Maternal and child immune profiles are associated with neurometabolite measures of early-life neuroinflammation in children who are HIV-exposed and uninfected: a South African birth cohort

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Abstract

Children who are HIV-exposed and uninfected (HEU) are at risk of neurodevelopmental delays, which may be partially due to maternal immune dysregulation during pregnancy. This study investigates associations between maternal and child immune profiles and early neurometabolite profiles in HEU and HIV-unexposed (HU) children from a South African birth cohort. A subgroup of 156 children (66 HEU, 90 HU) from the Drakenstein Child Health Study underwent magnetic resonance spectroscopy at age 2–3 years, and maternal and child serum markers were measured at multiple timepoints via immunoassays.

In HEU children, serum concentrations of maternal pro-inflammatory cytokines IL-5 (β=0.79, p=0.005) and IL-8 (β=0.64, p=0.02) were associated with myo-inositol ratios in parietal grey and white matter regions, respectively, while child serum MMP-9 at two years was associated with myo-inositol ratios in the midline parietal grey matter (β=1.30, p=0.03). The association of maternal anti-inflammatory cytokine IL-13 with glutamate ratios in the midline parietal grey matter was negative in HEU (β=-0.41, p=0.038) and positive in HU children (β=0.42, p<0.0001). These findings suggest maternal immune activation may affect neurometabolite profiles in HEU children.

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Introduction

An estimated 39 million people live with HIV worldwide, with approximately 20.8 million residing in Southern and Eastern Africa (1). Widespread access to antiretroviral therapy (ART) and vertical transmission prevention programmes have significantly reduced new HIV infections (1). As a result, the number of new infections among children under 14 years has decreased by over 60% since 2000 (2). This has led to the emergence of a rapidly growing population of children who are born to mothers living with HIV and remain uninfected themselves (HIV-exposed and uninfected [HEU]) (3,4). Children who are HEU are estimated at 16.1 million globally (1), with the highest number residing in South Africa (2). This group, while spared from HIV infection, faces unique health challenges, including increased mortality rates (5) and a higher risk of adverse neurodevelopmental outcomes compared to children who are HIV-unexposed (HU) (6).

Prenatal exposure to maternal HIV and ART may alter fetal immune programming, resulting in distinct immune profiles in children who are HEU compared to their HU peers (7,8). However, findings are mixed within and across geographical regions. Studies in Southern African cohorts have shown decreased pro- and anti-inflammatory cytokine levels in HEU children (9), while others found no differences (10). In contrast, Latin American studies reported elevated levels of inflammatory and monocyte activation markers in HEU compared to HU children (11,12). These inconsistencies highlight the need for further research to elucidate how HIV exposure affects immune and neurodevelopmental trajectories in children across different social and geographical contexts (7).

In HEU infants from a South African birth cohort, the Drakenstein Child Health Study (DCHS), increased serum inflammatory cytokines at 6 weeks of age predicted poorer motor and language development at two years (10). One proposed pathway linking maternal HIV to child immune profiles and neurodevelopmental outcomes is through the effect of maternal inflammation on the developing fetal brain. Pregnant women living with HIV may experience chronic immune activation despite ART (13,14), resulting in altered cytokine levels that can cross the placenta and impact glial cell integrity (15,16) and white matter development (17,18). Maternal immune activation has been associated with

65   altered brain growth and connectivity in the offspring, potentially increasing the risk for

66   neurodevelopmental disorders (19–21). It has been hypothesized that prenatal exposure to maternal

67   immune activation can predispose children who are HEU to exaggerated neuroinflammatory

68   responses when exposed to subsequent postnatal insults, potentially disrupting typical brain

69   development (18,22).

70   Given the critical role of the immune system in shaping brain development, a deeper understanding

71   of how immune profiles in children who are HEU change over time and their potential impact on brain

72   health is needed. Magnetic resonance spectroscopy (MRS) provides a clinically established method

73   for *in vivo* quantification of key brain metabolites such as myo-inositol and glutamate, which are linked

74   to neuroinflammation and neuronal function, respectively (23,24). Our research found elevated myo-

75   inositol ratios to creatine in the parietal white matter of South African HEU children at 2–3 years of

76   age, suggesting a neuroinflammatory response due to maternal HIV status (25). Other studies in older

77   children have also reported neurometabolite differences between HEU and HU groups (26–28). Taken

78   together, these findings suggest that HIV-associated changes to the maternal immune system may

79   contribute to altered child neurometabolite patterns, potentially leading to neurodevelopmental delays

80   observed in HEU children. To date, no studies have examined longitudinal changes in serum marker

81   levels through childhood nor their potential associations with neuroimaging findings in children who

82   are HEU. This approach is essential to delineate the temporal dynamics of immune profiles in this

83   population and their relationship to neurobiological outcomes.

84   Our study aimed to address this gap by investigating maternal and child peripheral blood immune

85   profiles from pregnancy through birth and early childhood, and their associations with brain metabolite

86   levels at age 2–3 years, in a cohort of South African HEU and HU children from the DCHS (29). We

87   hypothesized that mothers living with HIV and their HEU children would show altered levels of serum

88   markers compared to mothers without HIV and their HU children, and that these alterations would be

89   associated with child elevated levels of myo-inositol in the parietal white matter at age 2–3 years.

Results

Cohort and demographic characteristics

Out of the 1143 mother-child pairs enrolled in the DCHS, a group of 156 children underwent MRS imaging at age 2–3 years. Data was successfully acquired from 143 children in the parietal grey matter, 134 in the left parietal white matter, and 92 in the right parietal white matter. Following quality assessment, 9 participants were excluded due to low-quality spectra, resulting in a subset of 83 children (36 HEU, 47 HU) with high-quality data available for all three parietal brain regions.

