INFLAMMATORY BIOMARKERS AND BRAIN HEALTH INDICATORS IN CHILDREN WITH OVERWEIGHT AND OBESITY: THE ACTIVEBRAINS PROJECT

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3 **PROJECT**

4 **ABSTRACT**

5 **Introduction.** Chronic inflammation plays an important role on the pathogenesis of

6 several cardiovascular and metabolic diseases, as well as on brain function and

7 behaviour. The aim of the present study was to examine the associations between

8 inflammatory biomarkers and a wide range of brain health indicators (i.e., academic

9 performance, executive function, behavioural and emotional functioning, and brain

10 volume) in children with overweight/obesity.

11 **Methods.** A total of 107 children (10.0 ± 1.1 years, 41% girls) from the ActiveBrains

12 project were included in the analysis. Five inflammatory biomarkers were analysed in

13 plasma: white blood cell (WBC) count, interleukin-6 (IL-6), interleukin-1β, tumor

14 necrosis factor-α (TNF- α), and C-reactive protein (CRP). Academic performance was

15 assessed by Woodcock-Muñoz Tests of Achievement. Executive function was assessed

16 through the Design Fluency Test for cognitive flexibility, the Stroop test for cognitive

17 inhibition, and the Delayed Non-Match-to-Sample task for working memory.

18 Behavioural and emotional functioning was evaluated through the Behavior Assessment

19 System for Children (BASC) questionnaire. Total and regional brain volume was

20 assessed by magnetic resonance imaging.

21 **Results.** IL-6 was inversely associated with adaptive skills (β=-0.228; p=0.030), while

22 TNF-α was related to mathematics (β=-0.198; p=0.034). In addition, CRP was

23 positively associated with externalizing (β=0.246; p=0.046) and internalizing problems

24 (β=0.234; p=0.039), as well as the behavioural symptoms index (β=0.236; p=0.047).

25 However, these significant associations disappeared after multiple comparisons

26 correction. Inflammatory biomarkers were not associated with executive function and

27 total brain volumes. Regarding regional brain analyses, WBC was positively associated

28 with gray matter volume in the left middle temporal gyrus (β=0.387; p<0.001, k=44),

29 and CRP was positively associated with gray matter volume in the right superior

30 temporal gyrus (β=0.439; p<0.001, k=29). Additionally, when adjusting by total brain

31 volume, CRP was positively associated with gray matter volume in the right

32 supplementary motor cortex (β=0.453; p<0.001, k=51). Moreover, both, IL-6 (β=0.366;

33 p<0.001, k=81) and TNF-α (β=0.368; p<0.001, k=62) were positively associated with

34 white matter volume around the right inferior frontal gyrus pars opercularis, while CRP

35 was inversely associated with white matter volume around the left superior frontal gyrus

36 (β=-0.482; p<0.001, k=82). After adjusting by total brain volume, CRP was also

37 inversely associated with white matter volume in 3 additional clusters (β ranging

38 from -0.473 to -0.404; p<0.001, k=87).

39 **Conclusions.** Inflammation was slightly associated with brain health (i.e., academic

40 performance, behavioural and emotional functioning and regional brain volume) in

41 children with overweight or obesity. Further larger longitudinal and interventional

42 studies are warranted to elucidate the short-term and long-term effect of systemic low-

43 grade inflammation on children's brain health.

44 **Keywords:** inflammation, school performance, cognition, adaptive functioning, mental

45 health, brain structure.

INTRODUCTION

Childhood obesity has increased steadily in the past three decades becoming a serious worldwide health issue, with a prevalence rate of 23.8% of boys and 22.6% of girls in developed countries, and 12.9% of boys and 13.4% of girls in developing countries (Ng et al., 2014). Apart from weight gain, obesity has been closely linked to a cluster of disorders known as metabolic syndrome, resulting in subsequent systemic low-grade inflammation (Lumeng & Saltiel, 2011). Besides chronic low-grade inflammation plays an important role on the pathogenesis of several cardiovascular and metabolic diseases such as atherosclerosis, diabetes, autoimmune diseases, and cancer (Hotamisligil, 2006; Libby, 2006), there is emerging evidence suggesting an association between inflammation and brain health, including cognitive, behavioural and emotional functioning (Slopen, Kubzansky, & Koenen, 2013), as well as brain structure and function (Borsini, Zunszain, Thuret, & Pariante, 2015).

Inflammatory biomarkers circulating in blood could access the central nervous system through different pathways, which might affect brain health. Particularly, cytokines may cross the blood–brain barrier (BBB) via active transport mechanisms or via vagal nerve stimulation (Banks, Lynch, & Price, 2009). In addition, within the brain, inflammatory biomarkers could be expressed by astroglia, microglia, neurons and endothelial cells (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Indeed, in both, humans and animal models, overexpression of peripheral pro-inflammatory biomarkers has been associated with impaired synaptic plasticity, neurogenesis and neuromodulation (McAfoose & Baune, 2009), which in turn, may affect cognitive (e.g., reduction of spatial learning and memory skills), behavioural and emotional functioning, as well as brain structure (e.g., gray matter atrophy and lower tissue microstructure) (Borsini et al., 2015; Yirmiya & Goshen, 2011).

Prior research examining the relationship between peripheral inflammatory biomarkers and brain health indicators in humans have mainly focused on the two endpoints of the lifespan (i.e., preterm and elderly populations). For instance, in preterm infants, perinatal inflammation has been associated with both, structural (e.g. risk of white matter damage) and functional brain alterations (e.g. diparesis, and impaired cognitive functioning, mental and motor development) (Kuban et al., 2017, 2015; O'Shea et al., 2013; Rose, Vassar, Cahill-Rowley, Hintz, & Stevenson, 2015; Voltas et al., 2017). In elderly populations, inflammation has been closely related to neurodegenerative disorders such as Alzheimer's disease, dementia, and cognitive decline (Sartori, Vance, Slater, & Crowe, 2012), behavioural disorders (Rosenblat, Cha, Mansur, & McIntyre, 2014), and even with brain damage (Frodl & Amico, 2014).

Evidence in healthy midlife adults has shown an inverse association of inflammatory biomarkers with executive function (e.g., cognitive inhibition, working memory and attention) (Windham et al., 2014), behavioural and emotional functioning (Marteinsdottir, Ernerudh, Jonasson, Kristenson, & Garvin, 2016), and brain volume (Marsland, Gianaros, Abramowitch, Manuck, & Hariri, 2008; Marsland et al., 2015). However, in normal developing children and adolescents, the limited research available has only indicated that inflammatory biomarkers may negatively influence academic performance (Esteban-Cornejo et al., 2016), cognitive function (Cullen et al., 2017) and intelligence (Lee et al., 2016). Thus, further studies investigating the association of inflammation and brain health, including academic performance, cognition, behavioural and emotional factors, and brain measurements in children are needed.

