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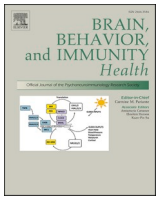

Neuroinflammatory markers at school age in preterm born children with neurodevelopmental impairments

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ABSTRACT

Background: Immune system activation in the neonatal period is associated with white matter injury in preterm infants. In animal studies, neonatal priming of the immune system leads to chronic activation of i.e. microglia cells and altered neuroinflammatory responses potentially years after preterm birth. This may contribute further to brain injury and neurodevelopmental impairment. It is unknown to what extend this also occurs in human.
Aim: To identify neuro-inflammatory markers at school age that relate to motor, cognitive and behavioral impairments in preterm born children in a pilot case-control study.
Methods: We included $n = 20$ preterm born children (GA < 28 weeks) in this study, of which n = 10 with motor, cognitive and behavorial impairments and $n = 10$ preterm born controls next to n = 30 healthy adult controls. In the preterm children, at 8–9 years, 39 inflammatory markers were assessed by Luminex assay in blood serum samples. Firstly, the preterm concentrations of these markers were compared to $n = 30$ adult controls. Then a univariate analysis was performed to determine differences in values between preterm children with and without impairment at school age. Finally, a principal component analysis and hierarchical clustering was performed to identify protein profiles in preterm born children that relate to impairment at school age.
Results: Inflammatory proteins in preterm children at school age differed from values of adult controls. Within the group of preterm children, we found significantly higher levels of GM-CSF in preterms with impairment ($p < 0.01$) and a trend towards significance for Gal1 and TRAIL ($p = 0.06$ and $p = 0.06$ respectively) when compared to preterms without impairment. In addition, differences in clustering of proteins between preterm children was observed, however this variance was not explained by presence of neurodevelopmental impairments.
Conclusion: The inflammatory profile at school age in preterm children is different from that of adult controls. The immune modulating cytokines GM-CSF, Gal1 and TRAIL were higher in preterm children with impairment than control preterm children, suggesting that immune responses are altered in these children. No specific cluster of inflammatory markers could be identified. Results indicate that even at school age, neuroinflammatory pathways are activated in preterm born children with neurodevelopmental impairments.

1. Introduction

Yearly, out of 15 million preterm born infants, 750.000 are born extremely preterm (<28 weeks gestation) (Torchin et al., 2015). These extremely preterm born infants are at risk for motor, cognitive and behavioral impairments at school age (Aho et al., 2021). High prevalence impairments include problems in fine motor skills, attention deficits, executive functioning and academic achievement (Geldof et al., 2012; Johnson and Marlow, 2014). Altered brain development, through disruption of normal in-utero brain maturation, is associated with long term neurodevelopmental impairments. In the third trimester, processes such as glial genesis, axon-dendrite outgrowth and synapse formation take place. These processes of brain maturation in preterm born infants are vulnerable to additional insults (Salmaso et al., 2014). Also, specific

; CNS, Central Nervous System; CP, Cerebral Palsy; GMH-IVH, Germinal Matrix Hemorrhage-Intraventricular Hemorrhage; IQ, Intelligence Quotient; NEC, Necrotizing enterocolitis; PC, Principal Component; PCA, Principal Component Analysis; ROP, Retinopathy Of Prematurity.

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types of brain injury in preterm infants are associated with neurodevelopmental impairments in later life. Examples are germinal matrix-intraventricular haemorrhage (GMH-IVH), but also white matter injury resulting from hypoxic and/or immunological insults (Lekic et al., 2015).

It is known that preterm infants show different immune responses than term born infants due to the immaturity of their immune system, with reduced innate and adaptive immunity (Melville and Moss, 2013). In preterm rats, Szaflarski et al. (1995) observed that during an hypoxic event an inflammatory response in the central nervous system (CNS) is induced in which cytokines and chemokines recruit immune cells (Szaflarski et al., 1995). In preterm infants, systemic inflammation is related to the development of white matter injury, i.e. in the case of sepsis or hypoxia (Galinsky et al., 2018; Prasad et al., 2021). It has been suggested that inflammatory responses may lead to microglial reactivity in the CNS, but also to chronic activation of microglia and astrocytes and recruitment of brain infiltrating $CD4^+$ T-lymphocytes up until months after preterm birth (Fleiss and Gressens, 2012). In previous research, Favrais et al. (2011) found that moderate perinatal systemic inflammation can alter the manner in which healthy white matter of the brain develops in new-born mice due to the damage done by the recruitment of immune cells (Favrais et al., 2011). This means that *priming* of the immune system that is observed in animal models after birth could have an ongoing negative effect on the integrity of the CNS later in life and could play an additional role in in the pathogenesis of brain injury in extremely preterm born infants (Hagberg et al., 2015). Neuroimmune crosstalk between the peripheral immune system and the CNS immune system is known to be an active manner of communication and plays a role in the white matter injury that is observed in preterm born children (Tian et al., 2012).

For the CNS, it is proposed that chronic activation of the immune system may predispose to future damage or counter repair or regeneration of the damaged tissue and therefore further worsen neurodevelopmental outcome (Dammann and Leviton, 2013).

