Corresponding author at: Center for Molecular Medicine, Karolinska University Hospital, Visningsgatan 18, 171 76 Stockholm, Sweden.
E-mail address: catharina.lavebratt@ki.se (C. Lavebratt).

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1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a common neurodevelopmental disorder with onset generally within childhood or early adolescence, and the disorder may continue throughout life (Pettersson et al., 2017; Wolraich et al., 2019). Diagnosis criteria include, but are not limited to, more than four inattentive or hyperactive-impulsive symptoms occurring before 12 years of age leading to impairments in at least two significant areas of functioning such as work/school, social skills and family settings (Pettersson et al., 2017; Wolraich et al., 2019). In children, ADHD is 3-9 times more common in boys than girls while in an adult population the sex ratio is more equal (Willcutt 2012; Simon et al., 2009). Autism spectrum disorder (ASD) is a common psychiatric comorbidity of ADHD with onset in childhood and adolescence (Antshel et al., 2016). ASD symptoms are in the aspects of social communication and interaction, as well as of restricted, repetitive patterns of behavior, interests or activities. Emotion dysregulation is a characteristic associated with several psychiatric disorders (e.g. ADHD, oppositional defiant disorder, anxiety and depression) (Bunford et al., 2018). Emerging evidence has shown that persons with neurodevelopmental disorders, such as ADHD and ASD, more often have chronic gastrointestinal (GI) disturbances, including constipation, diarrhea, and abdominal pain than the general population (McKeown et al., 2013; Wang et al., 2014; Rose et al., 2018). The immune system constitutes an important part of gut-brain axis, with circulating cytokines and other inflammatory molecules being key mediators of the axis (Petra et al., 2015; Rose et al., 2018). Neurobiologically, ADHD is characterized by structural and functional dysfunctions in a range of cortical and subcortical brain regions (Leffa et al., 2018). The genetic heritability of ADHD is high, and detected genetics variants are implicated mainly in neural development and plasticity, neuronal wiring and synaptic dopamine levels (Demontis et al., 2019). However, ADHD has a high comorbidity with immune-mediated conditions, like atopic dermatitis and asthma, raising a possibility of a neuropathological role of the immune system in ADHD (Leffa et al., 2018). Immune activation has been consistently reported for children with ASD, and has been proposed to have a neuropathological role (Osokine and Erlebacher 2017; Meltzer and Van de Water 2017). There are reports of elevated proinflammatory cytokines peripherally also in children with mood and anxiety disorders, and ADHD (Rosenblat et al., 2014; Furtado et al., 2019; Mitchell and Goldstein 2014). The strongest evidence for immune activation in the psychopathophysiology of adults is for major depressive disorder and schizophrenia (Schwarz et al., 2012; Dantzer et al., 2008; Miller et al., 2011; Khandaker et al., 2015), and there is support for immune dysregulation also in subgroups of bipolar disorder (Benedetti et al., 2020; Misiak et al., 2020; Millet et al., 2019).

For ADHD, a few studies have reported elevated peripheral blood proinflammatory marker levels in children (Mitchell and Goldstein 2014; Anand et al., 2017; Verlaet et al., 2019; Alasehiri et al., 2015; Darwish et al., 2019; Donfrancesco et al., 2016), but studies conducted in adults are few (Corominas-Royo et al., 2017). In children with ADHD, levels of soluble intercellular adhesion molecule 1 (sICAM-1) (25 patients vs. 18 controls), interleukin (IL)-6 (60 patients vs. 60 controls and 98 patients vs. 21 controls), IL-10 (58 patients vs. 36 controls) and high-sensitivity C-reactive protein (CRP) (98 patients vs 21 controls) were found elevated (Alasehiri et al., 2015; Darwish et al., 2019; Donfrancesco et al., 2016; Chang et al., 2020). Also, a few previous studies have examined inflammatory cytokine levels in relation to scores of symptoms being core in ADHD. Among them, levels of IL-6 and tumor necrosis factor (TNF)-α correlated positively with hyperactivity/impulsivity scores in children/adolescents with obesity (Cortese et al., 2019). Further, intervention with omega-3 supplements in children with ADHD improved the core symptoms (Chang et al., 2019, 2018), and associated with a parallel reduction in levels of CRP, IL-6 and hyperactivity (Hariri et al., 2012). However, two papers showed no significant association between ADHD symptom scores and CRP, IL-6 and TNF-α (Vogel et al., 2017; Darwish et al., 2019).

CRP and serum amyloid A (SAA) are among the most commonly investigated peripheral inflammatory markers in psychiatric disorders and are reported elevated in mood disorders and psychotic disorders (Horsdal et al., 2017; King et al., 2007; Kiecolt-Glaser et al., 2017). They are liver-derived acute phase proteins that respond rapidly to infectious and inflammatory stimuli and increase dramatically within hours. Also, SAA is implicated in several chronic inflammatory diseases, such as atherosclerosis, obesity and diabetes (Zhang et al., 2019; Uhlar and Whitehead 1999). ICAM-1 and VCAM-1 are cell adhesion molecules expressed predominantly by endothelial cells and are central to leukocyte-endothelial cell adhesion and leukocyte extravasation into the surrounding tissue facilitating inflammatory responses. ICAM-1 participates in binding leukocytes to the endothelial cell, and VCAM-1 participates in the subsequent leukocyte-endothelial cell interaction. sICAM-1 and sVCAM-1 are the soluble isoforms of ICAM-1 and VCAM-1, respectively, shed by the cells in proportion to membrane-bound levels and are thus detectable in blood. While VCAM-1 is expressed only by endothelial cells, ICAM-1 is expressed also by leukocytes and in brain by microglia and astrocytes (Blankenberg et al., 2003). Levels of ICAM-1, and its soluble form sICAM-1, have been associated with clinical features of schizophrenia, depression, and bipolar disorder and to specific neurodegeneration markers in dementia, and that may relate to ICAM-1’s key role in regulating blood-brain bar-
Table 1  Questionnaires included in this study.