Given that inflammation is one of the earliest consequences of obesity, which in turn, has also shown to alter brain health in children (AL Miller, Jong, & Lumeng, 2015; Sanders, Han, Baker, & Cobley, 2015), examining the influence of inflammation

96 on brain health indicators in the context of childhood obesity is of paramount

97 importance. To the best of our knowledge, this is the first study examining the influence

98 of inflammation on a wide range of brain health indicators, including gray and white

99 matter volumes in children. Thus, the aim of the present study was to examine the

100 associations of inflammatory biomarkers with brain health indicators (i.e., academic

101 performance, executive function, behavioural and emotional functioning, and total and

102 regional brain volume) in children with overweight/obesity.

METHODS

Participants

The present cross-sectional study is part of the ActiveBrains project (http://profith.ugr.es/activebrains), a randomized controlled trial aimed to analyse the effects of an exercise program on brain, cognitive and academic performance, as well as on selected physical and mental health outcomes in children with overweight/obesity. Additional information about the methodology of the project can be found elsewhere (Cadenas-Sánchez et al., 2016). The results presented in this cross-sectional analysis belong to the baseline data obtained between November 2014 and February 2016. We estimated that a sample of 100 participants would be required to provide statistical power of 80% with a level of significance of 0.05, assuming a dropout rate of 10%. All participants were recruited from schools and university hospitals of Granada (southern Spain). The final sample included 107 children with overweight or obesity (10.0 ± 1.1 years old; 41% girls) who had at least valid data for one inflammatory biomarker and one brain health indicator.

Parents or guardians were informed of the nature and characteristics of the study, and all signed an informed written consent. The ActiveBrains project was approved by the Human Research Ethics Committee of the University of Granada and was registered in ClinicalTrials.gov (identifier: NCT02295072).

Inflammatory biomarkers

After an overnight fast (at least 12 h), blood samples were drawn from the antecubital vein. Blood samples in tubes containing EDTA were spun immediately at 1000g for 10 min. Plasma was isolated and stored at -80°C until analysis in the Center of Biomedical Research (Granada, Spain). Five key inflammatory biomarkers analysed

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127   in plasma were included in this study: white blood cell (WBC, $10^3/\mu L$) count,

128   interleukin-6 (IL-6, pg/mL), interleukin-1β (IL-1β, pg/mL), tumor necrosis factor-α

129   (TNF-α, pg/mL), and C-reactive protein (CRP, mg/L). WBC count was analysed with

130   automated blood cell counters. IL-6, IL-1β and TNF-α were quantified by multiple

131   analyte profiling technology (MILLIPLEX® MAP Human High Sensitivity T Cell

132   Magnetic Bead Panel, EMD Millipore Corporation, Missouri, U.S.A.) using a kit plex

133   (HCYIL6-MAG Anti-Human IL-6 Beads set, HCYIL1B-MAG Anti-Human IL-1β

134   Bead, and HCYTNFA-MAG Anti-Human TNFα Beads set). The intra- and inter-assay

135   precision coefficients of variation (CVs) for IL-6 were 5% and 20%, respectively, and

136   sensitivity was 0.11 pg/mL. For both, IL-1β and TNF-α the intra- and inter-assay

137   precision CVs were 5% and 15%, respectively, with a sensitivity of 0.14 pg/mL for IL-

138   1β, and of 0.16 pg/mL for TNF-α. CRP was determined by turbidimetry.

139   *Academic performance*

140   Academic performance was assessed by the Spanish version of the Batería III

141   Woodcock-Muñoz Tests of Achievement, which has shown a high reliability and

142   validity (McGrew & Woodcock, 2001). Thirteen tests were individually administered in

143   one session of 100-120 min, and the obtained data were processed using the

144   Compuscore and profile software version 3.1 (Riverside Publishing Company, Itasca,

145   IL, USA). For the current study, a standard T-score based on an average of 100 and

146   standard deviations of 15 points was obtained for the following broad academic

147   performance indicators: mathematics (including calculation skills, problem solving and

148   the ability to subtract, sum, multiply or divide quickly), reading (including word

149   identification, reading speed and comprehension), writing (including spelling, quality of

150   written sentences and speed of writing), and total achievement (including mathematics,

151   reading and writing).

Executive function

The assessment of executive function was conducted individually for each child, and lasted approximately 45-60 min. Three main indicators were assessed: cognitive flexibility, cognitive inhibition and working memory.

Cognitive flexibility and cognitive inhibition were assessed using two paper-pencil based sub-tests of the Delis–Kaplan Executive Function System (D–KEFS) (Delis, Kaplan, & Kramer, 2001). For cognitive flexibility, we used the Design Fluency Test (DFT) (Delis et al., 2001), in which participants should connect dots using only four straight lines to design as many novel shapes as possible in periods of 60 seconds. The total number of correct drawn designs was registered and used in the analysis. For cognitive inhibition, we used a modified version of the Stroop test (Stroop, 1935) called the Stroop Colour Word Test, which includes measurements of 1) fundamental linguistic skills (i.e., namely speed of naming), and 2) inhibition, where colour-words are printed in a colour that differs from their meaning, and the task consists of naming the colour of the word and avoiding its reading. An interference score was obtained by subtracting completion times (2-1) (Moreno-López, Soriano-Mas, Delgado-Rico, Rio-Valle, & Verdejo-García, 2012). Because the Stroop interference scores are inversely related to cognitive inhibition, it was multiplied by -1.

Working memory was measured using a modified version of the Delayed Non-Match-to-Sample (DNMS) computerized task, that was previously developed to differentiate between manipulation (high memory load) and maintenance (low memory load) cognitive processes (Robinson et al., 2009). Each trial was presented on a computer screen using E-Prime and consisted of two phases: sample and choice. Sixteen practice trials plus 140 experimental trials were randomly presented. Participants had to remember 4 Pokémon cartoons (i.e., sample phase), and subsequently, to select the

177 cartoon that had not previously appeared (i.e., choice phase) between two different

178 targets. In the high memory load condition, in which 4 different stimuli were presented

179 before the choice phase, reaction time and response accuracy were registered. A ratio of

180 working memory was calculated as the quotient between reaction time and response

181 accuracy, and for analytic purposes, this ratio was multiplied by -1, so higher ratio

182 indicates better working memory.