It is unknown if and to what extent this inflammatory response is persistent until school age in preterm born *children* and which inflammatory proteins are involved in this response. In this pilot study, we aim to identify inflammatory markers at school age, for motor, cognitive and behavioral impairments in preterm born children. We hypothesize that previously identified proteins for neuro-inflammation are increased in preterm born children at school age with neurodevelopmental impairments, compared to controls. Identification of potential biomarkers at school age could reveal cellular pathways that play a role in neuro-inflammation and neurodevelopmental impairment.

2. Method

2.1. Subjects

This study is part of the 'Biomarkers for Outcome at School age in preterm born children' (BIOS) study. We included extremely preterm born children (<28 weeks of gestation), born between 2008 and 2012 that were admitted to the neonatal intensive care units of the Wilhelmina Children's Hospital. The BIOS study was approved by the medical ethical committee of the University Medical Center Utrecht (METC no. 17–645). Written informed consent was obtained from the parents to draw blood of all children included in this study. We registered if the children used the following types of medication at the moment of drawing blood or in the past week: corticosteroids (any route of administration), antibiotics or other immunosuppressive medicine, to control for confounding factors. None of the children used these medications.

Out of n = 102 children that were included until February 2022, we selected all children with significant neurodevelopmental impairment. Significant neurodevelopmental impairment was defined by one or more of the following criteria: presence of cerebral palsy (CP), intelligence quotient (IQ) < 85, or psychiatric disorder (according to the Diagnostic and Statistical Manual of Mental Disorders, DSM-V). In the cohort of n = 102 children, n = 10 had significant neurodevelopmental impairment according to these criteria. We then matched the cases to control infants from this cohort that had a favorable neurodevelopmental outcome (i.e. motor, cognitive, and behavioral outcome within the normal range) on gestational age and gender. We than randomly selected n = 10 controls. This resulted in a sample size of n = 20 children in this pilot study. Additionally, we incorporated the dataset of an existing control group of n = 30 healthy adult controls derived from Scholman et al. (2017) (Scholman et al., 2018).

2.1.1. Neurodevelopmental follow-up program

The preterm born children participated in an extension of a routine follow-up program. It entailed the assessment of motor performance, cognitive functions, and behavior at school age (8–9 years).

2.2. Motor outcome

On the basis of the reports of the routine follow-up program, we determined the presence or absence of CP following the guidelines of the Surveillance of Cerebral Palsy in Europe (SCPE) (Surveillance of Cerebral Palsy in Europe, 2000). At school age, motor development was determined by the Movement Assessment Battery for Children (Movement-ABC) (Smits-Engelsman et al., 2011) and a pediatric neurological examination.

2.3. Cognitive outcome

Intellectual development was assessed by the Wechsler Intelligence Scale for Children-V (Wechsler, 2018). Additionally, an extensive neuropsychological test battery was performed, that included tests for attention, visual perception, visuomotor integration, verbal memory, and executive function. Here, we report only on the IQ.

2.4. Behavioral outcome

During follow-up, we screened the behavior of the children by the Child Behavior Checklist (age 4–18) (Achenbach, 1991). In case of a subclinical or clinical score on this checklist, or when parents had worries about the behavior of their children, children were referred to a child psychiatrist for a formal psychiatric assessment according to the DSM-V.

2.4.1. Serum inflammatory markers

The blood of the children was drawn once, at 8–9 years of age during follow up. Blood samples were collected in day-time between 10:00–14:00. Inflammatory markers were determined in blood serum by using the Luminex technology from the laboratory of translational immunology (UMC Utrecht), as described before by Scholman et al. (2018) (Scholman et al., 2018). In short, Luminex is an Multi-Analyte Profiling (MAP) technique that allows to measure multiple analytes in one sample. Fluorescent beads with a unique fluorescent signal on the fluorescent spectrum are coated with a capture antibody. If added to the sample, it captures the protein of interest. Using a Luminex instrument, the beads are excited with one laser to determine the fluorescent signal and another laser determines the magnitude of the signal to determine the volume of the protein of interest (Bio-techne, 2022). We used a custom-made panel of 39 inflammatory markers (IL1-RA, IL-1α, IL-1β, IL-4, IL-6, IL-9, IL-10, IL-13, IL17, IL-18, IL-33, TNFα, TNFβ, IFNɣ, TWEAK, Follistatin, MIP-1α, MIP-1β, MCP3, Eotaxin, Eotaxin 3, IL-8, MIG, IP10, G-CSF, M-CSF, GM-CSF, VEGF, FasL, TRAIL, Gal1, Procalcitonin, PAI1, SerpinG1, RANTES, TIMP1, SerpinF2, MMP9 and CRP), that were previously related to neuro-inflammation in other diseases or in animal studies (Fleiss and Gressens, 2012; Favrais et al., 2011; Hagberg et al., 2015; Dammann and Leviton, 2013; Bona et al.,

Table 1
Baseline characteristics of preterm infants.