| Scale         | Target | Main area                | Items | Min-max per item | Scoring* | Subscales N | Reference                  |
|---------------|--------|--------------------------|-------|------------------|----------|-------------|----------------------------|
| SNAP-IV       | Child  | ADHD symptoms            | 18    | 0-3              | Mean     | 2           | (Stilling et al., 2016)    |
| ASRS          | Adult  | ADHD symptoms            | 18    | 0-4              | Mean     | 2           | (Kessler et al., 2005)    |
| WFIRS-PC      | Child  | Functional impairment    | 60    | 0-3              | Mean     | 7           | (2011)                    |
| WFIRS-SA      | Adult  | Functional impairment    | 69    | 0-3              | Mean     | 7           | (2011)                    |
| SCQ           | Child  | Autism symptoms          | 40    | 0-1              | Mean     | 3           | (Rutter et al., 2003)     |
| AQ            | Adult  | Autism symptoms          | 50    | 0-3              | Mean     | 5           | (Baron-Cohen et al., 2001)|
| DERS-16       | Adult  | Emotion dysregulation    | 16    | 1-5              | Sum-score| 5           | (Bjureberg et al., 2016)  |
| Swedish Wellbeing scale | Adult | Wellbeing                | 18    | 0-4              | Mean     | 0           | (Ström and Carlbring 2014)|
| GI-questionnaire | All    | GI symptoms              | 3     | 0-1              | Sum-score| 0           | Own-design                 |
| Food questionnaire | All    | Diet                     | 57    | NA               | Sum-score| 0           | (Kautto et al., 2014)     |

Scales: SNAP-IV parent report, Swanson, Nolan and Pelham scale; ASRS, Adult ADHD Self-Report Scale; WFIRS-PC/SA, The Weiss Functional Impairment Rating Scales; SCQ, Social Communication Questionnaire; AQ, Autism Spectrum Quotient; DERS-16, Difficulties in Emotion Regulation Scale; GI, gastrointestinal.

AQ includes the five subscales social skills, attention switching, attention to detail, communication and imagination [10 items each, mean score per item, score range 0-3]. DERS-16 includes the five subscales lack of emotional clarity [2 items, sum score range: 2-10], difficulties engaging in goal-directed behavior [3 items, 3-15], impulse control difficulties [3 items, 3-15], limited access to effective emotion regulation strategies [5 items, 5-25] and nonacceptance of emotional responses [3 items, 3-15].

* Sum score is the sum of all questions (items). Mean score is the sum score divided by the number of items. Generally, higher scale scores represent more symptoms, functional impairments, more emotion dysregulation or higher level of wellbeing.

rier (BBB) permeability (Müller 2019; Varararaj and Galea 2017). Elevated ICAM-1 expression in the BBB is associated with BBB hyper-permeability (Müller 2019). Also elevated sVCAM-1 has been reported to be associated with BBB breakdown (Haarmann et al., 2015). In fact, elevated levels of both ICAM-1 and VCAM-1 levels have been reported in the brains of individuals with unipolar or bipolar depression (Thomas et al., 2004, 2002). Thus, elevated levels of sICAM-1 and sVCAM-1 are indicative of vascular inflammatory activity and endothelial dysfunction (Müller 2019). Patients with ADHD are commonly treated with stimulants, e.g. methylphenidate and dexamphetamine, known to increase synaptic dopamine and noradrenaline levels. Chronic oral exposure to methylphenidate has at high clinically relevant doses been shown to cause microglia activation and neuroinflammation in cerebral cortex, hippocampus, thalamus and basal ganglia (Carias et al., 2018), and BBB hyperpermeability (Coelho-Santos et al., 2019) in rodent brain. Likewise, use of dexamphetamine has been reported to induce neuroinflammation in rodents (Valvassori et al., 2019; Thomas and Kuhn 2005). Another common medication in ADHD is atomoxetine. It is a noradrenaline reuptake inhibitor reported to have anti-inflammatory effects in the central nervous system (CNS) (O’Neill et al., 2020).

We hypothesized that CRP, SAA, sICAM-1, sVCAM-1 levels would be elevated in ADHD compared to controls and that higher levels would associate with severity of ADHD symptoms, functioning and medication with stimulants. Further, we hypothesized that the ADHD patients would manifest more GI symptoms and autistic traits, which would associate with elevated levels of the inflammatory markers. Finally, we considered the possibility of association between anxiety levels and age, sex, and body mass index (BMI).