Sociodemographic characteristics were generally comparable between mothers living with HIV (n=66) and those without HIV (n=90) in this cohort, with no significant differences in household income, education, employment, marital status, or most perinatal health behaviors (p>0.05). However, mothers living with HIV were older at delivery, had lower rates of depression and tobacco use during pregnancy, were less likely to be single mothers, and breastfed for a shorter duration than mothers not living with HIV. Children in HEU and HU groups had comparable birth weights and nutritional statuses (**Table 1**).

All mothers living with HIV received ART, either before conception (40.9%) or during pregnancy (59.1%). Most (86.4%) were on a first-line regimen of efavirenz, emtricitabine, and tenofovir, and nearly all (91.7%) received cotrimoxazole prophylaxis. All HEU children received post-exposure prophylaxis, either nevirapine alone (81.8%) or in combination with zidovudine (18.2%) (**Table 1**). In the subset of 83 children with complete MRS data, the only significant differences between HEU and HU groups were in maternal age at delivery and antenatal depression (**Supplementary Table 2A**). Overall, the complete MRS subset of participants was comparable to the initial group of 156 children (**Supplementary Table 2B**), ensuring representativeness for subsequent analyses.

Group differences in maternal and child serum marker concentrations

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Maternal markers during pregnancy: Out of 156 mothers whose children underwent MRS at age 2–3 years, 138 (88.5%, n=60 with HIV, n=78 not with HIV) had available serum marker data during pregnancy (**Supplementary Figure 1A**). Several differences were observed based on HIV status. Levels of pro-inflammatory cytokine GM-CSF and neuroinflammatory marker MMP-9 were significantly lower in mothers living with HIV compared to their peers, remaining after Benjamini-Hochberg (BH) adjustment for multiple comparisons (GM-CSF corrected p=0.034; MMP-9 corrected p=0.002) (**Supplementary Figure 2**, **Supplementary Table 3**). Prior to BH correction, mothers living with HIV were found to have lower circulating levels of the neuroinflammatory marker NGAL (uncorrected p=0.018) and higher levels of the monocyte activation marker CD14 (uncorrected p= 0.044) in comparison to their counterparts. For the remaining markers, no significant group differences were found.

Infant markers at 6 weeks of age: 97/156 infants (62.2%, n=41 HEU, n=56 HU) had available serum marker measurements at 6 weeks post-birth (**Supplementary Figure 1A**). NGAL levels were significantly lower in HEU compared to HU infants in the unadjusted analysis (p= 0.032), but this finding did not survive BH correction for multiple comparisons (**Supplementary Figure 2**, **Supplementary Table 3**).

Child markers at 2 years of age: In the 2-year timepoint, 111/156 children (71.2%, n=46 HEU, n=65 HU) had available serum marker data (**Supplementary Figure 1A**). Analyses indicated that levels of pro-inflammatory cytokine IL-1β (uncorrected p=0.032) and anti-inflammatory cytokine IL-12p70 (uncorrected p=0.048) were lower in HEU children compared to HU, but these differences did not remain significant after BH adjustment (**Supplementary Figure 2**, **Supplementary Table 3**).

Child serum marker trajectories

We used Linear Mixed-effects Models (LMMs) to examine longitudinal changes in circulating marker levels between ages 6 weeks and 2 years in 127/156 children (81.4%, n=56 HEU, n=71 HU) with available serum marker data at either of the two timepoints. To address the issue of missing data, Maximum Likelihood Estimation was applied in our LMMs, with use of the *lme4* package in R. Akaike

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139   Information Criterion (AIC) and Bayesian Information Criterion (BIC) values revealed that, out of three

140   covariance structures tested, the model with variance components fitted our data better than the

141   compound symmetry and unstructured covariance models.

142   No significant longitudinal differences were found between 6 weeks and 2 years of age in our group

143   of 127 participants or between HEU and HU subgroups (**Supplementary Figure 3**, **Supplementary**

144   **Table 4**).

145   **Cross-sectional associations between serum markers and brain metabolite ratios**

146   Linear models were run in a subset of 83 children (36 HEU, 47 HU) with high-quality MRS data for all

147   three parietal brain regions. Adjusted analyses revealed a significant influence of tissue composition

148   on total choline ratios across all parietal voxels, therefore, this metabolite was excluded from all

149   analyses. N-acetyl-aspartate ratios in the left parietal white matter were also excluded from our

150   analyses due to suboptimal tissue composition.

151   Results that were significant in unadjusted linear models, survived BH correction, and remained

152   significant after adjusting for potential confounders are reported in **Table 2**. For a full overview of all

153   results, see **Supplementary Figure 4** and **Supplementary Table 5**, as well as **Supplementary Table**

154   **6** for subsequent sensitivity analyses.

155   *Maternal serum marker levels and child neurometabolite ratios*

156   Of the 83 children with high-quality MRS data for all three parietal brain regions, 74 (89.2%, n=34

157   HEU, n=40 HU) had available data on maternal blood marker concentrations during pregnancy

158   (**Supplementary Figure 1B**).

159   Myo-inositol: Maternal IL-5 levels during pregnancy were found to be significantly associated with

160   myo-inositol ratios in the parietal grey matter of children who are HEU (β=0.79 p=0.0053), and

161   maternal IL-8 was found to be associated with myo-inositol ratios in the right parietal white matter of

162   the same group (β=0.64, p=0.0203). Maternal IL-5 was also found to be associated with the composite

163 factor reflecting a pattern of elevated myo-inositol across voxels in children who are HEU (β=0.84,

164 p=0.0075) (**Figure 1**).

165 Glutamate: Maternal IL-13 levels during pregnancy were positively associated with glutamate ratios

166 in the parietal grey matter of HU children (β=0.42, p<0.0001), and this relationship was significantly

167 modified by maternal HIV infection (β=-0.41, p=0.0380). In the same brain region, maternal MMP-9

168 levels showed an association with glutamate ratios of children who are HEU (β=-0.85, p=0.0231).