183 *Behavioural and emotional functioning*

184   The Behavior Assessment System for Children (BASC), level-2 for children

185 aged 6-12 years old, which has shown extensive psychometric properties in both non-

186 referred and clinical populations with reliabilities for the subscales ranging from 0.80 to

187 0.87, was completed by parents to assess negative behaviours and positive attributes

188 (Reynolds & Kamphaus, 2004). BASC responses cluster into 4 global dimensions of

189 behavioural and emotional functioning: externalizing problems (including aggressivity,

190 hyperactivity and behavioural problems), internalizing problems (including anxiety,

191 depression and somatization), adaptive skills (including adaptability, social skills and

192 leadership) and a behavioural symptoms index (including aggressivity, hyperactivity,

193 attention problems, atypical behaviours, anxiety and depression). For each indicator,

194 standard T-scores with an average of 50 and standard deviations of 10 points were used

195 in the analyses.

196 *Magnetic Resonance Imaging (MRI) procedure*

197   All images were collected on a 3.0 Tesla Siemens Magnetom Tim Trio scanner

198 (Siemens Medical Solutions, Erlangen, Germany) equipped with a 32-channel head coil.

199 High-resolution, T1-weighted images were acquired using a 3D MPRAGE

200 (Magnetization-Prepared Rapid Gradient-Echo) sequence. Acquisition parameters were:

201   repetition time (TR) = 2300 ms, echo time (TE) = 3.1 ms, inversion time (TI) = 900 ms,

202   flip angle = 9º, field of view (FOV) = 256 x 256, acquisition matrix = 320 x 320, 208

203   slices, resolution = 0.8 x 0.8 x 0.8 mm, and scan duration of 6 min and 34 s.

204   Imaging data were pre-processed using the Statistical Parametric Mapping

205   software (SPM 12; Wellcome Department of Cognitive Neurology, London, UK)

206   implemented in Matlab (The MathWorks, Inc, Natick, MA). Information about pre-

207   processing steps has been previously detailed (Esteban-Cornejo et al., 2017). First,

208   using the latest segmentation algorithm implemented in SPM12, we segmented T1-

209   weighted structural images of each participant into gray matter tissue, white matter

210   tissue, and cerebrospinal fluid (Ashburner & Friston, 2005). Second, segmented gray

211   matter/white matter tissues for all participants were used to generate a customized

212   template using Diffeomorphic Anatomical Registration Through Exponentiated Lie

213   algebra (DARTEL) (Ashburner, 2007). DARTEL estimates a best set of smooth

214   deformations from each participant's tissue to their common average and reiterates the

215   process until convergence. The resulting images were spatially normalized to Montreal

216   Neurological Institute (MNI) space with affine transformation to create the DARTEL

217   template. Subsequently, each participant's segmented images were normalized to the

218   DARTEL template via nonlinear transformation. In order to perform a volume change

219   correction, the normalized gray matter images were modulated with Jacobian

220   determinants derived from the spatial normalization (Ashburner & Friston, 2000).

221   Finally, the volumetric images were smoothed by convolving them with an isotropic

222   Gaussian kernel of 8 mm full-width at half-maximum (FWHM). Total gray and white

223   matter volumes were calculated from the non-normalized segmented images. Total

224   brain volume (TBV) was calculated by adding the volumes of gray and white matter.

225   *Covariates*

226   Sex, peak height velocity (PHV), parental education level, body mass index

227   (BMI) and TBV were included as covariates.

228   PHV was obtained from weight, height and seated height using Moore's

229   equations (Moore et al., 2015). Years from PHV were calculated from the chronological

230   age, and the difference in years was used in the analysis as a value of maturation.

231   Parental education level was used as a proxy of socioeconomic status. Parent

232   responses were combined as: neither of the parents had a university degree, one of the

233   parents had a university degree and both parents had a university degree.

234   Body weight was measured to the nearest 0.1 kg using an electronic scale

235   (SECA 861, Hamburg, Germany) lightly dressed and without shoes. Height was

236   measured to the nearest 0.1 cm using a wall-mounted stadiometer (SECA 225,

237   Hamburg, Germany). Measures were assessed in duplicate and average measures were

238   used for data analysis. BMI was calculated as weight/height square (kg/m^2) and BMI

239   categories were defined (i.e. overweight, obesity grade I, II, III) according to age- and

240   sex-specific BMI cut-off points (Cole & Lobstein, 2012).

241   *Statistical analysis*

242   Descriptive characteristics are presented as mean and standard deviations or

243   percentages. All variables were checked for normality using both graphical (normal

244   probability plots) and statistical (Kolmogorov-Smirnov test) procedures. Due to its

245   skewed distribution, CRP, IL-6, TNF-α, IL-1β, cognitive inhibition and working

246   memory ratio were normalized using Blom's formula before analysis (Blom, 1958). As

247   preliminary analyses showed no significant interactions of sex with inflammatory

248   biomarkers in relation to brain health indicators (all $p > 0.10$), all analyses were

249   performed for the whole sample.

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250   Multiple linear regression was used to analyse the association of inflammatory

251   biomarkers with academic performance, executive function, behavioural and emotional

252   functioning and total brain volumes adjusting for sex, PHV, parental education level,

253   and BMI. We conducted the Benjamini-Hochberg correction for assessing multiple

254   comparisons between inflammatory biomarkers and brain health indicators. Briefly, this

255   method uses ranked p-values to determine the cut-off, at which point the Type-I error

256   rate is below 0.05 (Benjamini & Hochberg, 1995). All the analyses were performed

257   using the IBM SPSS Statistics for Windows version 22.0 (Armonk, NY: IBM Corp),

258   and the level of significance was set at $P < 0.05$.

259   Statistical analyses of imaging data were performed using the General Lineal

260   Model approach implemented in SPM12. The association of each inflammatory

261   biomarker with gray matter volume was examined using five whole-brain voxel-wise

262   multiple regression models (one for each inflammatory biomarker), adjusted for sex,

263   PHV, parental education level and BMI. Additional analyses were conducted including

264   TBV. We performed similar whole-brain voxel-wise multiple regression models for

265   white matter volume. In addition, we extracted the eigenvalues from the peak

266   coordinates of each significant cluster.

267   The statistical threshold in the imaging analyses was calculated with AlphaSim,

268   as implemented in Resting-State fMRI Data Analysis Toolkit toolbox (RESTplus)

269   (Song et al., 2011). Parameters were defined as follows: cluster connection radius

270   (rmm) = 5mm and the actual smoothness of the data after model estimation,

271   incorporating a gray mask volume of 128190 voxels or a white matter mask volume of

272   302567 voxels, respectively. The voxel-level alpha significance (threshold, $p<0.001$

273   uncorrected) along with the appropriate cluster size for controlling for multiple

274   comparisons in each analysis were indicated in the results. The resulting cluster extents

275   were further adjusted to account for the non-isotropic smoothness of structural images,

276   in accordance with Hayasaka et al. (2004).