2. Experimental procedures

2.1. Participants and measurement

All patients and healthy controls in this case-control study were recruited through a clinical trial of a dietary intervention at predefined psychiatric out-patient clinics in Stockholm, Sweden and through advertisement in a local newspaper. Of the participants (248 patients and 72 controls), 156 patients and 61 controls provided blood samples at intervention baseline between October 2016 and July 2018 and were included in this study. All patients had a prior ADHD-diagnosis (based on criteria from ICD-10 or DSM-5) and were 5-55 years old. Those on medication had stable pharmacological treatment in the last four weeks before recruitment. Healthy controls in this study had no ADHD diagnosis and were family member of (n = 39) or non-related to the recruited patients (n = 22). Participants with a GI-diagnosis other than irritable bowel syndrome, antibiotic treatment the last six weeks, or a diagnosis of diabetes or celiac disease were excluded. After providing informed consent, all participants were interviewed regarding delivery route, breastfeeding, pharmacological treatment, symptoms of infection such as sore throat and fever, and all adults provided weight/height. All participants were asked to fill in questionnaires on ADHD symptoms, daily function, GI symptoms, autism symptoms, and, for adults only, emotion dysregulation (Table 1). The interviews and sampling were conducted by research nurses experienced in psychiatric care. Participants with a symptom of infection were asked to come back for reassessment when symptoms were gone.
2.2. Questionnaires and scoring

In this study, we included both children (5-18 years) and adults (19-55 years). For children up to 12 years of age, the questionnaires (Table 1) were filled in by or with support from parents. For ADHD symptoms, the validated questionnaires Swanson, Nolan and Pelham Parent Rating Scale (SNAP-IV) 18 item, and Adult ADHD Self-Report Scale (ASRS) were administered for children and adults, respectively. The Weiss Functional Impairment Rating Scales (WFIRS) is a validated instrument to measure functional impairment in individuals with ADHD. For children, parents rated functioning using WFIRS-parent reported for child (PC), whereas for adults WFIRS-self reported (SA) was applied. Autistic symptoms were assessed using the validated Social Communication Questionnaire (SCQ) for children and the Autism Spectrum Quotient (AQ) for adults. Difficulties of emotion regulation were measured in adults by the validated Difficulties in Emotion Regulation Scale (DERS-16).

The GI-questionnaire used to assess the GI symptoms was own-designed based on the Rome IV criteria for diagnosis of GI disorders. The GI-questionnaire consisted of three parts, stool consistency measured using Bristol Stool Scale, subjective level of pain, and finally number of defeactions per week. Each part was scored 0 to 1, providing a sum-score of the GI questionnaire from 0 to 3, higher score indicating more GI symptoms. (1) The Bristol Stool Scale includes seven different stool types ranked from 1 (constipation) to 7 (diarrhea). Types 1, 2, 6 and 7 were interpreted as having an unsatisfactory stool consistency and provided 1 point to the sum-score. (2) Pain was scored from 0 to 3 in how often the participant experienced abdominal pain the last two weeks (0=never, 1=sometimes, 2=often, 3=very often), answers 2 and 3 gave 1 point to the sum-score and answer 1 gave 0 point. (3) Average number of defeactions per week (from 0 to 10 times per week) was scored as follows: less than 2 times per week was interpreted as GI disturbances and provided 1 point to the sum-score. A food-frequency questionnaire, on participant’s food intake information 4 weeks retrospectively, was used to identify putative major differences in nutrient intake between ADHD patients and controls. The food questionnaire used in this study was based on the ETICS diet study questionnaire and consisted of 57 items representing common food units or common food groups (Kautto et al., 2014). The answer options ranged from “2 times or more per day” to “never in the last four weeks”. The frequency intake of the food items were converted into nutrient intake per participant based on the Swedish national food agency’s nutrition content database’s portion sizes and nutrient compositions adjusted for age and sex (Livsmedelsverket, 2015).

The Swedish Wellbeing scale was used to assess self-reported subjective wellbeing among adults (Ström and Carlbö 2014; Broacner 2015; fbanken.se 2020) recruited in the second half of the study (n = 57). Difference between the scale responders and the non-responders with regard to age, BMI, sex, ADHD medication and ASD score was not detectable (Table S2). The scale has been validated in a Swedish study of 107 psychiatric patients and 163 non-clinical persons (Broacner 2015), and showed good internal consistency (Cronbach’s α = 0.93 and ω = 0.95, respectively), high test-retest reliability (intraclass-correlation coefficient = 0.80, test-retest time interval: 7 ± 1 days) and high validity. The validity was determined by comparison with the validated scales Patient Health Questionnaire (PHQ-9) (Hanea et al., 2012) and Generalized Anxiety Disorder 7-item scale (GAD-7) (Spitzer et al., 2006). The scoring strategy of all the questionnaires included in this study are described in Table 1.