169 Additionally, maternal YKL-40 was positively associated with child glutamate ratios in the right parietal

170 white matter of HU children (β=0.52, p=0.0046) and maternal HIV infection modified this relationship

171 (β=-0.90, p=0.0024). Child sex was identified as a significant covariate in right parietal white matter

172 analyses.

173 N-acetyl-aspartate: Maternal MMP-9 levels during pregnancy were found to be associated with N-

174 acetyl-aspartate ratios in the parietal grey matter of children who are HEU (β=-1.01, p=0.0081).

175 *Infant serum marker levels and child neurometabolite ratios*

176 Data on blood marker concentrations at 6 weeks of age were available for 52 out of 83 children

177 (62.7%, n=23 HEU, n=29 HU) (**Supplementary Figure 1B**). Infant IL-1β levels were found to be

178 associated with glutamate ratios in the left parietal white matter of HU children at age 2–3 years

179 (β=0.28, p=0.0259), and maternal HIV infection modified this relationship (β=-0.76, p=0.0113).

180 *Child serum marker levels and neurometabolite ratios at age 2–3 years*

181 Serum marker data at 2 years of age were available for 63 out of 83 participants (75.9%, n=28 HEU,

182 n=35 HU) (**Supplementary Figure 1B**). In the parietal grey matter, levels of the neuroinflammatory

183 marker MMP-9 showed an association with myo-inositol ratios of children who are HEU (β=1.29,

184 p=0.0308). The same was found for NGAL levels and glutamate ratios in the HIV-exposed group

185 (β=1.00, p=0.0364). Additionally, NGAL was also found to be associated with the composite factor

186 reflecting a bilateral white matter pattern of glutamate in the HEU group (β=0.84, p=0.0193).

187 **Mediation effects**

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188   Mediation analyses using structural equation modelling were conducted to investigate whether serum

189   markers found to display significant associations with neurometabolites (**Table 2**) would mediate the

190   relationship between maternal HIV infection and child brain metabolite ratios at 2 years of age. A

191   series of models were evaluated, each assessing a distinct serum marker-neurometabolite pair

192   (**Supplementary Table 7**).

193   Across all models, goodness-of-fit indices consistently indicated a good fit to the data. However,

194   neither the direct paths between maternal HIV infection and child neurometabolite ratios nor the

195   indirect effects through candidate markers were statistically significant. This was consistent across all

196   models (**Supplementary Table 7**).

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Discussion

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198   In this study, we investigated the associations of maternal HIV status with maternal and child serum

199   markers and brain metabolite ratios to creatine in a South African cohort of young children. We found,

200   firstly, differences in immune marker concentrations during pregnancy by maternal HIV status and,

201   secondly, associations between maternal, infant, and child serum markers with child neurometabolite

202   ratios in parietal brain regions at age 2–3 years that differed by HIV exposure. These findings suggest

203   potential pathways through which maternal HIV status may influence early brain development in

204   children and provide insights into how *in utero* changes to maternal immune markers may shape early

205   neurometabolite profiles, potentially through alterations in maternal and child immune systems.

206   Mothers living with HIV in our cohort had lower levels of the neuroinflammatory marker MMP-9 and

207   pro-inflammatory cytokine GM-CSF compared to mothers not living with HIV, consistent with findings

208   from a larger sample of the same cohort (9). Similarly, decreased MMP-9 levels have been reported

209   in adults living with HIV in other South African settings (30), suggesting a broader trend across

210   populations. While differences in infant and child serum marker levels did not persist after correction

211   for multiple comparisons, we observed lower NGAL levels in HEU infants and lower IL-1β and IL-

212   12p70 in HEU children at 2 years of age before correction. This may indicate that some serum marker

213   differences present at birth diminish over time, whereas others emerge later in childhood. Consistent

214   with this, a recent study on the effects of maternal inflammation on newborn serum profiles found that

215   by six months of age, many HEU serum markers that initially differed had normalized to levels

216   comparable to HU infants (31). Despite this, our longitudinal analysis from six weeks to two years did

217   not reveal significant differences in serum marker trajectories between HEU and HU children.

218   Our association analyses revealed interactions between maternal HIV status, serum marker

219   concentrations, and child myo-inositol ratios. In children who are HEU, increases in maternal pro-

220   inflammatory cytokines IL-5 and IL-8 were associated with higher child myo-inositol ratios in the

221   parietal grey and white matter regions, respectively. A similar pattern was observed for child MMP-9

222   levels at age 2 years and myo-inositol ratios in the parietal grey matter at the same timepoint,

223 supporting our hypothesis that maternal and child immune markers are associated with early-life

224 elevated myo-inositol in the parietal white matter of children who are HEU. These results build on our

225 previous work, which identified group differences in myo-inositol ratios in the parietal white matter of

226 this cohort, as well as an association between maternal HIV status and a cross-regional pattern of

227 elevated myo-inositol (25). Clinically, myo-inositol is recognized as a marker of neuroinflammation

228 and gliosis (23,24); thus, increases in myo-inositol may potentially result from maternal immune

229 dysregulation during pregnancy. Inflammatory patterns of myo-inositol have also been linked to

230 cognitive impairment in children living with HIV (26,32).