Clinical characteristics	Neurodevelopmental impairment (n = 10)	Control (n = 10)
Gestational age (weeks), SD	26.4 (1.2)	25.8 (1.2)
Birth weight (g), SD	902 (256)	887 (191)
Female, no. (%)	3 (30)	5 (50)
Neurological development		
CP	n = 3	–
Low IQ (<85)	n = 7	–
Psychiatric disorder	n = 4	–
Apgar score (5 min), SD	7 (1)	6 (2)
Cerebral pathology		
Cystic PVL[a]	n = 1	n = 0
Mild GMH-IVH[b]	n = 2	n = 2
Severe GMH-IVH[b]	n = 3	n = 2
Late on-set morbidity		
NEC	n = 1	n = 0
ROP grade 1–2	n = 2	n = 3
ROP grade 3–5	n = 0	n = 1
BPD	n = 4	n = 2
Sepsis	n = 3	n = 2

Abbreviations: BPD-bronchopulmonary dysplasia; CP- cerebral palsy; GMH-IVH- germinal matrix haemorrhage-intraventricular haemorrhage; IQ-intelligence quotient; NEC- necrotising enterocolitis; PVL-periventricular leukomalacia; ROP- retinopathy of prematurity; SD−standard deviation.

[a] Defined as grade 2 or worse.

[b] Mild was defined as grade I or II, severe as grade III or with parenchymal extension.

1999; Assarsson et al., 2014). The determination of the analytes by Luminex technology was done in a validated manner, as described by Scholman et al. (2018) (Scholman et al., 2018). If protein measurements values showed >3 values that were out-of-range than the protein was excluded (i.e. the lowest or highest analyte concentration that can be quantified with acceptable (predefined goals for bias and imprecision) precision and accuracy in our Luminex assays, calculated using a signal to noise ratio (S:N) of 10:3 (Bioplex FLEXMAP 3D) multiplied by the SD of 10 replicates) (Bio-techne, 2022). If the protein had ≤3 values that were out-of-range, the lowest or highest analyte concentration that can be quantified, specific for the protein, was included in the analysis.

2.4.2. Statistical analysis

We firstly examined if the inflammatory markers of preterm born children at school age differed from the inflammatory markers of the adult controls. Subsequently we performed a univariate analyses to examine differences between individual proteins in the preterm impairment group (n = 10) versus the preterm controls (n = 10). Additionally, we performed a profile analysis to define what combination of proteins explain differences in volumes across the entire cohort. The univariate analysis was performed by using SPSS (version 26.0.0.1) and the profile analysis by using R-studio (version 4.1.0).

2.5. Univariate analysis

To define differences between individual proteins between groups we performed a student t-test for normally distributed proteins and a Mann-Whitney *U* test for not normally distributed proteins. The distribution of the volumes of the proteins was examined with a Kolmogorov-Smirnov test.

2.6. Inflammatory profile analysis

A hierarchical analysis and a principle component analysis (PCA) was performed to visualize the biomarker expression profiles from patients and controls. With these analyses we aimed to determine if combinations of proteins explain variability in values and separate patients from controls.

Table 2
Concentrations of analytes (pg/mL) in blood serum of preterm children compared to adult controls.

	Preterm children at school age (n = 20)		Adults (n = 30)	
	Mean (SD) or Median (IQR)[a,b]	Min-Max	Mean (SD) or Median (IQR)[a,b]	p-value
IL-RA	170.12 (237.42)[b]	4.20–1165.58	3.20 (35.24)[b]	<0.01
IL-1α	2.09 (3.36)[b]	0.65–21.68	4.23 (4.05)[b]	n.s.
IL-10	2.91 (1.15)[b]	1.69–35.45	0.75 (1.33)[b]	<0.01
IL-13	11.16 (8.99)[b]	2.92–132.01	9.41 (39.66)[b]	n.s.
IL-18	170.08 (106.94)[b]	121.89–465.48	75.57 (53.58)[b]	<0.01
TNF-α	0.87 (0.79)[b]	0.11–3.23	0.01 (0)[b]	<0.01
TWEAK	4938.48 (1127.09)[a]	2630.98–6831.43	14,625.04 (5118.94)[b]	<0.01
MIP-1α	37.31 (15.30)[a]	9.12–74.78	7.60 (6.61)[b]	<0.01
MIP-1β	95.35 (41.23)[a]	26.26–172.26	48.44 (24.41)[a]	<0.01
MCP3	45.70 (48.76)[b]	5.50–1156.24	1.43 (12.82)[b]	<0.01
Eotaxin	76.28 (37.23)[a]	19.17–151.04	76.27 (33.69)[a]	n.s.
Eotaxin3	5.98 (2.94)[1]	0.70–12.31	n.r.	–
IL-8	5.51 (4.75)[b]	2.96–63.42	12.76 (9.19)[b]	<0.01
MIG	5.86 (4.88)[b]	2.84–52.24	93.21 (17.82)[b]	<0.01
IP-10	114.88 (65.82)[b]	62,90–717.64	250.36 (129.65)[b]	<0.01
GM-CSF	6.11 (2.20)[a]	2.4–9.9	8.00 (0)[b]	n.s.
VEGF	202.48 (143.46)[a]	45.19–529.38	353.65 (474.90)[b]	n.s.
TRAIL	89.24 (52.70)[b]	12.93–114.18	n.r.	–
GAL1	29,927.33 (6851.80)[a]	21,070.94–45,653.21	18,253.94 (5909.00)[a]	<0.01
PAI1	27,767.74 (8205.76)[a]	12,366.74–41,496.42	n.r.	–
SerpinG1	486,182.67 (118,811.73)[a]	278,195.77–756,826.51	n.r.	–
RANTES	84,698.74 (45,204.87)[a]	35,979.42–227,842.35	462,401.88 (99,091.02)[b]	<0.01
TIMP1	115,248.66 (24,748.48)[a]	69,473.33–167,703.59	510,125.00 (61,900.00)[b]	<0.01
SerpinF2	3,175,173.26 (1,496,067.80)[a]	368,365.20–7,477,000	n.r.	–
MMP9	4,120,255 (1,479,418.46)[a]	1,885,300–7,702,400	1,729,017.18 (936,180.58)[a]	<0.01
CRP	1,007,515.29 (2,567,605.80)[b]	70,718.47–20,481,000	n.r.	–

NR; not reported.

