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Hanna Kallankari

PERINATAL FACTORS AS PREDICTORS OF BRAIN DAMAGE AND NEURODEVELOPMENTAL OUTCOME

STUDY OF CHILDREN BORN VERY PRETERM

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE,
INSTITUTE OF CLINICAL MEDICINE,
DEPARTMENT OF PAEDIATRICS;
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OULU UNIVERSITY HOSPITAL

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Study of children born very preterm

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 12 of the Department of Paediatrics, on 23 January 2015, at 12 noon

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Abstract

Children born preterm are prone to acute brain insults related to subsequent neurodevelopmental impairments. However, the role of specific biomarkers and perinatal clinical factors in the pathogenesis of brain injury and neurodevelopmental sequelae has remained poorly understood.

The present study evaluated whether specific immunoproteins at birth predict the risk of intraventricular hemorrhage (IVH) and whether their receptors are localized at the bleeding site. We further investigated whether children who went on to develop cerebral palsy (CP) could be identified on the basis of blood immunoproteins collected during the perinatal period. The association between single nucleotide polymorphisms in the *chemokine CCL18* gene and susceptibility to CP was also studied. Finally, we investigated the association of pre- and postnatal factors with cognitive outcomes in very preterm-born schoolchildren without impairments.

The present study revealed that a low concentration of CCL18 in cord blood was an independent risk factor of IVH in very preterm infants. The CCL18 receptor, CCR3, was detectable in the periventricular area and in the neurons of the hippocampus in preterm infants already at 23 weeks of gestation. We also identified a cluster of cord blood cytokines that was associated with the risk of CP. In addition, inflammatory cytokine levels were associated with CP risk on days 1 and 7 after birth. The genetic study showed that both IVH and the *CCL18* polymorphism independently and additively had an influence on CP susceptibility.

Our study further demonstrated that schoolchildren born very preterm without CP or cognitive impairment had poorer performance in visuospatial–sensorimotor skills and in attention–executive functions than term-born children. Fetal growth restriction was an independent risk factor of compromised neurocognitive outcome in very preterm children predicting difficulties in language, memory and learning.

In conclusion, specific cytokines and cytokine clusters serve as biomarkers of different pathways involved in damage to the brain structures and in the pathogenesis of CP. In addition, genetic factors can affect these processes. Further, fetal growth restriction and prematurity play important roles in neurocognitive development later in life.

Keywords: brain, cerebral palsy, cytokines, fetal growth restriction, genetic predisposition, intraventricular hemorrhage, neurocognitive development, very premature birth

Kallankari, Hanna, Perinataalisten tekijöiden vaikutukset aivojen vaurioitumiseen sekä neurologiseen ja kognitiiviseen kehitykseen hyvin ennenaikaisesti syntyneillä lapsilla.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Lastentaudit; Medical Research Center; Oulun yliopistollinen sairaala
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Tiivistelmä

Hyvin ennenaikaisina syntyneet lapset ovat alttiita akuuteille aivovaurioille sekä myöhemmin ilmeneville kehityshäiriöille. Eri välittäjäaineiden sekä raskaudenaikaisten ja syntymänjälkeisten kliinisten tekijöiden vaikutusta aivojen vaurioherkkyyteen sekä neurologiseen ja neurokognitiiviseen kehitykseen ei kuitenkaan ole tutkittu riittävästi.

Tässä tutkimuksessa tarkasteltiin, ennustaako jokin napaverestä tutkituista sytokiineista aivoverenvuotoa hyvin ennenaikaisesti syntyneillä vastasyntyneillä. Lisäksi selvitettiin, onko sytokiinin spesifinen reseptori osoitettavissa vuotoherkällä alueella aivoissa. Tutkimme myös, ennustaako jokin napaveren immunoproteiini-profiilin komponentti CP-vamman syntyä joko itsenäisesti tai yhdessä muiden perinataalisten riskitekijöiden kanssa sekä lisääkö tietyn sytokiinin (CCL18) geneettinen vaihtelu CP-vamman riskiä hyvin ennenaikaisesti syntyneillä lapsilla. Lisäksi selvitimme, vaikuttavatko raskaudenaikaiset tekijät ja vastasyntyneisyyskauden sairaudet neurokognitiiviseen kehitykseen kouluiässä.

Tämän tutkimuksen mukaan napaveren matala CCL18-kemokiinipitoisuus oli itsenäinen aivoverenvuodon riskitekijä. CCR3-reseptori, johon CCL18 sitoutuu, oli osoitettavissa sekä vuotoherkällä alueella että hermosoluissa 23. raskausviikon iästä lähtien. Havaitsimme myös, että tietyt napaveren sytokiiniryppäät ja yksittäisten tulehdusvastevälittäjäaineiden pitoisuudet 1. ja 7. elinpäivänä olivat yhteydessä CP-riskiin. Lisäksi havaitsimme yhteyden CCL18-kemokiinin geneettisen vaihtelun ja aivoverenvuodon sekä CP-vamman kehittymisen välillä.

Tutkimuksemme mukaan hyvin ennenaikaisesti syntyneet koululaiset, joilla ei ollut CP- tai kehitysvammaa, suoriutuivat täysiaikaisina syntyneitä verrokkeja heikommin visuaalista hahmotusta ja sensomotoriikkaa sekä tarkkaavuutta ja toiminnanohjausta mittaavissa testeissä. Lisäksi havaitsimme sikiöaikaisen kasvuhäiriön ennustavan itsenäisesti heikkoa suoritusta kieltä, muistia ja oppimista testaavissa tehtävissä ennenaikaisesti syntyneillä lapsilla.