231 Maternal levels of anti-inflammatory IL-13 were positively associated with child glutamate ratios in the

232 parietal grey matter of HU children; however, the association was not observed in the HEU group. In

233 contrast, levels of neuroinflammatory MMP-9 in mothers living with HIV were negatively associated

234 with child ratios of this neurotransmitter in the same brain region. Further, infant pro-inflammatory IL-

235 1β at 6 weeks, and neuroinflammatory NGAL at 2 years, were both associated with glutamate ratios

236 in the parietal grey matter of HEU children. Glutamate is a key excitatory neurotransmitter, essential

237 for synaptic plasticity and neurodevelopment (23,24). Its regulation is influenced by astrocytes, which

238 help maintain glutamate balance and prevent excitotoxicity (33). It has been reported that the anti-

239 inflammatory cytokine IL-13 may support astrocyte function and promote cognitive processes by

240 fostering synaptic plasticity and neuroprotection (34,35). HEU children in our study may therefore be

241 at risk of disrupted IL-13-associated regulation of glutamate, potentially contributing to impaired

242 synaptic plasticity and increased vulnerability to neuroinflammatory processes. This disruption could

243 be a potential pathway to explain the neurodevelopmental delays observed in HEU children (6). The

244 relationship between marker concentrations and child neurometabolite ratios was not uniform across

245 HEU and HU groups. It is plausible that maternal immune dysregulation due to HIV creates a unique

246 prenatal environment that sensitizes the fetal brain to subsequent neuroimmune challenges.

247 Despite the robust associations observed between serum markers and neurometabolites, our

248 mediation analyses did not identify any markers that significantly mediated the relationship between

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249   maternal HIV and child brain metabolite profiles at 2–3 years. This suggests that while certain markers

250   are altered in mothers living with HIV, and are associated with brain metabolite patterns, they do not

251   fully account for the impact of maternal HIV exposure on child brain development. The mediatory role

252   of maternal immune activation on child brain health across diverse infection and exposure contexts

253   has been recently reviewed (36). In the context of typical child development, maternal immune

254   activation —measured via circulating IL-6 during pregnancy— was found to mediate neurometabolite

255   ratios and motor development in newborns (37). In our study, maternal IL-6 was associated with child

256   myo-inositol ratios in the right parietal white matter voxel prior to multiple comparison correction.

257   These differences highlight the variability in how maternal immune dysregulation may influence child

258   brain development and suggest that pathways beyond immune markers, such as antiretroviral drug

259   exposure (38,39) or other environmental and psychosocial factors (40,41), may also contribute to the

260   observed neurometabolite alterations in HEU children.

261   The main strengths of this study include the use of a well-characterized South African cohort and the

262   comprehensive assessment of maternal and child immune profiles at multiple timepoints, combined

263   with high-quality MRS data. Our findings provide a nuanced understanding of how maternal HIV

264   infection affects maternal and child immune profiles and their relationship to early brain development.

265   However, several limitations should be noted. First, although our sample size was larger than those

266   in many previous studies, it was relatively small for some subgroup analyses, which may have limited

267   the power to detect smaller effects. And second, residual confounding cannot be ruled out, particularly

268   given the complex interplay between immune, neurobiological, and environmental factors. We

269   conducted sensitivity analyses to account for potential confounders, including maternal depression

270   and alcohol use during pregnancy as both have been associated with poorer cognitive outcomes in

271   children who are HEU (40,41), and found that our results held.

272   Future studies will focus on delineating the temporal evolution of neurometabolite and immune profiles

273   in children who are HEU, integrating neurodevelopmental assessments to capture the potential clinical

274   impacts of prenatal HIV exposure. Investigating the combined effects of maternal immune activation,

275   ART regimens, and psychosocial factors may help identify the underlying mechanisms driving the

276   observed neurometabolite alterations. Such insights could inform targeted interventions to mitigate

277   neurodevelopmental risks in HEU children.

278   In conclusion, this study demonstrates that maternal HIV status is associated with distinct alterations

279   in maternal and child immune profiles, which in turn influence neurometabolite patterns in early

280   childhood. Our findings suggest that perinatal HIV exposure and its immunological impact may create

281   a unique risk profile for HEU children, emphasizing the need for optimal HIV management in

282   pregnancy to mitigate immune dysregulation. Further research is needed to elucidate

283   neuroinflammatory patterns in HEU children and their long-term consequences for neurodevelopment.

284   Finally, these findings highlight the need for strategies that support optimal neurodevelopment in this

285   vulnerable population.

286

Methods

Participants

This study included a sub-sample of mothers and their children from the DCHS, a population-based birth cohort study conducted in a peri-urban area of the Western Cape, South Africa, with the aim of identifying early-life factors that influence child health, development, and illness (42–44). The cohort comprises a low socioeconomic community with a high prevalence of HIV infection and related risk factors. Pregnant women were enrolled between 2012 and 2015 during their second trimester and have been followed, along with their children, from birth through childhood. Eligible women were at least 18 years old, in their second trimester (gestational period of 20-28 weeks), planned to attend one of the two recruitment clinics, and intended to remain in the area. Written informed consent was obtained from all participants and renewed annually.

For this study, serum markers were measured in a subset of mothers during pregnancy (n=138; 60 living with HIV and 78 without HIV) and in their children at 6 weeks (n=97; 41 HEU and 56 HU) and 2 years of age (n=111; 46 HEU and 65 HU). Selection of makers to include in our panel was based on published evidence from pediatric HIV exposure studies reporting alterations in circulating blood markers (**Supplementary Table 1**). A subgroup of children (n=156; 66 HEU and 90 HU) also participated in a longitudinal neuroimaging sub-study at the Cape Universities Body Imaging Centre (CUBIC) (**Supplementary Figure 1**). Participants included in this report were children aged 2–3 years who had undergone neonatal imaging (44), as well as additional children selected based on maternal HIV and alcohol use during pregnancy, and a randomly selected comparison group. Exclusion criteria included medical comorbidities (e.g., congenital abnormalities, genetic syndromes, neurological disorders), low Apgar scores (<7 at 5 minutes), neonatal intensive care admission, maternal use of illicit drugs during pregnancy, child HIV infection, and magnetic resonance imaging (MRI) contraindications such as cochlear implants (45).