2.3. Measurement of inflammatory markers

Peripheral blood, without prior fasting, was collected in EDTA tubes between 8 a.m. and 4 p.m. Immediately after collection, the tube was centrifuged at 1700 g (3500 rpm) for 20 min and plasma was directly aliquoted into 3 sterile cryotubes and stored at –80 °C until analysis. The levels of CRP, SAA, sICAM-1 and sVCAM-1 in plasma were measured in April-May 2019 by a sandwich immunoassay using human 4-spot multiplex Mesoscale Discovery VPLEX Vascular Injury Panel 2 Human Kit (Cat. #K1519HD, Mesoscale Diagnostics, Maryland, USA), according to the manufacturer’s instructions. In each plate, standard curves were generated using the manufacturer-provided calibrators in duplicate and vascular injury control 1 and 2 were used as inter-plate controls. In total, five 96-well plates were run. All standard curves had a robust correlation (R2 > 0.999). The inter-plate coefficient of variation (CV) from inter-plate controls (same controls in each plate) and intra-plate CV from calibrators for the four analytes were: CRP 4.6% and 2.4%, SAA 11.0% and 1.5%, sICAM-1 8.9% and 5.3%, sVCAM-1 6.6% and 1.6%. The calculated lower limit of detection from five plates for each marker is the following ranges: CRP 2.39–3.35 μg/L, SAA 8.00–13.03 μg/L, sICAM-1 1.00–1.61 μg/L, sVCAM-1 5.43–8.43 μg/L. Each plasma sample was run in single well. Samples of both ADHD patients and controls were distributed equally in all the five plates. All values obtained from the plasma samples were within the detection range. All plasma samples had undergone two freeze/thaw cycles. Analyte data from two patients with ADHD were excluded because of suspected acute infections based on high CRP and SAA levels, leaving n = 154 patients with ADHD and 61 healthy controls in the statistical analysis. No other sample analyzed in this study had a distinct acute inflammation (CRP < 15 mg/L). A few outlier data points (nCRP = 3, nSAA = 2) were excluded, defined by levels more than 50 × interquartile range (IQR) from the median.

2.4. Statistical analysis

Non-normally distributed variables were assessed using non-parametric Mann-Whitney U test or Spearman’s rank correlation test and normally distributed variables were assessed using Student’s t-test or general linear modeling. The concentrations of analytes were generally not normally distributed and were therefore naturally logarithm-transformed to facilitate the consideration of covariates in parametric tests. To select the covariates for the association analysis between analyte levels and groups or symptom scores, stepwise regression with backward elimination based on AIC (Akaike’s Information Criteria) was performed. In comparison between patients and controls, age and sex were tested for covariate status. In analysis only within ADHD patients, age, sex, ADHD medication and medication with anti-inflammatory melatonin (Carrascal et al., 2018; Tarocco et al., 2019) were tested in children, whereas in adults other drugs with reported anti-inflammatory properties (antidepressants (Wijedocha et al., 2018), antipsychotics (Bobermin et al., 2018), anxiolytics (Lazzarini et al., 2001; Chen et al., 2018), sleeping pills (Tilgada et al., 2011), proton-pump inhibitors (Naganaram et al., 2020) and statins (Pinal-Fernandez et al., 2018)) were combined with melatonin in the variable named other adult drugs (OAD), and tested together with age, sex, ADHD medication and BMI. BMI and these drug data were unavailable for children. The relationships between the levels of analytes and symptom scores among ADHD patients were determined applying general linear models. Statistical significance was defined as α = 0.025 (Bonferroni correction for two independent analyte groups). When there was an association between analyte levels and total questionnaire scores, association analyses were conducted with the subscales as well. Suggestive significance was defined as α = 0.05. The statistical power was 80% to detect a level difference between patients and controls of CRP = 1.14e+05 pg/mL, SAA = 3.16e+06 pg/mL, sICAM-1 = 1.39e+05 pg/mL, sVCAM-1 = 1.16e+04 pg/mL at α = 0.025. All statistical analyses were per-
formed using the R Studio version 1.2.5033 (R Studio Inc, Boston, USA).

3. Results

3.1. Increased levels of sICAM-1 and sVCAM-1 in patients with ADHD

Two ADHD patients were excluded because of high CRP and SAA levels indicating ongoing acute infection. Characteristics of the remaining participants, being 154 patients with ADHD and 61 healthy controls, are shown in Table 2. Levels of sICAM-1 and sVCAM-1 were higher in participants with ADHD as compared to healthy controls, indicating vascular inflammatory activity, while no difference between these groups was found for CRP and SAA levels (Fig. 1(A)). None of these statistically analyzed participants, including both ADHD patients and controls, had acute systemic inflammation as all had CRP levels below 15 mg/L, and the vast majority had CRP levels (162/212) below 2 mg/L (= 2e+06 pg/mL) and SAA levels (205/213) below 10 mg/L (= 1e+07 pg/mL), which indicates no low-grade systemic inflammation (Markanday 2015; Targoska-Stepniak and Majdan 2014). Among ADHD patients, levels for the three markers CRP, sICAM-1, sVCAM-1 differed between children (n = 49) and adults (n = 105) (Fig. S1(A)). sICAM-1 and sVCAM-1 levels were higher in children (p<0.01; p<0.01), while CRP levels were higher in adults (p<0.01). Among adults, levels of SAA were higher in females than males among (p<0.001; p<0.001) (Fig. S1(B)). After adjusting for age and/or sex, the sICAM-1 and sVCAM-1 levels were still increased in ADHD patients compared to controls (p<0.001; p<0.001) (Fig. 1(B)). The differences in sICAM-1 and sVCAM-1 levels between ADHD patients compared to controls were found also when analyzing only adults (p<0.01; p<0.01), while the few children controls (n = 4, all being siblings to patients) allowed only an indication of sICAM-1 level difference between children with ADHD and children controls (p<0.01). We detected no difference in levels of the analytes between family controls and controls unrelataed to the ADHD patients (Fig. S2).

Correlation coefficients between any two of the markers are shown in Fig. 2, indicating that CRP and SAA were highly correlated (r = 0.70, p = 1.2e-32), and sICAM-1 and sVCAM-1 were highly correlated (r = 0.74, p = 6.6e-39). Analysis in ADHD patients only, and controls only, showed similar correlation patterns (for ADHD patients: rCRP-SAA = 0.69, p<0.001 and rSICAM-1-SVCAM-1 = 0.76, p<0.001; and for controls: rCRP-SAA = 0.74, p<0.001 and rSICAM-1-SVCAM-1 = 0.56, p<0.001) (Fig. S3).