RESULTS

Descriptive characteristics of the study population, including inflammatory biomarkers and brain health indicators, are presented in **Table 1**. Participants showed a BMI of 26.7 kg/m^2, being 26% overweight and 74% obese. Overall, boys and girls showed similar values of inflammatory biomarkers. Academic performance ranged from 102.1 to 114.2 ($\pm$ ~12), cognitive flexibility scored 20.0, cognitive inhibition scored -40.6, and working memory ratio was 15.2. In addition, participants presented behavioural and emotional functioning indicators ranging from 49.0 to 54.4, and a total brain volume of 1199 cm^3 (793 cm^3 of gray matter).

The results of the multiple linear regression models showing the associations of inflammatory biomarkers with brain health indicators after adjustment for sex, PHV, parental education level, and BMI are shown in **Table 2**. IL-6 was inversely associated with adaptive skills (β=-0.228; p=0.030), and TNF-α was inversely related to mathematics (β=-0.198; p=0.034). In addition, CRP was positively associated with externalizing (β=0.246; p=0.046) and internalizing problems (β=0.234; p=0.039), as well as, with the behavioural symptoms index (β=0.236; p=0.047). However, these significant associations disappeared after multiple comparisons correction. Inflammatory biomarkers were not associated with executive function and total brain volumes.

Figure 1 displays the brain regions showing positive associations between inflammatory biomarkers and gray matter volume after adjustment for sex, PHV, parental education level and BMI. WBC was positively associated with gray matter volume in the left middle temporal gyrus (β=0.387, t=4.20; p<0.001, k=44; Figure 1A). In addition, CRP was positively associated with gray matter volume in the right superior temporal gyrus (β=0.439, t=4.37; p<0.001, k=29; Figure 1B). IL-6, IL-1β and TNF-α

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were not positively associated with regional gray matter volume. Furthermore, there were no statistically significant inverse associations between any inflammatory biomarker and gray matter volume in any region of the brain. Similar results were found after adjusting by TBV (**Table S1**). Additionally, CRP was positively associated with gray matter volume in the right supplementary motor cortex (β=0.453, t=3.88; p<0.001, k=51).

Figure 2 presents the brain regions showing positive and inverse associations between inflammatory biomarkers and white matter volume after adjustment for potential confounders. IL-6 (β=0.366, t=4.00; p<0.001, k=81) and TNF-α (β=0.368, t=3.98; p<0.001, k=62) were positively associated with white matter volume around the right inferior frontal gyrus pars opercularis (Figure 2A). CRP was inversely associated with white matter volume around the left superior frontal gyrus (β=-0.482, t=-5.11; p<0.001, k=82; Figure 2B). Furthermore, there were no other statistically significant positive or inverse associations between any inflammatory biomarker and white matter volume in any region of the brain. Results persisted after including TBV as a covariate (**Table S2**). Furthermore, CRP was inversely associated with white matter volume bilaterally around the superior frontal gyrus pars orbital (β=-0.404, t=-3.76 and β=-0.473, t=-4.17; p<0.001, k=87), and around the right middle cingulum (β=-0.424, t=-3.77; p<0.001, k=87).

DISCUSSION

The main findings of the present study indicated that inflammatory biomarkers were slightly associated with brain health indicators in children with overweight/obesity. Higher levels of IL-6 and TNF-α were associated with lower adaptive skills and mathematics, respectively, while CRP was positively associated with externalizing and internalizing problems, as well as with the behavioural symptoms index. However, these significant associations disappeared after correcting for multiple comparisons. In addition, inflammation was not associated with executive function and total brain volumes. Unexpected, a whole-brain analytical approach revealed that higher levels of WBC and CRP were associated with greater regional gray matter volume in the left middle temporal gyrus, and in the right superior temporal gyrus, respectively. Moreover, higher levels of IL-6 and TNF-α were associated with greater white matter volume around the right inferior frontal gyrus pars opercularis, while CRP was the only inflammatory biomarker showing an expected and inverse association with white matter volume around the left superior frontal gyrus. These results contribute to the current knowledge by suggesting that inflammation, one of the earliest consequences of obesity, is not only associated with behavioural and emotional functioning, but also with regional brain volumes in children with overweight/obesity.

This is the first study that examines the association between inflammatory biomarkers and a wide range of brain health indicators, including academic performance, executive function, behavioural and emotional functioning, as well as total and regional brain volumes. Regarding academic performance, to date only one study has examined the association between inflammatory biomarkers and academic achievement in a sample of 494 healthy children and adolescents showing that WBC, IL-6 and CRP (but not TNF-α) were inversely associated with school grades (i.e., math,

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language, the mean of math and language, and grade point average), independently of adiposity (Esteban-Cornejo et al., 2016). In this sense, we found an isolated association between TNF-α and mathematics, which disappeared after correcting for multiple comparisons. Therefore, the fact that previous research did not use multiple comparisons correction, along with differences in methodological issues (e.g., school grades vs. academic performance standardized questionnaire), and the multifactorial nature of academic performance which involves both, cognitive and non-cognitive traits, could partially explain these inconsistent findings.

Importantly, prior research has suggested that while cytokines such as IL-6 seem not to affect proliferation and gliogenesis, with no effects on cognitive functioning, TNF-α plays a substantial role in the inhibition of neurogenesis, which may affect cognition (Borsini et al., 2015). However, our results indicate a lack of association between inflammatory biomarkers and executive function in children with overweight/obesity. These findings are in consonance with previous interventional (Grigoleit et al., 2010) and prospective (Jonker et al., 2014) studies conducted in youths. For instance, Grigoleit et al. (2010) conducted an interventional study in healthy young men showing that the administration of lipopolysaccharides increased circulating neutrophils and plasma cytokine levels, without affecting memory performance, selective attention or executive function. Likewise, Jonker et al. (2014) showed that high sensitive-CRP in adolescents was not associated with memory and executive functioning two years later.

In contrast, an emerging body of literature has suggested an inverse association between inflammation and executive function in preterm infants (Kuban et al., 2017; O'Shea et al., 2013; Rose et al., 2015), children (Huang et al., 2016; Lee et al., 2016), adolescents (Cullen et al., 2017), and adult populations (Marsland et al., 2015; Sartori et

371   al., 2012; Windham et al., 2014). In youths, no previous studies have focused on

372   population with overweight/obesity, which hinders comparisons among studies. In a

373   study conducted in normal-weight children from impoverished countries, higher

374   inflammation was closely linked to lower general intelligence, independently of

375   nutritional and socioeconomic status (Lee et al., 2016). Another study conducted in

376   normal-weight children, of which 60% presented obstructive sleep apnea, revealed that

377   high levels of inflammatory biomarkers were related with decreased executive functions

378   (Huang et al., 2016). In addition, a more recent research suggested that TNF-α and

379   interferon-γ (but not several interleukins) were inversely associated with memory in a

380   general population of children (Kyriklaki et al., 2019). In adolescents, Cullen et al.