Sociodemographic data collection

312   Maternal HIV status was determined through routine testing during pregnancy and re-checked every

313   12 weeks, following the Western Cape PMTCT guidelines at the time (46). HEU children were tested

314   at 6 weeks, 9 months, and 18 months using PCR, rapid antibody, or ELISA tests and confirmed HIV-

315   negative at 18 months or after breastfeeding cessation if this extended beyond then. HU children were

316   defined as those born to mothers without HIV infection. Mothers living with HIV received ART

317   according to PMTCT guidelines, and HEU children received post-exposure prophylaxis from birth (47).

318   Maternal CD4 cell count and viral load data during pregnancy were obtained from clinical records and

319   the National Health Laboratory Service system, using the lowest maternal CD4 cell count within one

320   year before birth and three months after birth to maximize data availability.

321   Sociodemographic and maternal psychosocial data were collected between 28 and 32 weeks of

322   gestation using interviews and standardized questionnaires adapted from the South African Stress

323   and Health study (42,43). Infant birthweight and nutrition markers were recorded following WHO Z-

324   score guidelines (48). Maternal alcohol use during pregnancy was assessed using the Alcohol,

325   Smoking, and Substance Involvement Screening Test (ASSIST), and retrospectively recorded as a

326   binary measure of moderate-severe alcohol use (44). During pregnancy, maternal smoking was self-

327   reported and assessed by ASSIST, and maternal depression was assessed using the Edinburgh

328   Postnatal Depression Scale, with depression subsequently categorized as a binary variable based on

329   a predefined cutoff.

330   **Immune assays**

331   Serum samples were collected from mothers at 26–28 weeks of pregnancy and from children at 6

332   weeks and 2 years of age (42). Concentrations of GM-CSF, INF-γ, IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8,

333   TNF-α, IL-4, IL-10, IL-12p70, and IL-13 were measured using a Milliplex® Luminex 13-plex kit

334   (#HSTCMAG28SPMX13; Merck) according to the manufacturer's instructions (9). Plates were read

335   on a Luminex system (Bio-Plex 200 System; commercial provider Bio-Rad). ELISA assays (R&D

336   Systems, Minneapolis, USA) were used to measure neutrophil gelatinase-associated lipocalin

337 (NGAL), matrix metalloproteinase-9 (MMP-9), chitinase-3-like kinase (YKL-40), and soluble markers

338 of monocyte activation (sCD14, sCD163) (9). All samples were assayed in duplicate.

339 Neuroinflammatory markers were selected due to their roles in HIV-related neuropathology. NGAL is

340 involved in neuroinflammation (49) and microglial activation (50) and has been linked to reduced brain

341 volumes and cognitive impairment in a South African cohort of adults living with HIV (30,51).

342 Circulating MMP-9 levels have been found to be significantly lower in adults with HIV compared to

343 controls (51). YKL-40 has been associated with axonal injury (52) and cognitive impairment (53) in

344 cohorts of patients living with HIV from Sweden and the US, respectively.

Magnetic Resonance Spectroscopy protocol

346 Participants in the neuroimaging sub-study underwent a multimodal MRI protocol without sedation at

347 the Cape Universities Body Imaging Centre, University of Cape Town, between January 2016 and

348 September 2018. Imaging was performed using a 3 Tesla Siemens Skyra MRI scanner (Erlangen,

349 Germany) with a 32-channel head coil (45). Children were scanned during natural sleep after obtaining

350 informed consent from their parent/legal guardian and ensuring a comfortable and secure positioning

351 using pillows, blankets, and ear protection. A trained study staff member remained in the scanner

352 room throughout the session in case the child awoke.

353 The MRS acquisition protocol has been previously described (25). Briefly, single-voxel MRS was used

354 to target the midline parietal grey matter as well as bilateral parietal white matter. Spectral quality was

355 maintained using automatic shimming and manual adjustments to reduce spectral linewidths. High-

356 resolution T1-weighted structural images were also acquired for voxel placement.

Magnetic Resonance Spectroscopy data processing

358 MRS data were processed using LCModel software (version 6.3-1) for metabolite quantification, as

359 previously described (25,54). Voxels were registered to T1-weighted structural images and

360 segmented into grey matter, white matter, and cerebrospinal fluid to correct for partial volume effects.

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361   Metabolite ratios to total creatine were calculated for myo-inositol, glutamate, N-acetyl-aspartate, and

362   total choline.

363   Spectral quality was visually inspected and assessed using signal-to-noise ratio (SNR) and full width

364   at half maximum (FWHM) given by LCModel. Spectra with FWHM values greater than 0.08 or SNR

365   values lower than 10 were considered of low quality and therefore excluded. Metabolite ratios were

366   compared across brain regions to assess neuroinflammation (myo-inositol), neuronal function

367   (glutamate), neuronal health (n-acetyl-aspartate), and white matter maturation (total choline) (23–25).

368   **Statistical analysis**

369   Sociodemographic characteristics of the mother-child pairs were reported as mean (±SD) and

370   frequencies (%). Histograms were used to visually inspect the distribution of continuous variables.

371   Differences between HEU and HU children were assessed using independent *t*-tests or Wilcoxon

372   rank-sum tests for normally and non-normally distributed continuous data, respectively. Categorical

373   variables were compared using Chi-square (X^2) tests.

374   Serum marker concentrations were log-transformed to meet assumptions of normality. Preliminary

375   group comparisons were performed using independent *t*-tests or Wilcoxon tests, depending on data

376   distribution. Longitudinal trajectories of child peripheral blood markers from 6 weeks to 2 years of age

377   were analyzed via LMMs with Maximum Likelihood Estimation with use of the *lme4* R package. This

378   approach allowed us to account for intra-individual variability and repeated measures, without

379   requiring complete cases or imputation, making it suitable for handling missing data across timepoints.