Table 2 Clinical characteristics of the participants at baseline.

|                  | Children |                  | Adults |                  |
|------------------|----------|------------------|--------|------------------|
|                  | ADHD patients | Controls | ADHD patients | Controls |
|                  | (n = 49) | (n = 4) | (n = 105) | (n = 57) |
| Age [years]      | 13 (11-14) | 13 (12.8-13.2) | 36 (29-43) | 38 (34-43) |
| BMI [kg/m²]      | 23.8 (22.2-26.6) | 23.6 (21.6-27.6) | 23.8 (22.2-26.6) | 23.6 (21.6-27.6) |
| Sex              | Female | Male | Female | Male |
| ADHD             | Yes | No | Yes | No |
| Medication       | 17 (35) | 4 (100) | 17 (35) | 4 (100) |
| Antibiotic drugs | 14 (29) | 0 (0) | 14 (29) | 0 (0) |
| Melatonin        | 16 (33) | 0 (0) | 16 (33) | 0 (0) |
| Other prescribed drug for adults | 54 (51) | 3 (5) | 54 (51) | 3 (5) |

Results are given as median (25th-75th percentile [IQR]) or as number (% of subjects);.

1 ADHD ICD-10 diagnosis: F90.0 (18.8%), F90.08 (57.8%), F90.0C (17.5%), F90.0X (2.6%), F90.1 (0.7%), F90.8 (0.7%), F98.8 (2.0%);

2 ADHD medications for children include the stimulants Methylphenidate (n = 14), Lisdexamfetamine (n = 10), the nonstimulant Atomoxetine (noradrenalin re-uptake inhibitor, n = 4) and Methylphenidate Plus Atomoxetine (n = 3), and for adults they include Methylphenidate (n = 34), Lisdexamfetamine (n = 34), Dexamphetamine (n = 12), Atomoxetine (n = 3) and Methylphenidate Plus Atomoxetine (n = 1).

3 number of antibiotic drug use in the last two years (no one was on antibiotic drug use last 6 weeks);.

4 other prescribed drugs for adults include Antidepressants, Antipsychotics, Anxiolytics, Sleeping pills (mainly antihistamines), Proton-pump inhibitors and Statins;.

5 participants on medication currently or in last 3 months as “Yes”, not on medication currently or in the last 3 months as “No”. BMI = body mass index.

* difference between adult ADHD patients and adult controls at α = 0.05.
3.2. Associations between proinflammatory marker levels and ADHD medication, ADHD symptom score and functioning

We then examined whether there was any association for analyte levels with ADHD medication and clinical symptom and functioning scores among ADHD patients, in children and adults separately. For children, those currently on methylphenidate, dexamphetamine or atomoxetine in monotherapy had suggestively or significantly elevated sICAM-1 and sVCAM-1 levels compared to those without treatment using any of these drugs (Fig. S4). We detected no difference in sICAM-1 and sVCAM-1 levels between these different medications and therefore combined the different medications, although the number of children per medication was small. In children, those with current ADHD medication had higher sICAM-1 ($p = 0.0037$) and sVCAM-1 ($p = 0.0053$) levels than those without medication, while no such difference was found in adults (Fig. 3). Children with current ADHD medication had higher sICAM-1 and sVCAM-1 levels than adults with ADHD medication ($p_{sICAM-1} = 3.5e-09$, $p_{sVCAM-1} = 1.9e-09$), while no difference was detected
between children and adults without ADHD medication. No child ended treatment the last 3 months. The sICAM-1 and sVCAM-1 levels for those on stimulants between 3 and 24 months prior to the study were in similar range as for those who were never on medication during these 24 months (Fig. S5(A)).

There was no detectable difference of ADHD symptom score between participants on ADHD medication and those without ADHD medication ($\rho_{\text{children}} = 0.20$, $\rho_{\text{adults}} = 0.075$; Fig. S5(B)). We did not detect any analyte level association with inattention or hyperactivity-impulsivity symptom score (total scale score) (Fig. S6(A) and S7(A)) or overall functioning score (Fig. S6(B) and S7(B)). Further, younger age among children associated with higher sICAM-1 and sVCAM-1 levels ($\rho_{\text{sICAM-1}} = 0.0081$, $\rho_{\text{sVCAM-1}} = 0.0044$) (Fig. S8(A)). In adults, apart from the aforementioned association between sex and SAA, higher BMI was associated with higher levels of CRP and SAA ($\rho_{\text{CRP}} = 1.2e-05$, $\rho_{\text{SAA}} = 7.2e-06$) (Fig. S9(C)).