381   (2017) found that salivary CRP was inversely related to letter fluency and cognitive

382   inhibition, but not to memory. Collectively, although inflammatory biomarkers may

383   also play a key role on cognitive processes in youths, the negative influence of

384   inflammation on cognitive functioning seems to become more evident during the early

385   and late stages of the human lifespan. Thus, differences in age, ethnicity population, as

386   well as in the study design and methodologic technics (e.g., blood vs. salivary analysis),

387   together with the fact that it is likely that our study was underpower to determinate

388   statistically significant results, may be the responsible for the divergent results.

389   Therefore, further studies in young populations are warranted to clarify the role of

390   inflammation on cognition, and specifically in populations with overweight/obesity,

391   since adiposity is a key factor on this relationship (AL Miller et al., 2015).

392   According to behavioural and emotional functioning, IL-6 was inversely

393   associated with adaptive skills, while CRP was directly associated with externalizing

394   and internalizing problems, as well as with the behavioural symptoms index in children

395   with overweight/obesity. However, in our relatively small sample, these significant

396 associations disappeared when correcting for multiple comparisons. These findings

397 partially concur with prior research in infants (Voltas et al., 2017), children (Brambilla,

398 Monteleone, & Maj, 2004; Cicchetti, Handley, & Rogosch, 2015; Slopen et al., 2013)

399 and adolescents (Belem da Silva et al., 2017). For example, in children with major

400 depressive disorders, although TNF-α was negatively correlated to depressive

401 symptoms, IL-1β was positively correlated with both, depressive and anxiety symptoms

402 (Brambilla et al., 2004). In addition, elevated levels of CRP were related to higher

403 internalizing problems in recently maltreated children, but not in non-maltreated

404 children (Cicchetti et al., 2015). However, the cross-sectional design of the

405 abovementioned studies conducted in children and adolescents cannot determine

406 causality, making reverse causation equally plausible; behavioural functioning could

407 also influence inflammation. In fact, prior research showed that internalizing and

408 externalizing problems at age 8 were associated with higher concentrations of IL-6 and

409 CRP at age 10, respectively (Slopen et al., 2013). Interestingly, in another study,

410 adolescents with internalizing behaviours presented higher levels of IL-6, when

411 compared with their healthy peers (Belem da Silva et al., 2017). Thus, longitudinal and

412 interventional studies examining the relationship between inflammation and behavioural

413 functioning in young populations are needed to elucidate the direction of causality.

414   The reasons underlying why inflammatory biomarkers were associated with

415 behavioural and emotional functioning cannot be elucidated in the present study.

416 Nevertheless, we suggested some mechanisms that could be implicated in this

417 association. First, several cytokines convert tryptophan to kynurenine, as well as amino

418 compounds into acidic compounds, through different molecular processes reducing

419 levels of serotonin (Rosenblat et al., 2014; Zhang, Terreni, De Simoni MG, & Dunn,

420 2001), which may affect mood, and consequently, behavioural and emotional

22

421   functioning. Second, cytokines can activate the hypothalamic-pituitary-adrenal axis,

422   releasing specific hormones such as cortisol, an important component of the stress

423   response (AH. Miller, Haroon, Raison, & Felger, 2013), which might contribute to

424   behavioural dysfunctions. Third, inflammatory biomarkers also activate microglia,

425   which in turn, promotes apoptosis of functional neuronal pathways, leading to poor

426   brain functioning, and behavioural and emotional problems (Ekdahl, 2012). Last,

427   inflammation could affect brain structure and function through the impairment of

428   neuroplasticity, which might also contribute to behavioural and emotional disorders

429   (AH. Miller et al., 2013). Thus, we speculate that inflammation may influence specific

430   molecular processes altering brain function, and possibly leading to behavioural and

431   emotional dysfunctions.

432   Importantly, to our knowledge this is the first neuroimaging research aimed to

433   test the associations of inflammatory biomarkers with total brain volumes, and regional

434   gray matter and white matter volumes using a whole-brain analytical approach, which

435   hampers comparisons among studies. Our results revealed no association of

436   inflammatory biomarkers with total gray matter, total white matter and total brain

437   volume. Regarding the regional brain volumes findings, we found positive and negative

438   isolated associations of inflammatory biomarkers with gray and white matter volumes in

439   some small clusters. Surprisingly, higher concentrations of WBC and CRP were

440   associated with greater gray matter volume in the left middle temporal gyrus, and in the

441   right superior temporal gyrus, respectively. Additionally, when considering TBV, CRP

442   was also positively associated with gray matter volume in the right supplementary

443   motor cortex. Furthermore, higher concentrations of IL-6 and TNF-α were associated

444   with greater white matter volume around the right inferior frontal gyrus pars

445   opercularis. Conversely, higher levels of CRP were associated with lower white matter

volume in 1 cluster around the left superior frontal gyrus. After considering TBV, CRP

was also inversely associated with white matter volume bilaterally around the superior

frontal gyrus pars orbital and in the middle cingulum. Prior evidence from animal

models has shown that brain morphology may be particularly vulnerable to

inflammation-related processes (Yirmiya & Goshen, 2011). In parallel to animal work,

increased attention has been paid to the relationship between inflammation and brain

structure in adult humans. Findings from a previous study in healthy midlife adults

showed that higher levels of IL-6 were inversely associated with hippocampal gray

matter volume, as well as, with gray matter volume in the medial prefrontal cortex and

in the right cerebellum (Marsland et al., 2008). In a more recent study, both IL-6 and

CRP showed inverse associations with cortical gray and white matter volumes,

hippocampal volume, and cortical surface area (Marsland et al., 2015). In line with our

findings, research in healthy elderly adults showed that higher levels of IL-6 and CRP

were associated with greater white matter hyperintensities, and lower total gray matter

and hippocampal volumes (Satizabal, Zhu, Mazoyer, Dufouil, & Tzourio, 2012).

Similarly, Taki et al. (2013) found an inverse association between high sensitivity-CRP

and regional gray matter volume in the left temporal cortex, while Bettcher et al. (2012)

showed an inverse association of CRP with left medial temporal lobe volumes. Thus,

brain structure has shown to be particularly vulnerable to the effects of age.

We speculate that the few isolated and mixed associations between

inflammation and regional brain volumes found in our study are probably due to the fact

that in children brain is not as vulnerable as in preterm infants and older adults, and

inflammation has not significant adverse effects on brain yet. In addition, the fact that

inflammatory biomarkers at physiologically normal levels can act as both, anti- and pro-

inflammatory substances, could explain the positive and inverse associations of

471   inflammation with regional brain volume found in our study. Therefore, more studies

472   are needed to elucidate the developmental period at which inflammation produces

473   detrimental effects on brain structure, as well as, to assess the short-term and long-term

474   effect of systemic low-grade inflammation on brain.