[a] Mean reported for normally distributed variables.

[b] Median reported for non-normally distributed variables.

Table 3
Concentrations of analytes (pg/mL) in blood serum of preterm children with and without impairment.

	Neurodevelopmental impairment (n = 10)	Control (n = 10)	
	Mean (SD) or Median (IQR)[a,b]	Mean (SD) or Median (IQR)[a,b]	P-value
IL-RA	234.37 (219.06)[b]	150.98 (275.06)[b]	n.s.
IL-1α	2.79 (6.81)[b]	1.88 (2.66)[b]	n.s.
IL-10	3.20 (7.53)[b]	2.71 (1.02)[2]	n.s.
IL-13	10.82 (22.63)[b]	14.05 (8.80)[b]	n.s.
IL-18	142.90 (109.72)[b]	199.45 (130.07)[b]	n.s.
TNF-α	0.96 (0.91)[b]	0.71 (0.71)[b]	n.s.
TWEAK	4907.97 (1088.72)[a]	4968.99 (1222.46)[a]	n.s.
MIP-1α	38.48 (19.75)[a]	36.13 (10.05)[a]	n.s.
MIP-1β	87.40 (34.93)[a]	103.30 (47.21)[a]	n.s.
MCP3	39.48 (63.98)[b]	53.35 (50.54)[b]	n.s.
Eotaxin	68.89 (39.94)[a]	83.66 (34.79)[a]	n.s.
Eotaxin3	6.42 (3.21)[a]	5.54 (2.73)[a]	n.s.
IL-8	7.14 (5.31)[b]	4.70 (3.22)[b]	n.s.
MIG	5.86 (6.89)[b]	5.86 (7.72)[b]	n.s.
IP-10	108.57 (109.29)[b]	130.76 (102.66)[b]	n.s.
GM-CSF	6.77 (2.62)[a]	5.44 (1.54)[a]	**p < 0.01**
VEGF	172.40 (111.00)[a]	232.56 (170.64)[a]	n.s.
TRAIL	92.16 (35.37)[b]	56.21 (62.50)[b]	**0.063**
GAL1	32,756.72 (7192.29)[a]	27,097.95 (5437.18)[a]	**0.063**
PAI1	26,045.98 (7797.91)[a]	29,487.51 (8644.66)[a]	n.s.
SerpinG1	473,617.67 (114,774.87)[a]	498,747.67 (127,578.64)[a]	n.s.
RANTES	80,617.66 (32,576.34)[a]	88,777.83 (56,706.36)[a]	n.s.
TIMP1	106,578.08 (22,374.51)[a]	123,917.25 (25,007.68)[a]	n.s.
SerpinF2	3,067,036.52 (1,973,402.41)[a]	3,283,310.00 (897,117.41)[a]	n.s.
MMP9	4,079,980.00 (1,512,270.63)[a]	4,160,530.00 (1,526,426.36)[a]	n.s.
CRP	784,510.88 (1,691,849.11)[b]	1,175,850.00 (3,040,546.38)[b]	n.s.

Ns; not significant.
[a] Mean reported for normally distributed variables.
[b] Median reported for non-normally distributed variables.

3. Results

3.1. Patient characteristics

Out of the neurodevelopmental impairment group of 10 patients, 1 patient had solely CP, 2 had both CP and intellectual impairment (IQ < 85), 2 had both a psychiatric disorder and intellectual impairment, 2 a psychiatric disorder without a report of having an intellectual impairment and 3 patients had an intellectual impairment only. The patient characteristics are summarized in Table 1. Considering the neonatal complications, the rate of necrotizing enterocolitis (NEC), sepsis and GMH-IVH was higher in the impairment group than in the controls. This was not the case for retinopathy of prematurity (ROP).

In Table 2, biomarker concentrations of the 39 inflammatory markers over the entire cohort of preterm children (n = 20) are given and compared to healthy adult values. In 13 proteins >3 values were out-of-range beyond detection and were not included in the analyses (IL-1β, IL-4, IL-6, IL-9, IL-17, IL-33, TNF-β, IFN-γ, Follistatin, GSCF, MCSF, FASL and Procalcitonin). Therefore, the remaining 26 proteins were included for analyses, of which 18 proteins had no out-of-range values, IL-13, MIP-1α and TRAIL had 1 out-of-range value, IL-1α protein had 2 out-of-range values and IL-RA, TNF-α and MCP-3 had 3 out-of-range values. All out-of-range values were below the lowest analyte concentration that can be quantified, non were above the highest analyte concentration.