### 3.3. Associations between proinflammatory marker levels and common comorbid traits among patients with ADHD

None of the participants had an ASD diagnosis, however, autistic traits scored using AQ scale in adults were overrepresented in ADHD compared to controls ($\rho_{\text{adults}} = 4.4e-08$, Fig. 4(A)). Among children, where all controls were siblings to patients, no clear overrepresentation of overall autistic traits scored using SCQ scale was seen ($\rho_{\text{children}} = 0.11$). Among child patients only, the overall autistic symptom scores were higher in participants on current ADHD medication than in those without ADHD medication ($\rho_{\text{children}} = 0.021$, $\rho_{\text{adults}} = 0.60$; Fig. S5(B)) raising the question of whether or not

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**Fig. 3** Levels of CRP, SAA, sICAM-1 and sVCAM-1 in patients with ADHD stratified by current ADHD medication. (A) Children; (B) Adults. Y-axes (log10-scale) represent unadjusted analyte levels; differences were tested using nonparametric Mann-Whitney U test; for SAA in children and adults and CRP in adults, levels were adjusted by sex; effect size was assessed for each analyte ($R^2_{\text{CRP}} = 0.018$, $R^2_{\text{SAA}} = 0.007$, $R^2_{\text{sICAM-1}} = 0.172$, $R^2_{\text{sVCAM-1}} = 0.159$ for children; $R^2_{\text{CRP}} = 0.002$, $R^2_{\text{SAA}} = 0.001$, $R^2_{\text{sICAM-1}} = 0.010$, $R^2_{\text{sVCAM-1}} = 0.025$ for adults); the red lines in CRP and SAA represent 2e+i06 pg/mL (= 2 mg/L) and 1e+i07 pg/mL (=10 mg/L), respectively, the cutoffs for non-acute inflammation. Each dot represents a participant. ADHD medication includes methylphenidate, dexamphetamine, atomoxetine and for adults also lisdexamphetamine.

**Fig. 4** Autism symptoms and gastrointestinal (GI) symptoms and in ADHD patients and controls. (A) Y-axis represents unadjusted autism symptom score among adults scored using the Autism Spectrum Quotient (effect size: $R^2 = 0.19$). (B) Y-axis represents unadjusted GI symptom score. Both children and adults were scored using the same GI questionnaire and are included. Age-adjusted scores were used for statistical analysis (effect size adjusted $R^2 = 0.065$). The difference between controls and ADHD patients was analyzed using a nonparametric Mann-Whitney U test. Each dot represents a participant.
Proinflammatory mediators in ADHD

Fig. 5 Analytes (CRP, SAA, sICAM-1 and sVCAM-1) and total scales of comorbid traits in adults. Association analysis using general linear models of (A) analytes and autistic traits (total scale scores); (B) analytes and emotion dysregulation (total scale scores); (C) analytes and gastrointestinal symptoms (total scale scores). Y-axes represent residuals of natural logarithm (ln)-transformed analyte levels adjusted by the covariates listed. Each dot in the plot represents the residual from a participant. The x-axes represent total scale scores. \( \text{ar}^2 \): adjusted variance explained by the model. P-value: for the symptom score (x-axis). The following possible covariates were explored: age, sex, ADHD medication (AM), other adult drugs (OAD) and body mass index (BMI).

those ADHD patients with more severe autistic symptoms had higher levels of proinflammatory markers, particularly sICAM-1 and sVCAM-1. In children, we did not detect any linear association between analyte levels and overall autistic symptom score without or with covariate adjustment (Fig. 5(C)). In adults, however, sICAM-1 levels were increased with higher overall autistic symptom scores (adjusted \( \text{ar}^2 = 0.12, p = 0.022 \) (Fig. 5(A)). Since there was a sICAM-1 association to overall score of the AQ scale for adults, we assessed association between sICAM-1 levels and the subdomains in this scale (social skill, attention switching, attention to detail, communication, imagination). Elevated sICAM-1 levels were associated with impairments in attention to detail and imagination (\( \text{ar}^2 = 0.14, p = 0.0091 \) and \( \text{ar}^2 = 0.19, p = 0.00034 \), respectively) (Fig. 5(A)).

We carried out analysis between analyte levels and scores of the emotion dysregulation scale DERS-16 validated for adults only. There was a suggestive positive association between CRP levels and overall DERS-16 scores (\( \text{ar}^2 = 0.25, p = 0.025 \) (Fig. 5(B)). Analysis of CRP levels in relation to the DERS-16 subscales (clarity, goals, impulse, strategies, and non-acceptance), showed that higher CRP levels were associated with more limited access to effective emotion regulation strategies (\( \text{ar}^2 = 0.27, p = 0.0059 \)) and suggestive associated with the impulse control difficulties (\( \text{ar}^2 = 0.24, p = 0.047 \) (Fig. 5(B)).

The patients with ADHD had more GI symptoms than healthy controls had (age-adjusted \( p = 1.9 \times 10^{-4} \) for children and adults combined, Fig. 4(B)). In adults, CRP levels were positively associated with GI symptom score \( \text{ar}^2 = 0.24, p = 0.014 \), while SAA levels showed a suggestive positive association with GI symptom score (\( \text{ar}^2 = 0.25, p = 0.036 \) (Fig. 5(C)). In children, there was no association between GI symptom score and analyte levels (\( p > 0.066 \); Fig. 5(D)).

The adult patients recruited in the second half of the study \( (n = 57) \) responded to the Swedish Wellbeing scale. We could not detect any linear association between the wellbe-
Fig. 6 Analytes (CRP and sICAM-1) and subscales of comorbid traits in adults. Association analysis using general linear models of (A) analytes and subscales of autistic traits: attention to detail, imagination, and social skill where higher scores indicate more symptoms; (B) analytes and DERS-16 subscales of emotion dysregulation: limited access to effective emotion regulation strategies, impulse control difficulties, and difficulties engaging in goal-directed behavior, where higher scores indicate more difficulties. Y-axes represent residuals of natural logarithm (ln)-transformed analyte levels adjusted by the covariates listed. Each dot in the plot represents residual from a participant. The x-axes represent sub-scale scores. \(r^2\): adjusted variance explained by the model. \(P\)-value: for the symptom subscale score (x-axis). The following possible covariates were explored: age, sex, ADHD medication (AM), other adult drugs (OAD) and body mass index (BMI).