475   **Limitations and strengths**

476   The current study has some limitations that must be mentioned. The cross-

477   sectional design of our analyses prevents us from inferring causal relationships. In

478   addition, our analyses need replication in a larger sample size in order to elucidate the

479   associations between inflammation and brain health in children with

480   overweight/obesity. However, the strengths of the study comprise the inclusion of a

481   wide range of brain health indicators assessed through validated tools, as well as the use

482   of a whole-brain analytical approach.

483   **Conclusions**

484   Our findings reveal that inflammation is slightly associated with brain health,

485   particularly with behavioural and emotional functioning, as well as with regional brain

486   volumes, in children with overweight or obesity. Further larger longitudinal and

487   interventional studies in children with overweight/obesity are warranted to elucidate the

488   pathways by which inflammation is linked to brain health, as well as the short-term and

489   long-term effect of systemic low-grade inflammation and obesity on brain health.

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Table 1. Descriptive characteristics of the study sample.

	All	**Boys**	**Girls**
N	107	63	44
Physical characteristics			
Age (years)	10.0 ± 1.1	10.2 ± 1.2	9.9 ± 1.1
Peak height velocity (years)	-2.3 ± 1.0	-2.7 ± 0.8	-1.7 ± 1.0
Weight (kg)	55.9 ± 11.0	56.8 ± 11.0	54.5 ± 11.1
Height (cm)	144.1 ± 8.4	144.8 ± 7.9	143.0 ± 9.1
Body mass index (kg/m^2)	26.7 ± 3.6	26.9 ± 3.7	26.4 ± 3.4
Overweight/Obesity grade I/II/III (%)	26/44/20/10	25/48/16/11	27/39/25/9
Parental education university level: Neither/One/Both parents (%)	64/19/17	70/16/14	57/23/20
Inflammatory biomarkers			
White blood cell (10^3/μL) (n = 101)	7.3 ± 1.7	7.2 ± 1.7	7.6 ± 1.6
Interleukin-6 (pg/mL) [a] (n = 96)	1.7 ± 1.2	1.7 ± 1.3	1.8 ± 1.2
Interleukin-1β (pg/mL) [a] (n = 101)	1.6 ± 0.9	1.5 ± 0.9	1.7 ± 1.0
Tumor necrosis factor-α (pg/mL) [a] (n = 102)	4.1 ± 1.5	3.9 ± 1.6	4.3 ± 1.4
C-reactive protein (mg/L) [a] (n = 77)	3.2 ± 3.1	3.4 ± 3.4	2.9 ± 2.5
Academic performance [b] (n = 106)			
Mathematics	102.1 ± 11.0	102.8 ± 11.8	101.0 ± 9.7
Reading	108.6 ± 12.7	109.0 ± 11.0	108.0 ± 15.1
Writing	114.2 ± 12.9	113.2 ± 12.1	115.7 ± 14.0
Total achievement	109.7 ± 11.9	109.7 ± 11.0	109.6 ± 13.2
Executive function			
Cognitive flexibility [c]	20.0 ± 6.5	20.7 ± 6.9	19.1 ± 6.0
Cognitive inhibition [a, d]	-40.6 ± 17.2	-38.8 ± 15.0	-43.2 ± 19.8
Working memory [a, e]	15.2 ± 6.7	15.0 ± 6.8	15.4 ± 6.8
Behavioural and emotional functioning [f] (n=106)			
Externalizing problems	49.0 ± 8.8	49.1 ± 9.1	48.7 ± 8.3
Internalizing problems	54.4 ± 14.1	52.9 ± 14.0	56.5 ± 14.1
Adaptive skills	49.5 ± 11.1	48.8 ± 10.7	50.6 ± 11.7
Behavioural symptoms index	50.8 ± 11.1	50.8 ± 11.7	50.9 ± 10.2
Brain volumes (cm^3) [g] (n=99)			
Total gray matter	793.0 ± 66.4	819.1 ± 56.5	754.5 ± 61.4
Total white matter	406.0 ± 47.5	425.8 ± 42.4	376.7 ± 38.9
Total brain volume	1199.0 ± 106.4	1244.9 ± 88.9	1131.2 ± 93.7

Values are mean ± standard deviation or percentages.

[a] Values were normalized using the Blom's formula before analysis, but non-transformed values are

presented in the table.

[b] Measured by the Bateria III Woodcock-Muñoz Tests of Achievement. Values based on standard T-scores with an average of 100 and standard deviations of 15 points.

[c] Measured by the Design fluency test as the total number of correct drawn designs.

[d] Measured by Stroop test as the subtraction of the completion times of two tasks.

[e] Measured by the Delayed nonmatch-to-sample task. Calculated as the quotient between reaction time and response accuracy.

[f] Measured by the Behavior Assessment System for Children (BASC). Values based on standard T-scores with an average of 50 and standard deviations of 10 points.

[g] Measured by Magnetic Resonance Imaging.

Table 2. Associations between inflammatory biomarkers and brain health indicators in children with overweight/obesity.

		White blood cell				Interleukin-6 [a]				Interleukin-1β [a]				Tumor necrosis factor-α [a]				C-reactive protein [a]		
	n	R^2	β	p	n	R^2	β	P	n	R^2	β	p	n	R^2	β	p	n	R^2	β	p
Academic performance [b]	100				95				100				101				77			
Mathematics		0.176	-0.022	0.811		0.188	-0.029	0.762		0.160	-0.054	0.582		0.210	-0.198	**0.034**		0.159	0.094	0.403
Reading		0.106	0.078	0.423		0.131	0.016	0.875		0.144	-0.096	0.333		0.176	-0.033	0.731		0.013	0.100	0.409
Writing		0.076	-0.029	0.773		0.107	-0.063	0.528		0.102	-0.116	0.251		0.092	-0.012	0.904		0.011	-0.018	0.885
Total achievement		0.162	0.020	0.831		0.202	-0.031	0.742		0.190	-0.108	0.268		0.194	-0.099	0.288		0.072	0.073	0.535
Executive function	101				96				101				102				77			
Cognitive flexibility [c]		0.261	0.119	0.181		0.243	0.038	0.679		0.259	-0.071	0.433		0.253	-0.038	0.673		0.252	-0.089	0.399
Cognitive inhibition [a, d]		-0.019	-0.083	0.426		-0.003	-0.079	0.401		-0.015	-0.117	0.207		-0.017	-0.049	0.597		-0.026	-0.003	0.978
Working memory [a, e]		0.062	-0.051	0.603		0.066	0.006	0.949		0.064	-0.012	0.904		0.077	-0.100	0.314		0.007	-0.012	0.919
Behavioural and emotional functioning [f]	100				96				100				101				76			
Externalizing problems		-0.010	0.154	0.141		-0.026	0.080	0.452		-0.032	-0.028	0.790		-0.022	-0.100	0.343		0.038	0.246	**0.046**
Internalizing problems		0.100	-0.002	0.981		0.105	0.143	0.152		0.075	0.038	0.698		0.082	-0.076	0.447		0.184	0.234	**0.039**
Adaptive skills		-0.032	0.000	0.998		0.024	-0.228	**0.030**		-0.022	-0.051	0.625		-0.023	0.087	0.409		-0.024	-0.080	0.523
Behavioural symptoms index		0.035	0.087	0.393		0.060	0.152	0.138		0.030	0.012	0.906		0.037	-0.079	0.437		0.105	0.236	**0.047**
Brain volumes [g]	93				89				94				95				74			
Total gray matter		0.299	0.020	0.821		0.305	0.110	0.229		0.289	-0.038	0.674		0.295	0.072	0.412		0.331	0.106	0.302
Total white matter		0.370	-0.012	0.891		0.350	0.072	0.412		0.357	0.019	0.829		0.365	0.083	0.322		0.415	-0.076	0.428
Total brain volume		0.374	0.007	0.930		0.373	0.102	0.241		0.358	-0.016	0.857		0.368	0.083	0.324		0.408	0.033	0.729