The inflammatory proteins of preterm children at school age showed significant differences in all values compared to the adult controls, except for IL-13. Some cytokines were significantly lower, whilst others on the other hand were higher.

In Table 3 the differences between the neurodevelopmental impairment group and control group are given.

3.1.1. Serum markers: univariate analysis

A univariate analysis showed that out of the 26 analytes that we detected, GMCSF was significantly higher in the impaired group versus the controls (mean 6.77 versus 5.44, p = 0.006). TRAIL and Gal1 showed a trend towards significance (median 92.16 vs 56.21, p = 0.063 and mean 32,756.72 vs 27,097.95, p = 0.063 respectively). Here, we did not correct for multiple-testing. The distribution of the analytes is shown in.

3.1.2. Inflammatory profile analysis

With R-studio we mapped which subjects showed similar expression in the 39 analytes by an hierarchical analysis, independently of the group they belong to (see Fig. 1). All 39 analytes are included here, considering that we report relative volumes rather than absolute values. The heatmap (Fig. 2) shows no clear separation between the preterm children with neurodevelopmental impairment (X1-X10) and preterm controls (X11-20).

3.1.3. Principal component analysis

To understand which proteins explained the most variance of the concentrations of the analytes, we performed a principle component analysis (PCA, Fig. 3). Although it seems that the neurodevelopmental impairment group (triangles) are more defined by principal component (PC) 2 and controls (circles) more by PC 1, this analysis shows that the neurodevelopmental impairment group and the control group are situated in both clusters defined by the PCs. This means that the clusters show only mild differences between the two groups or that the clusters are not defined by the difference between the defined groups, but possibly by other (currently unknown) factors.

4. Discussion

In this pilot case-control study we aimed to identify neuro-inflammatory markers related to motor, cognitive and behavioral impairments in extremely preterm born children at school age. Compared to the level of inflammatory proteins in adults, preterm children at school age have a different inflammatory profile with certain cytokines being significantly higher and others being lower. This suggests that immune responses are altered in these children. In addition, the immune modulating cytokines GM-CSF, Gal1 and TRAIL were higher in preterm children with impairment compared to preterm children without. No specific cluster of inflammatory markers was identified. Results indicate that even at school age, neuro inflammatory pathways seem activated in preterm born children with neurodevelopmental impairments.

The inflammatory profile we observed in preterm children at school age differed from the inflammatory profile in adults. Certain cytokines where significantly higher, whilst other were lower compared to the adult controls. The present study is, to the best of our knowledge, the first to report on immune profiles at school age in preterm born children. It is the question whether the reported differences in our study can be attributed to preterm birth, or that the difference in age between the cases and controls plays a role. A study of Decker et al. (2017) showed a significant effect of age on cytokine measurements in healthy children. They found that with increasing age, concentrations of cytokines IL-1RA, IP-10 and TNF- α significantly decreased (Decker et al., 2017). Sack et al. (1998) also found that healthy children have higher values of TNF-α than adults (Sack et al., 1998). Interestingly, in our cohort of preterm children we found the opposite regarding the protein of IP-10, were we found higher concentrations of IP-10 in adults compared to preterm children, suggesting that being born preterm affects these concentrations. Additionally, for IL-1α, IL-13, Eotaxin, GM-CSF and VEGF we found no significant differences between adult and preterm