380   Three covariance structures —variance components, compound symmetry, and unstructured— were

381   considered to determine the best model fit using AIC and BIC values.

382   Linear regression models with robust standard errors and BH correction for multiple comparisons were

383   applied to investigate cross-sectional associations between serum markers and brain metabolite ratios

384   in HEU and HU children at age 2–3 years. For MRS data, a factor analysis approach was used to

385   construct weighted linear combinations of all four metabolite ratios across three parietal brain voxels,

386   creating four metabolite patterns —composite factor scores—, as previously described (25). Separate

387 models were then run to investigate cross-sectional associations between serum and metabolite

388 patterns. Adjusted linear models were run for associations that remained statistically significant after

389 BH correction. Potential confounders were chosen a priori due to their reported influence in

390 neurometabolite or neurobehavioral outcomes in children, and included child age (23,24), and child

391 sex (55,56). Given that the percentage of white matter in white matter voxels was suboptimal in our

392 study (≈52), tissue composition was also included in the adjusted analysis (25). Sensitivity analyses

393 were conducted for any sociodemographic or clinical variables that showed significant differences

394 between HEU and HU groups.

395 Mediation analyses were performed on serum markers and neurometabolites with statistically

396 significant associations in the adjusted linear models, using the *lavaan* R package for structural

397 equation modelling. Bootstrapping was used to compute confidence intervals for the indirect effect,

398 providing robust estimates that did not rely on the assumption of a normal distribution. This approach

399 was particularly valuable in our sample, where small subsample sizes limit statistical power.

400 Results were considered significant at a p-value threshold of < 0.05 (two-tailed). Statistical analyses

401 were conducted using R (version 4.4.2) with RStudio software. The analysis plan was pre-registered

402 in the Open Science Framework (57).

Ethics approval and consent

The studies involving human participants were reviewed and approved by the Faculty of Health Sciences, Human Research Ethics Committee, University of Cape Town (401/2009; 525/2012 & 044/2017), by Stellenbosch University (N12/02/0002), and by the Western Cape Provincial Health Research committee (2011RP45). Written informed consent to participate in this study was provided by the participants' parent/legal guardian.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

CBC: methodology, formal analysis and interpretation, visualization, writing – original draft, review & editing. **FR**: methodology, formal analysis, supervision, and writing – review & editing. **TK**: methodology, data curation and writing – review & editing. **JA**: methodology, data curation and writing – review & editing. **SS**: investigation and writing – review & editing. **KN**: methodology and writing – review & editing. **SJ**: methodology and writing – review & editing. **NH**: project administration and writing – review & editing. **HZ**: conceptualization, methodology, resources, and writing - review & editing. **DS**: conceptualization, methodology, investigation, resources, supervision, and writing – review & editing. **KD**: conceptualization, methodology, investigation, resources, supervision, and writing – review & editing. **CW**: conceptualization, methodology, investigation, data curation, supervision, and writing – review & editing. **PN**: conceptualization, methodology, investigation, data curation, supervision, and writing – review & editing.

All authors approved the final version.

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Data Availability

429 The Drakenstein Child Health Study is committed to the principle of data sharing. De-identified data

430 will be made available to requesting researchers as appropriate. Requests for collaborations to

431 undertake data analysis are welcome.

432 More information can be found on our website http://www.paediatrics.uct.ac.za/scah/dclhs. A

433 comprehensive Statistical Analysis Report, as well as the R code used for this study analysis, are

434 openly accessible on GitHub: https://github.com/Cescualito/UCT_DCHS_PhD.

435

Funding

447

Acknowledgments

Mutseyekwa and Judy Gatei, and research assistant Joavine Fourie for their work, the clinical and administrative staff of the Western Cape Government Health Department at Paarl Hospital and at the clinics for support of the study. We are also grateful for the assistance provided by the Cape Universities Body Imaging Centre (CUBIC) team, particularly Petty Samuels, Ingrid Op't Hof and Mazwi Maishi.

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624

625 **Tables**

626 **Table 1. Sociodemographic characteristics of children invited for MRS at age 2–3**

627 **years, according to HIV exposure**

	CHEU (n=66) Median (IQR) or n/N (%)	CHU (n=90) Median (IQR) or n/N (%)	P value
Child age at date of scan (in months)	34.00 (2.00)	35.00 (2.00)	0.07
Sex			0.43
Female	25/66 (37.9%)	41/90 (45.6%)	
Male	41/66 (62.1%)	49/90 (54.4%)	
Monthly household income (in ZAR)			0.16
<R1,000	18/66 (27.3%)	19/90 (21.1%)	
R1,000–5,000	45/66 (68.2%)	59/90 (65.6%)	
>R5,000	3/66 (4.5%)	12/90 (13.3%)	
Maternal education			0.60
Primary	3/66 (4.5%)	6/90 (6.7%)	
Some secondary	43/66 (65.2%)	49/90 (54.4%)	
Completed secondary	17/66 (25.8)	29/90 (32.2%)	
Tertiary	3/66 (4.5%)	6/90 (6.7%)	
Employed mother	16/66 (24.2%)	26/90 (28.9%)	0.64
Maternal relationship status (partnered)	38/66 (57.6%)	36/90 (40.0%)	**0.045**
Maternal age at delivery (in years)	29.09 (6.63)	25.71 (8.36)	**0.0001**
Gestational age at delivery (in weeks)	39.00 (2.75)	39.00 (2.00)	0.07
Premature birth (<37 weeks' gestation)	10/66 (15.2%)	10/90 (11.1%)	0.61
Child birthweight (in grams)	3180.00 (652.50)	3180.00 (685.00)	0.48
Exclusive breastfeeding for 5 or more months	11/66 (16.7%)	7/90 (7.8%)	0.14
Exclusive breastfeeding duration (in months)	0.81 (2.76)	1.84 (2.08)	**0.0008**
Nutritional status at 2 years old			
Stunting (height-for-age Z-score < -2)	8/56 (14.3%)	10/82 (12.2%)	0.45