4. Discussion

4.1. Elevated sICAM-1 and sVCAM-1 levels in ADHD and in children with ADHD medication

Our case-control study found elevated plasma levels of the inflammatory adhesion molecules sICAM-1 and sVCAM-1 in adult patients with ADHD, indicating overrepresentation of an endothelial dysfunction and vascular inflammatory activity in ADHD. Further, among children with ADHD, those with current ADHD medication had higher sICAM-1 and sVCAM-1 levels than children without ADHD medication and than adults with ADHD medication. We found no difference in levels between children and adults without ADHD medication. Due to a small sample size (n = 4 children controls), we could not determine if children without ADHD medication had higher levels than controls.

Previous possible support for a vascular inflammatory activity in ADHD include epidemiological studies reporting increased risk for type 2 diabetes (T2DM) in pediatric ADHD (Chen et al., 2013), and increased risk for T2DM and hypertension in adults with ADHD (Chen et al., 2018), likely through an unhealthy lifestyle and increased BMI (McClernon and Kollins 2008; Chen et al., 2017). ICAM-1 and VCAM-1 levels are often elevated in insulin resistance and cardiometabolic disorders (Song et al., 2007; Fathollahi et al., 2018). Maybe elevated plasma sICAM-1 and sVCAM-1 levels could be possible early markers for later cardiometabolic risk in ADHD. A small case-control study (25 patients vs. 18 controls) showed elevated blood levels of sICAM-1 in children with ADHD (Alasehiri et al., 2015). Levels of CRP, SAA and sVCAM-1 in relation to ADHD have to our knowledge not previously been reported. Further support for an immune activity component in ADHD include small-medium size genetic association studies, and small case-control studies reporting elevated IL-6 and IL-10 levels in children with ADHD (Darwish et al., 2019; Mitchell and Goldstein 2014; Donfrancesco et al., 2016; Leffa et al., 2018). However, there are very few previous reports on inflammatory markers in adults with ADHD, where plasma levels of IL-6 and TNF-\(\alpha\) in un-medicated adults with ADHD were reported to be at control levels (Corominas-Roso et al.,
Nevertheless, in ADHD rat models, being spontaneously hypertensive, increased levels of proinflammatory cytokines were found in serum and spleen (Kozlowska et al., 2019). Further, ADHD has a high comorbidity with immune-mediated conditions, like atopic dermatitis and asthma (Leffa et al., 2018). A strong genetic correlation was found between ADHD and asthma with cross-disorder genome-wide significant loci. In fact, a small causal effect of ADHD on asthma was found (Zhu et al., 2019). sICAM-1 and sVCAM-1 are also associated with hypertension (Chae et al., 2001).

One common side effect of stimulants used in treatment of ADHD, e.g. methylphenidate and amphetamines, is slightly increased heart rate and blood pressure (Mick et al., 2013). However, it is inconclusive whether or not stimulant use in ADHD is associated with a small increased risk of cardiovascular events (Shin et al., 2016; Dalsgaard et al., 2014; Winterstein 2013). These stimulants have also been shown to induce neuroinflammation in rodents at chronic high therapeutic doses (Coelho-Santos et al., 2019). Atomoxetine, however, has been reported to have anti-inflammatory effects in the CNS (O’Sullivan et al., 2010; O’Neill et al., 2020; Valvassori et al., 2019), so our indications of elevated sICAM-1 and sVCAM-1 in children not only for stimulants but also for atomoxetine treatment may seem unexpected. Notably however, we do not know if these elevated levels reflect the BBB status or derive only from other sites less essential for CNS protection.

4.2. Proinflammatory marker levels in ADHD and in relation to comorbidities

As previously reported from other cohorts, autistic symptoms and GI symptoms were more common among the ADHD patients compared to controls. However, due to a small set of control children (n = 4) we could not show this in a children-only analysis. As not only sICAM-1 and sVCAM-1 levels, but also autistic symptom scores were higher in children on current stimulant medication compared to those without such medication, we tested if the analyte level elevations in those medicated could be because they had a more severe disorder. However, in the children we could not detect any linear association between the analyte levels and overall autistic symptom scores. A limitation, however, is that we could assess those medicated only after the medication had started. But, uniquely among the adult ADHD patients, higher sICAM-1 levels were associated with increased comorbid autistic symptoms in the domains attention to detail and imagination. Also comorbid mood disorders are common in ADHD and both ICAM-1 and VCAM-1 have been reported upregulated in depressive disorder and bipolar disorder (Thomas et al., 2002, 2004). The adult participants were scored using a wellbeing scale with items reflecting depressive symptoms. We could not detect any association between the wellbeing mean score and any of the analytes measured suggesting that the relationship between sICAM-1 levels and autistic symptom was not explained by depressive symptoms. Importantly, these findings need replication and elucidation of causality. Levels of proinflammatory cytokines have consistently been reported elevated in patients with ASD (Mitchell and Goldstein 2014), although sICAM-1 and sVCAM-1 levels have been reported decreased in ASD (Kamen et al., 2013; Onore et al., 2012). Elevated CRP levels, on the other hand, were in our study associated with more severe GI symptoms in adults with ADHD, whereas elevated SAA levels showed a suggestive association with more GI symptoms. CRP levels have been reported to positively correlate with GI symptoms in children with asthma (Zhang et al., 2018). Lower SAA levels were found in the mucosal healing process in patients with Crohn’s disease (Yarur et al., 2017). Among both ADHD patients and healthy controls, there were high correlations between CRP and SAA levels, and between sICAM-1 and sVCAM-1 levels, in this sample of subjects without acute inflammation. Previously, correlations between CRP and SAA levels and between sICAM-1 and sVCAM-1 levels are well established (Sjoholm et al., 2009; Yarur et al., 2017; Nielsen et al., 2007), but not in ADHD. Moreover, increased CRP levels were associated with a more severe emotion dysregulation in total scale and the strategies-subscale among adults. A similar association between CRP levels and emotion dysregulation was previously reported in women with T2DM (Powers et al., 2016).