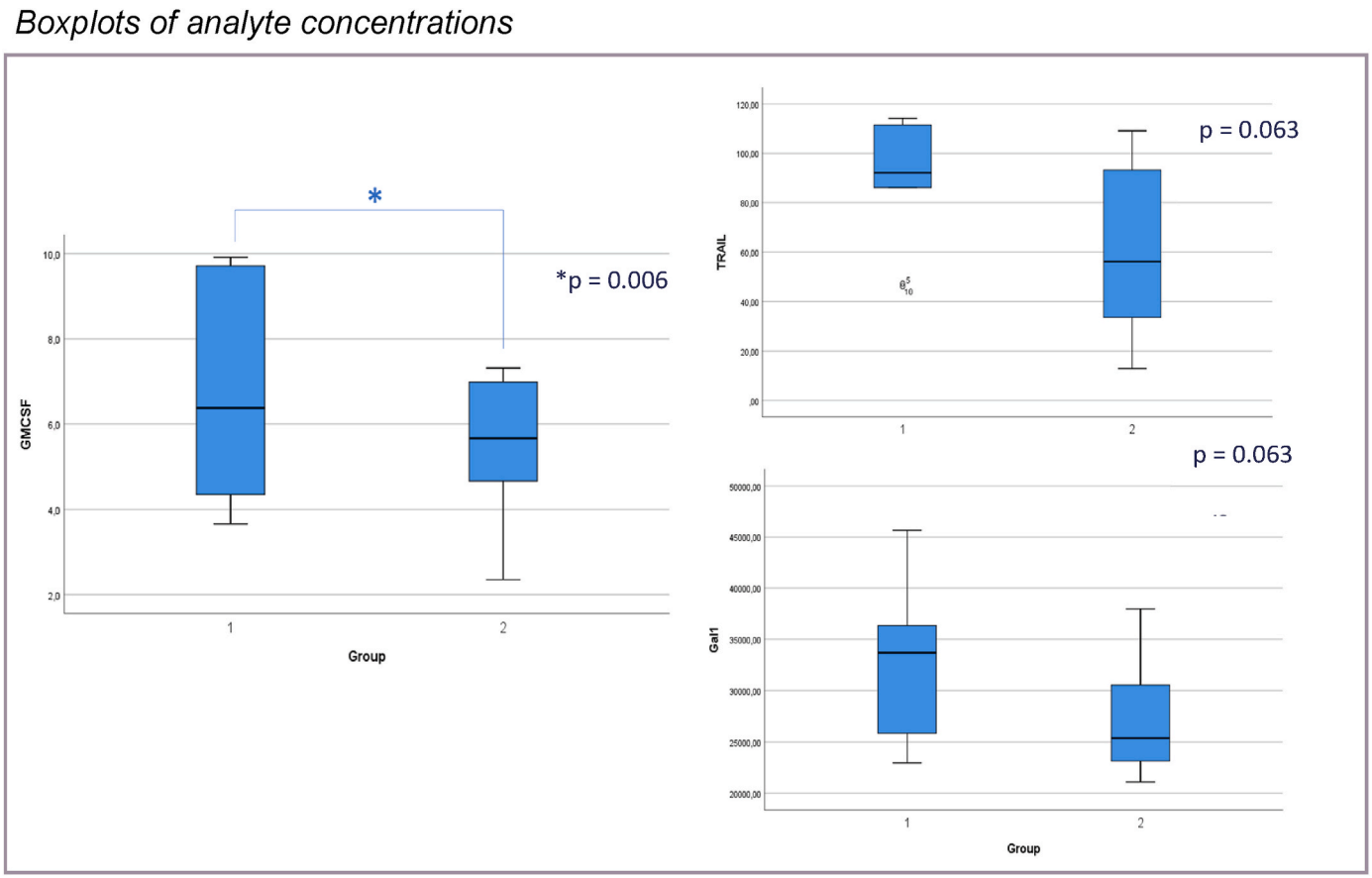

Fig. 1. Boxplots of concentration (pg/mL) of GM-CSF, TRAIL and Gal1 across group 1 (impairment) and group 2 (control).

children's inflammatory values.

In our study, GM-CSF (granulocyte macrophage colony-stimulating factor) concentrations were increased in preterm children with neurodevelopmental impairment. This pro-inflammatory protein is involved in myeloid cell development and dendritic cell differentiation and survival (Egea et al., 2010). Spath et al. (2017) also showed that a dysregulated production of this protein induces spontaneous immunopathology in a mouse model of CNS inflammation (Spath et al., 2017). The higher concentrations of GM-CSF measured in children with impairment, might be a marker of this so called 'tertiary phase of damage' in the brain (Fleiss and Gressens, 2012). Also, TRAIL (TNF-related apoptosis inducing ligand) was increased in the impairment group. This protein plays a role in the innate immune response to infection and in apoptosis signaling in CNS cells (Shepard and Badley, 2009; Huang et al., 2005). On the contrary, the *anti*-inflammatory protein Gal1 was also increased in the impairment group. Gal1 deactivates activated microglia and protects from inflammation-induced neurodegeneration (Starossom et al., 2012). Perhaps this is a reflection of an activated 'repair' mechanism. Nevertheless, these contradicting functions of GM-CSF, TRAIL and GAL1 in relation to the damage to the CNS indicate that the cellular pathogenic pathways involved are complex.

A study of Lin et al. measured TNF- α levels in the plasma of preterm children at school age with CP (Lin et al., 2010). They found significant higher levels of TNF- α expression in 32 preterm children with CP compared to preterm controls, both before and after lipopolysaccharide stimulation. We could not identify differences in TNF- α levels between preterm children with impairments and controls. Our sample size was smaller, but also the type of neurodevelopmental impairment was different, since all of their included children had motor disability (CP) whilst our study also included children with isolated intellectual and psychiatric disorders.

In the principle component analyses, we identified clustering of certain inflammatory markers at school age, however they could not be related to presence neurodevelopmental impairments. The neurodevelopmental impairment group (illustrated by triangles in Fig. 3) falls into both clusters. However, what is seen in the PCA is that four subjects of the neurodevelopmental impairment group were more defined by a specific combination of PC1 and PC2. This could be an indication of a subgroup within the neurological impairment group. In this study, we took motor, cognitive and behavioral impairments together in the neurodevelopmental impairment group. However, it could be that different types of impairments are related to a different inflammatory profile. Also, other factors not reported on (such as episodes of neonatal sepsis) could play a role in this clustering of inflammatory markers at school age.

Interestingly, subject X9 in the heatmap shows a different profile on almost all inflammatory proteins compared to the entire cohort. This patient was not ill at the time blood was drawn and had no history of medical diseases. With the current knowledge we do not have an explanation for these higher values.