Underweight (weight-for-age Z-score < -2)	3/57 (5.3%)	2/82 (2.4%)	0.44
Wasting (weight-for-length Z-score < -2)	3/57 (5.3%)	2/82 (2.4%)	0.44
Maternal anaemia during pregnancy	22/66 (33.3%)	26/90 (28.9%)	0.68
Maternal smoking during pregnancy	9/66 (13.6%)	32/90 (35.6%)	**0.004**
Maternal alcohol use during pregnancy	7/63 (11.1%)	18/89 (20.2%)	0.13
Maternal depression during pregnancy	3/48 (6.2%)	27/80 (33.8%)	**<0.0001**
Maternal hospitalization during pregnancy	5/64 (7.8%)	5/90 (5.6)	0.22
Maternal HIV diagnosis timepoint			
Before pregnancy	27/66 (40.9%)		
During pregnancy	39/66 (59.1%)		
Maternal lowest CD4 cell count during pregnancy[§]			
≤500 cells/mm^3	24/49 (49.0%)		
>500 cells/mm^3	25/49 (51.0%)		
Highest maternal viral load during pregnancy			
(undetectable) <40 copies/mL	44/53 (83.0%)		
40–1000 copies/mL	3/53 (5.7%)		
>1000 copies/mL	6/53 (11.3%)		
Antiretroviral therapy initiation			
Before pregnancy	27/66 (40.9%)		
During pregnancy	39/66 (59.1%)		
First-line antiretroviral therapy			
Efavirenz + Emtricitabine + Tenofovir (FDC)	57/66 (86.4%)		
Lamivudine + Efavirenz + Tenofovir	3/66 (4.5%)		
Lamivudine + Zidovudine + Nevirapine	2/66 (3.0%)		
Lamivudine + Zidovudine + Lopinavir/Ritonavir	1/66 (1.5%)		
Lamivudine + Zidovudine + Efavirenz	1/66 (1.5%)		
Zidovudine monotherapy	1/66 (1.5%)		
Others	1/66 (1.5%)		
Cotrimoxazole prophylaxis	55/60 (91.7%)		

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Infant prophylaxis	
Nevirapine monotherapy	54/66 (81.8%)
Nevirapine + Zidovudine	12/66 (18.2%)

628 Data are median (IQR) or n/N (%). Percentages calculated out of available data. Continuous data was

629 assessed for normality using Shapiro-Wilk tests. Comparisons between CHEU and CHU were made

630 using Wilcoxon Rank Sum (Mann Whitney U) tests for continuous data, and X^2 tests for categorical

631 data. **DCHS**: Drakenstein Child Health Study; **CHEU**: Children who are HIV-Exposed and Uninfected;

632 **CHU**: Children who are HIV-Unexposed; **ZAR**: South African Rand; **FDC**: Fixed Dose Combination.

633 Missing data: delays in child administration of routine vaccination (n=2 in the HEU group, n=1 in the

634 HU group); nutritional conditions at 2 years old (n=10 in the HEU group, n=8 in the HU group);

635 maternal alcohol use during pregnancy (n=3 in the HEU group, n=1 in the HU group); maternal

636 depression during pregnancy (n=18 in the HEU group, n=10 in the HU group); maternal lowest CD4

637 cell count during pregnancy (n=17); maternal highest viral load during pregnancy (n=13);

638 cotrimoxazole prophylaxis (n=6). §The lowest maternal CD4 cell count within 1 year before birth and
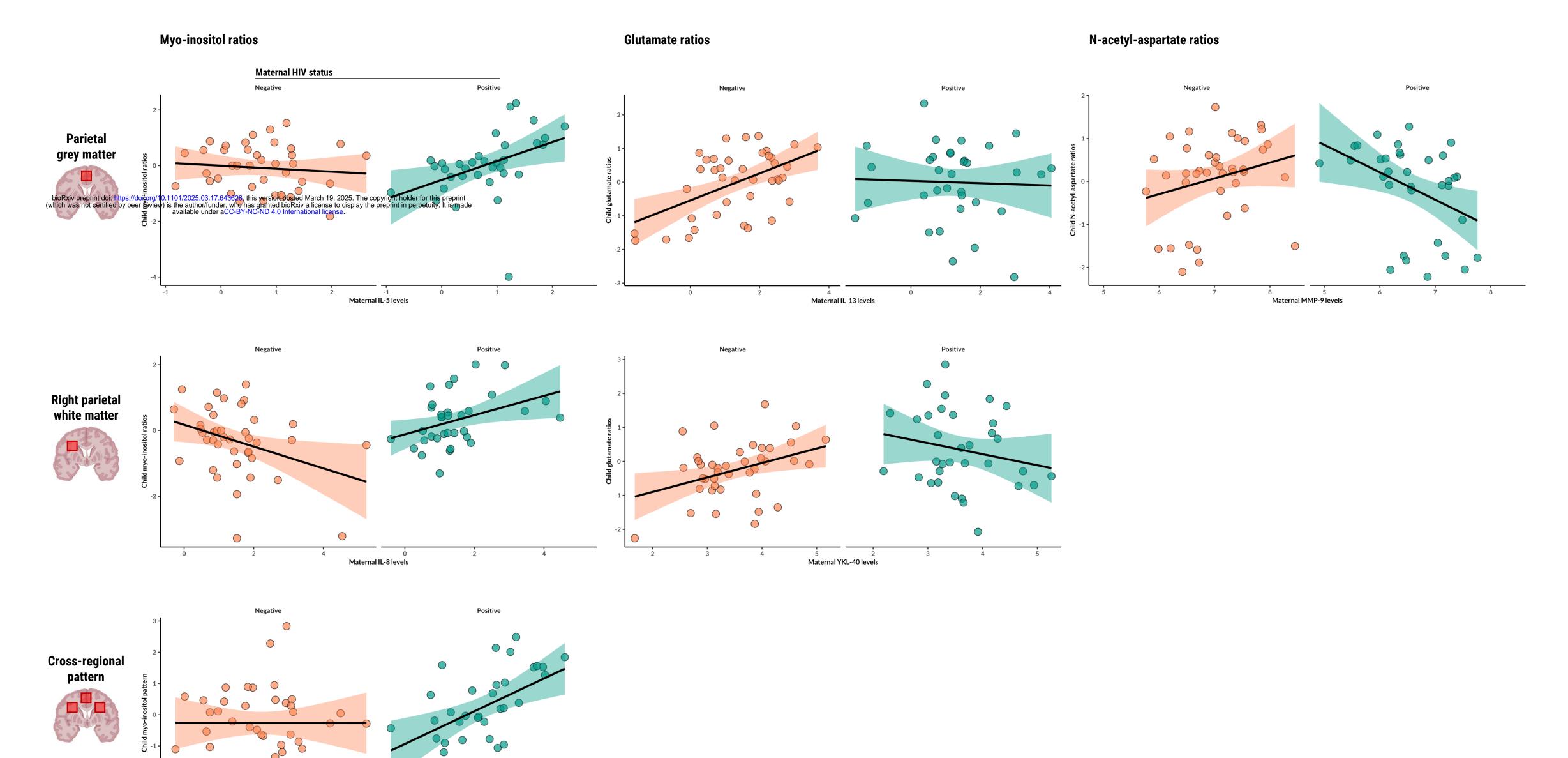
639 3 months after birth was used to maximise numbers.