Analyses were adjusted by sex, peak height velocity, parental education university level (neither/one/both of them) and body mass index. Statistically significant associations that are shown in bold

disappeared when p values were adjusted for multiple comparisons using the Benjamini-Hochberg method.

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Blom's normalized values were used in the analysis.

Measured by the Bateria III Woodcock-Muñoz Tests of Achievement. Values based on standard T-scores with an average of 100 and standard deviations of 15 points.

Measured by the Design fluency test as the total number of correct drawn designs.

Measured by Stroop test as the subtraction of the completion times of two tasks. The original score was multiplied by -1 to invert the variable, so that a higher score indicates higher cognitive inhibition.

Measured by the Delayed nonmatch-to-sample task. Calculated as the quotient between reaction time and response accuracy. The original score was multiplied by -1 to invert the variable, so that a highe

core indicates higher working memory.

Measured by the Behavior Assessment System for Children (BASC). Values based on standard T-scores with an average of 50 and standard deviations of 10 points.

Measured by Magnetic Resonance Imaging.

706 **Fig. 1.** Brain regions showing positive associations of (A) white blood cell and (B) C-reactive protein with gray matter volume in children with

707 overweight/obesity. Analyses were adjusted by sex, peak height velocity (years), parental education university level (neither/one/both) and body

708 mass index (kg/m^2). Each inflammatory biomarker was introduced in a separate model. Maps were thresholded using AlphaSim at p<0.001 with

709 k = 44 voxels for WBC and k = 29 for CRP, and surpassed Hayasaka correction. Anatomical coordinates (X, Y, Z) are given in Montreal

710 Neurological Institute (MNI) Atlas space. The colour bar represents t-values, with lighter pink colour indicating higher significant association.

711 Images are displayed in neurological convention; therefore, the right hemisphere corresponds to the right side in coronal displays. β =

712 standardized regression coefficient. [a] Blom's normalized values were used in the analysis. *p<0.001

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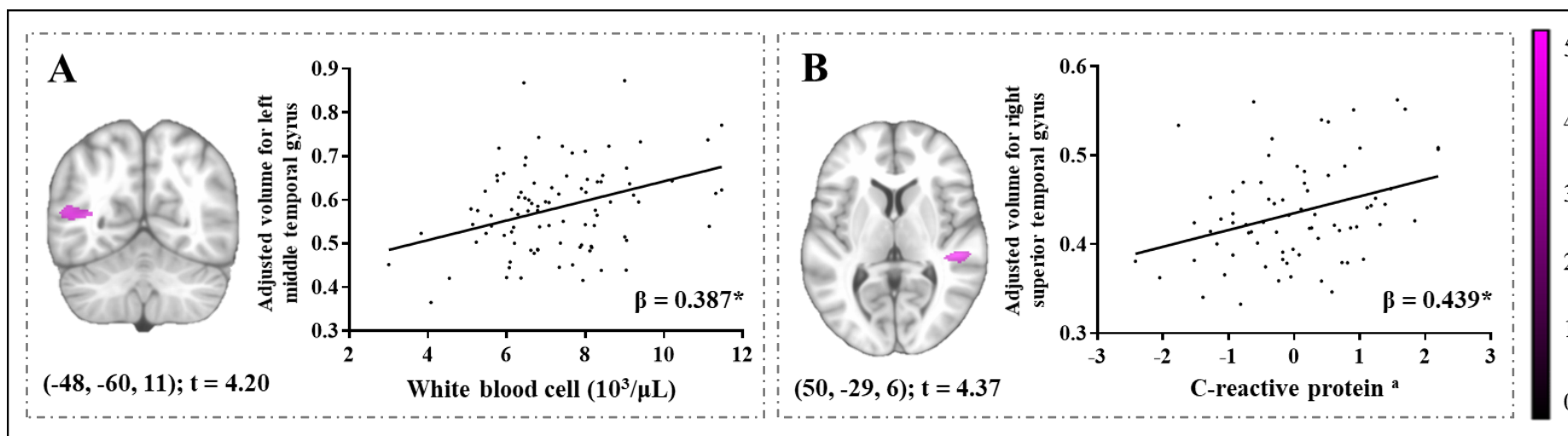
Fig. 2. Brain regions showing (A) positive associations of interleukin-6 and tumor necrosis factor-α with white matter volume and (B) negative association of C-reactive protein with white matter volume in children with overweight/obesity. Analyses were adjusted by sex, peak height velocity (years), parental education university level (neither/one/both) and body mass index (kg/m^2). Each inflammatory biomarker was introduced in a separate model. Maps were thresholded using AlphaSim at $p<0.001$ with $k = 81$ voxels for IL-6, $k = 62$ for TNF-α, and $k = 82$ for CRP, and surpassed Hayasaka correction. Anatomical coordinates (X, Y, Z) are given in Montreal Neurological Institute (MNI) Atlas space. The colour bar represents t-values, with lighter green colour indicating higher significant association. Images are displayed in neurological convention; therefore, the right hemisphere corresponds to the right side in coronal displays. β = standardized regression coefficient. [a] Blom's normalized values were used in the analysis. *$p<0.001$.
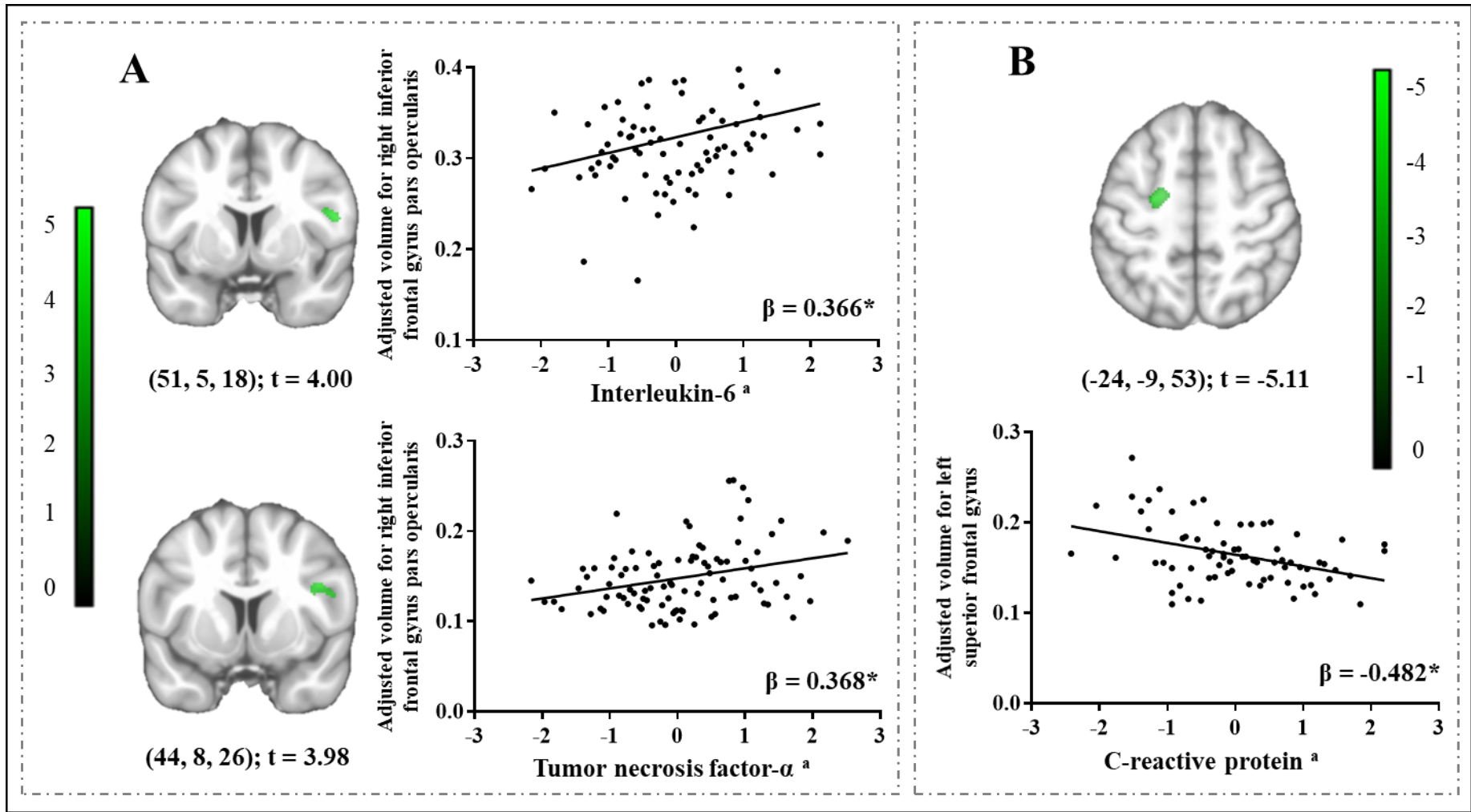
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Table S1. Brain regions showing independent associations of inflammatory biomarkers with gray matter volume in children with overweight and obesity.