Some considerations need to be taken into account when interpreting our results. Firstly, we identified some interesting differences between inflammatory protein values in preterm born children compared to term born adult controls, however, studies that measure inflammatory protein values longitudinally with age are lacking. Therefore, the comparison of inflammatory protein values of preterm born children with term-born adults should be interpreted with caution. Secondly, taking the small sample size of this pilot into account, it is of interest that we found differences in concentrations of inflammatory markers at school age. We chose not correct for multiple comparison since the expected differences in proteins are not random, however this needs to be taken into account when interpreting the results. Studies with larger sample sizes will be

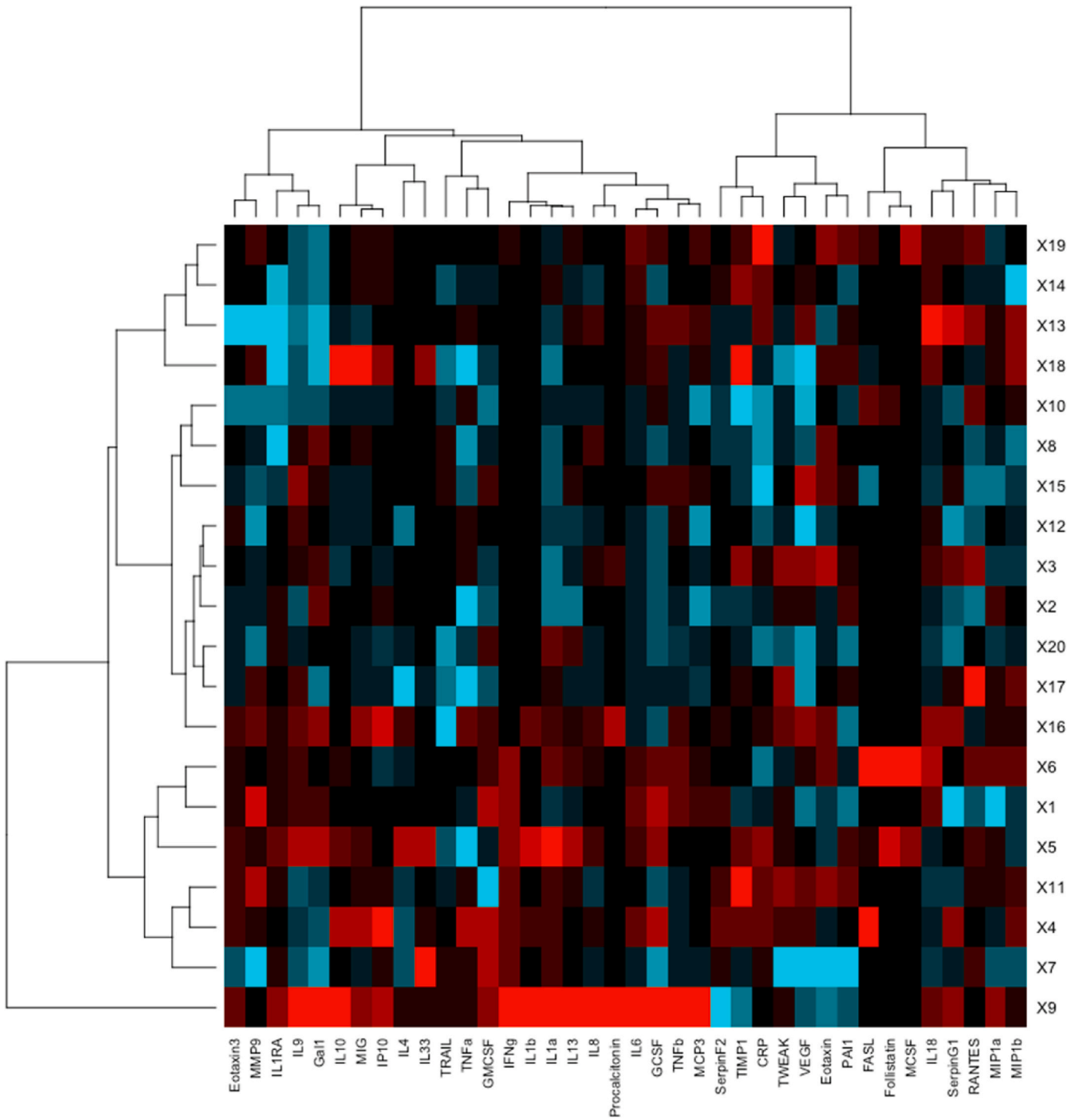

Fig. 2. Hierarchical analysis plotting the 39 proteins measured in blood serum along the x-axis against the individual subjects (with neurodevelopmental impairment: X1-X10, controls: X11-X20). Red indicates relatively high volumes of the protein, blue indicates relatively low volumes and black median levels {Color should be used for this figure in print}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

necessary to confirm our findings. Furthermore, some inflammatory proteins that were measured that could serve as biomarkers were not possible to detect because they fell out of the quantifiable range. Additionally, some values that were reported by using Luminex were extrapolated values of fluorescence-concentration curves. They are included in this study, however, there might be a certain margin of error in these values.

For future studies, it would be of interest to study inflammatory markers in a longitudinal manner in order to see if the detected differences at school age already exist early after birth. Also, stratifying outcome in motor, cognitive and behavioral impairment subgroups might be relevant. This may lead to a further understanding of the cellular pathways involved in brain injury and neurodevelopmental impairment in preterm children and potential intervention strategies with immune modulating therapies.