640

Table 2. Associations between maternal and child immune marker concentrations and child neurometabolite ratios

Timepoint	n CHU	n CHEU	Brain region	Metabolite ratios	Marker type	Marker	β	95% CI	P-value	BH	β	95% CI	P-value
			MRS data at age 2–3 years		**Immune marker data**		**Unadjusted analysis**				**Adjusted analysis**		
Pregnancy	40	34	PGM	Glutamate	Anti-inflammatory	IL-13	-0.44	-0.80 to -0.07	0.019	0.030	-0.41	-0.80 to -0.02	**0.038**
					Neuroinflammatory	MMP-9	-0.85	-1.58 to -0.13	0.022	0.044	-0.85	-1.57 to -0.12	**0.023**
				Myo-inositol	Pro-inflammatory	IL-5	0.79	0.25 to 1.33	0.005	0.047	0.79	0.24 to 1.34	**0.005**
				N-acetyl-aspartate	Neuroinflammatory	MMP-9	-1.01	-1.74 to -0.28	0.008	0.013	-1.01	-1.74 to -0.27	**0.008**
			RPWM	Glutamate	Neuroinflammatory	YKL-40	-0.75	-1.37 to -0.14	0.018	0.032	-0.90	-1.47 to -0.33	**0.002**
				Myo-inositol	Pro-inflammatory	IL-8	0.62	0.11 to 1.14	0.018	0.009	0.64	0.10 to 1.17	**0.020**
			All regions	Myo-inositol pattern*	Pro-inflammatory	IL-5	0.83	0.24 to 1.43	0.006	–	0.84	0.23 to 1.44	**0.007**
6 weeks	29	23	LPWM	Glutamate	Pro-inflammatory	IL-1β	-0.86	-1.37 to -0.35	0.001	0.015	-0.76	-1.34 to -0.18	**0.011**
2 years	35	28	PGM	Glutamate	Neuroinflammatory	NGAL	1.00	0.12 to 1.88	0.027	0.039	1.00	0.07 to 1.94	**0.036**
				Myo-inositol		MMP-9	1.31	0.24 to 2.38	0.017	0.012	1.29	0.12 to 2.45	**0.031**
			L & RPWM	Glutamate pattern*	Neuroinflammatory	NGAL	0.78	0.13 to 1.43	0.019	0.044	0.84	0.14 to 1.54	**0.019**

Linear regression models with robust standard errors and multiple comparison correction. Models were adjusted for child age, child sex, and voxel tissue composition. Table displays statistically significant results detected in CHEU as compared to CHU. *Pattern refers to cross-regional neurometabolite factor loadings previously reported in the same cohort, identified with factor analysis (25). **CHEU**: Children who are HIV-Exposed and Uninfected; **CHU**: Children who are HIV-Unexposed; **MRS**: Magnetic Resonance Spectroscopy; **β**: Effect size; **BH**: Benjamini-Hochberg corrected p-value; **PGM**: Parietal Grey Matter; **LPWM**: Left Parietal White Matter; **RPWM**: Right Parietal White Matter.

648 **Figure legends**

649 **Figure 1. Associations between maternal or child serum markers and child neurometabolite**

650 **ratios**

651 Scatter plots displaying statistically significant associations between maternal or child serum markers

652 (IL-5, IL-8, IL-13, MMP-9, YKL-40) and child neurometabolite ratios to total creatine (myo-inositol,

653 glutamate, N-acetyl-aspartate) in specific parietal brain regions, as found in adjusted linear regression

654 analyses. Regression lines represent the direction and strength of the associations, with 95%

655 confidence intervals shaded. Data points are colour-coded based on maternal HIV status.

Myo-inositol ratios

Glutamate ratios

N-acetyl-aspartate ratios

Maternal HIV status

Parietal grey matter

Right parietal white matter

Cross-regional pattern