Brain Regions (mm³)	Model 1							Model 2						
	x	y	z	t	Cluster size	Hem	β	x	y	z	t	Cluster size	Hem	β
Positive associations														
White blood cell														
Middle temporal gyrus	-48	-60	11	4.20	241	L	0.387	-48	-60	11	4.16	179	L	0.410
C-reactive protein														
Superior temporal gyrus	50	-29	6	4.37	185	R	0.439	50	-29	6	5.25	305	R	0.549
Supplementary motor cortex	-	-	-	-	-	-		2	17	60	3.88	232	R	0.453

Model 1: Analyses were adjusted by sex, peak height velocity, parental education university level and body mass index. Maps were thresholded using AlphaSim at p<0.001 with k = 44 voxels for white blood cell and k = 29 for C-reactive protein, and surpassed Hayasaka correction. Model 2: Model 1 + total brain volume. Maps were thresholded using AlphaSim at p<0.001 with k = 29 voxels for white blood cell and k = 51 for C-reactive protein, and surpassed Hayasaka correction. Each inflammatory biomarker was introduced in a separate model. Anatomical coordinates (X, Y, Z) are given in Montreal Neurological Institute (MNI) Atlas space. Hem, hemisphere; R, right; L, left. β: standardized regression coefficient.

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Table S2. Brain regions showing independent associations of inflammatory biomarkers with white matter volume in children with overweight and obesity.

Brain Regions (mm³)	Model 1							Model 2						
	x	y	z	t	Cluster size	Hem	β	x	y	z	t	Cluster size	Hem	β
Positive associations														
Interleukin-6														
Inferior frontal gyrus pars opercularis	51	5	18	4.00	101	R	0.366	50	5	20	3.96	84	R	0.392
Tumor necrosis factor-α														
Inferior frontal gyrus pars opercularis	44	8	26	3.98	108	R	0.368	44	8	26	3.86	80	R	0.371
Negative associations														
C-reactive protein														
Superior frontal gyrus	-24	-9	53	-5.11	302	L	-0.482	-21	-9	51	-5.29	600	L	-0.529
Superior frontal gyrus pars orbital	-	-	-	-	-	-		-15	54	-15	-3.76	161	L	-0.404
Superior frontal gyrus pars orbital	-	-	-	-	-	-		20	38	-15	-4.17	236	R	-0.473
Middle cingulum	-	-	-	-	-	-		12	6	33	-3.77	119	R	-0.424

Model 1: Analyses were adjusted by sex, peak height velocity, parental education university level and body mass index. Maps were thresholded using AlphaSim at $p<0.001$ with k = 81 voxels for interleukin-6, k = 62 for tumor necrosis factor-α, and k = 82 for C-reactive protein, and surpassed Hayasaka correction. Model 2: Model 1 + total brain volume. Maps were thresholded using AlphaSim at $p<0.001$ with k = 72 voxels for interleukin-6, k = 53 for tumor necrosis factor-α, and k = 87 for C-reactive protein, and surpassed Hayasaka correction. Each inflammatory biomarker was introduced in a separate model. Anatomical coordinates (X, Y, Z) are given in Montreal Neurological Institute (MNI) Atlas space. Hem, hemisphere; R, right; L, left. β: standardized regression coefficient.

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