Our study explored the analytes of a manufacturer-designed highly validated four-marker panel, because of these markers’ joint roles in vascular inflammation, tissue damage and cell adhesion. Since the strong correlations between levels of the pairs CRP/SAA and sICAM-1/sVCAM-1, the multiple comparisons were corrected for two tests. We included age, sex, ADHD medication, medication with anti-inflammatory melatonin, and for adults also BMI and other drugs with reported anti-inflammatory effects as covariates in the data analysis. That the SAA levels were elevated in female compared to male adults with ADHD and healthy controls was found also previously in healthy individuals and those without T2DM (Sjöholm et al., 2009), and sex-dependent effects of perinatal inflammation on brain development related to neuropsychiatric disorders, including ADHD, has been reported (Ardalan et al., 2019). Meanwhile, we found that sICAM-1 and sVCAM-1 levels were higher in younger individuals. There are known intrinsic age-associated changes in the immune system and thus age-related differences in immune marker levels (Simon et al., 2015). In adults, apart from the aforementioned association between sex and SAA, higher BMI was associated with higher levels of CRP and SAA, although almost all cases had levels indicating no systemic inflammation. High BMI or obesity are known to contribute to inflammation and cardiovascular disorders.

4.3. Limitations

The sample size for children with an ADHD diagnosis was small (n = 49) and that might explain why associations between inflammatory markers and comorbid traits were found only in adults (n = 105). Moreover, since we had only n = 4 children controls we could not detect differences between children with ADHD and children controls. However, the sample size of adults with ADHD was large compared to previous studies of inflammatory plasma markers in ADHD, still the results need to be replicated. Further, there were two categories of controls each with small sample size.
(\(n_{\text{family-member}}=39\) and \(n_{\text{un-related}}=22\)), however, as we could not detect any difference in the inflammatory marker levels between the two categories (\(p>0.32\)), they were combined. Of note, to adequately elucidate whether relatives of those with ADHD have elevated analyte levels or not, larger sample sizes and considerations of type of relatedness are required. Of common comorbidities in ADHD we screened only for autism in children, and in adults only for autism, emotion dysregulation and wellbeing. We could not adjust for effects of BMI on analyte levels in children, since for them BMI was not measured. However, we found no association for BMI with sICAM-1 or sVCAM-1 in the adults. Our attempt to adjust for medications with anti-inflammatory effects (melatonin, antidepressants, antipsychotics, anxiolytics, sleeping pills, proton-pump inhibitors and statins) in the analysis of sICAM-1 in adults is incomplete as we lack information on the specific drug names and drugs specifically targeting inflammation. Diet may affect the levels of the four markers studied (Verlaet et al., 2014). However, comparing the diet between the ADHD patients and healthy controls regarding fat, protein, carbohydrates and energy we did not detect any significant difference (Table S1). Circadian rhythmicity in sICAM-1 and sVCAM-1 levels over the sampling times used (8 a.m. to 4 p.m.) is likely minor but cannot be excluded as these levels were reported stable between 7:30 a.m. and 2:30 p.m. but dropped at 6 p.m. and returned to morning-afternoon levels at 9 p.m. (Wipfler et al., 2013). The GI questionnaire was self-designed based on the standard Bristol Stool Scale but not validated in other studies. Finally, we measured the levels of inflammatory markers only in plasma as cerebrospinal fluid samples were not available.

In conclusion, adults with ADHD had increased plasma levels of sICAM-1 and sVCAM-1. In children with ADHD, the sICAM-1 and sVCAM-1 levels were higher among those with current ADHD medication than among children and adults without ADHD medication. In adults with ADHD, higher sICAM-1 levels were associated with more comorbid autistic symptoms. Thus, our results suggest that vascular inflammatory activity is overrepresented in ADHD, relates to current ADHD medication in children, and relates to certain comorbid autistic symptoms in adults. The site of the likely upregulated membrane-bound adhesion molecules is however unknown. CRP levels were positively associated with GI symptoms possibly indicating involvement of the gut-brain axis. Replication studies are warranted. Identification of a putative immune activity component in ADHD, linked to ADHD medication and/or comorbid autistic traits, may have pathophysiological and therapeutic implications.

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Contributors

All authors revised and approved the final version of the manuscript. Formulating the research question(s): CL, LY, MG; Designing the study: CL, MG; Carrying it out: LY, ES, ÅS, MG, CL; Analysing the data: LY, MStiernborg; Writing the article: LY, CL

Conflict of Interest

The authors have no competing financial interests in relation to the work described.

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Supplementary materials

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