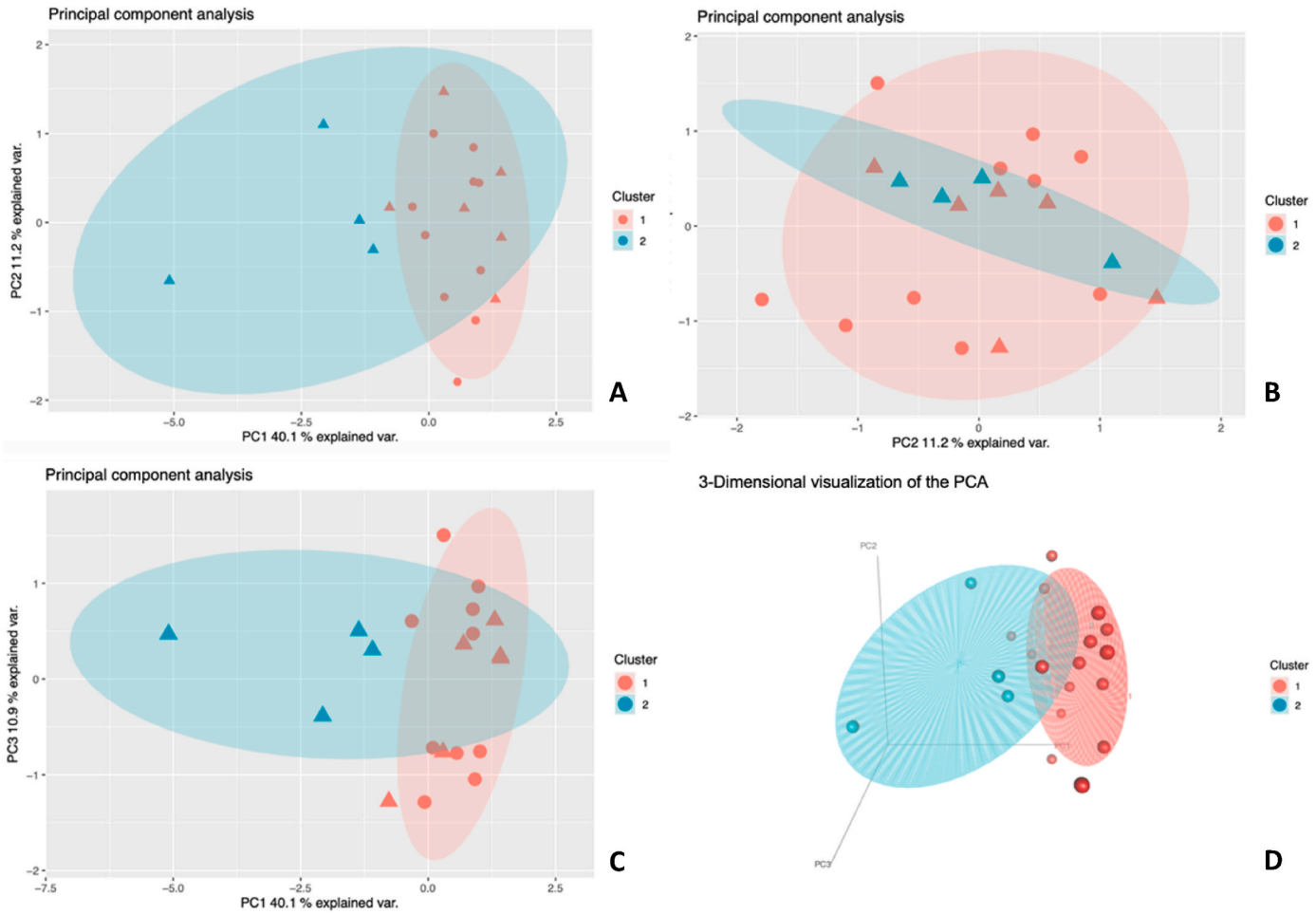

Fig. 3. A: PC1 plotted to PC2. B: PC2 plotted to PC3. C: PC3 plotted to PC1. D: visual representation of PC1, PC2 and PC3. The neurodevelopmental impaired group are shown by the triangles, the control by circles in A B and C. **PC1**: IFNg, TNFb, IL6, IL9, IL1RA, GCSF, MCP3, IL1b, IL1a, IL13. **PC2**: IL1RA, GCSF, TNFb, MCP3, IFNg, IL1a, IL6, IL33, IL8, CRP. **PC3**: CRP, IL9, IL33, TNFb, IL1RA, IL10, FASL, IFNg, MIG, IP10 {Color should be used for this figure in print}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

5. Conclusion

This study showed that preterm born children at school age have a different inflammatory profile than healthy adult controls. The immune modulating cytokines GM-CSF, Gal1 and TRAIL were higher in preterm children with impairment than without. This might suggest that immune responses are altered in these children. No specific cluster of inflammatory markers was identified. Results indicate that even at school age, neuro inflammatory pathways seem activated in preterm born children with neurodevelopmental impairments.

CRediT authorship contribution statement

S. Van der Zwart: Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **E.F. Knol:** Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. **P. Gressens:** Conceptualization, Supervision, Validation, Writing – review & editing. **C. Koopman:** Data curation. **M. Benders:** Conceptualization, Funding acquisition. **E. Roze:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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