Tietyt sytokiinit ja sytokiiniryppäät ovat yhteydessä aivovauriomekanismeihin. Nämä mekanismit saattavat yhdessä perinnöllisen alttiuden kanssa vaikuttaa myös CP-vamman syntyyn. Sikiöaikainen kasvuhäiriö ja ennenaikaisuus vaikuttavat lapsen myöhempään neurokognitiiviseen kehitykseen.

Asiasanat: aivot, aivoverenvuoto, CP-oireyhtymä, hyvin ennenaikainen syntymä, neurokognitiivinen kehitys, perinnöllinen alttius, sikiöaikainen kasvuhäiriö, sytokiinit

To my loved ones

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Do I dare
Disturb the universe?
In a minute there is time
For decisions and revisions which a minute will reverse (Eliot TS 1920).

Oulu, November 2014 Hanna Kallankari

Abbreviations

BBB	blood–brain barrier
BPD	bronchopulmonary dysplasia
BW	birth weight
CA	chorioamnionitis
CART	classification and regression trees
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CI	confidence interval
CNS	central nervous system
CP	cerebral palsy
cPVL	cystic periventricular leukomalacia
DAMP	damage-associated molecular pattern molecules
ELBW	extremely low birth weight (<1000g)
ELGA	extremely low gestational age (<28 weeks)
FGR	fetal growth restriction
FTF	Five to Fifteen questionnaire
FU	fluorescence unit
GA	gestational age
GABA	gamma-aminobutyric acid
GM	germinal matrix
HCA	histologic chorioamnionitis
IL	interleukin
IQ	intelligence quotient
IUGR	intrauterine growth restriction
IVH	intraventricular hemorrhage
LD	linkage disequilibrium
MRI	magnetic resonance imaging
OL	oligodendrocyte
OR	odds ratio
PHI	periventricular hemorrhagic infarction
PRR	pattern recognition receptor
RDS	respiratory distress syndrome
SGA	small for gestational age
SNP	single nucleotide polymorphism
SVZ	subventricular zone

TNF	tumor necrosis factor
US	ultrasound
VLBW	very low birth weight (<1500 g)
VLGA	very low gestational age (<32 weeks)
WM	white matter
WMI	white matter injury

List of original articles

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I Kallankari H, Kaukola T, Ojaniemi M, Herva R, Perhomaa M, Vuolteenaho R, Kingsmore SF, Hallman M (2010) Chemokine CCL18 predicts intraventricular hemorrhage in very preterm infants. Ann Med 42: 416–25.

II Kaukola T, Kallankari H, Tuimala J, Olsén P, Tammela O, Kingsmore SF, Hallman M (2013) Perinatal immunoproteins predict the risk of cerebral palsy in preterm children. Ann Med 45: 57–65.

III Kallankari H, Huusko JM, Kaukola T, Ojaniemi M, Mahlman M, Marttila R, Kingsmore SF, Haataja L, Lavoie PM, Synnes A, Hallman M (2014) Cerebral palsy and polymorphism of the chemokine *CCL18* in very preterm children. Manuscript.

IV Kallankari H, Kaukola T, Olsén P, Ojaniemi M, Hallman M (2014) Very preterm birth and foetal growth restriction are associated with specific cognitive deficits in children attending mainstream school. Acta Paediatr. doi: 10.1111/apa.12811.

Contents

1 Introduction

Major advances in perinatal treatment practices including antenatal corticosteroids, early surfactant treatment, and high technology for pre- and postnatal monitoring, have improved the survival rates of very preterm infants (Saigal & Doyle 2008). However, due to their physical vulnerability very preterm infants are prone to acute brain insults linked to subsequent neurologic, neurocognitive, and behavioral sequelae. Some of the disabilities become evident only later in childhood and during school years (Aarnoudse-Moens *et al.* 2009, Anderson 2014, Lind *et al.* 2011, Marlow *et al.* 2007).

The degree of prematurity has been considered the major cause of morbidity in preterm infants (Bhutta *et al.* 2002, Moore *et al.* 2012, Serenius *et al.* 2013). The etiology of short- and long-term outcomes is, by far, more complicated. Besides structural immaturity and functional deficits in the premature brain cytokines, hormones, and growth factors may influence defense against brain damage (Douglas-Escobar & Weiss 2012). In some studies, gestational age (GA) had a modest or no influence on the developmental outcome (Reidy *et al.* 2013), whereas factors during the antenatal period, such as intrauterine inflammation or growth restriction (Hagberg *et al.* 2012, Morsing *et al.* 2011), severity of postnatal disease (Kaukola *et al.* 2009), or environmental factors beyond the neonatal period (Charkaluk *et al.* 2010), were more dominant outcome predictors among children born preterm. Prematurity may disrupt fetal programming by epigenetic or other mechanisms, leading to defects in brain volume or abnormalities in white matter (WM) (Counsell *et al.* 2008, Peterson *et al.* 2000, Skranes *et al.* 2007). It may well be that a complex cascade of both pre- and postnatal factors, genetic factors included, influence brain development and later neurocognitive sequelae in very preterm infants.

The aim of the present study was to gain a deeper understanding of factors leading to brain damage and neurodevelopmental impairment among children born very preterm. The more detailed knowledge of risk factors and protective factors could be utilized in planning interventions to prevent impaired brain development and to alleviate later neurocognitive sequelae in this high-risk population.

2 Review of literature

2.1 Preterm birth

2.1.1 *Definitions*

The World Health Organization identifies prematurity as birth before 37 completed gestational weeks. Prematurity can be further subdivided according to GA, classifying infants born at 34–36 weeks as late preterm, those born at 32–33 weeks as moderately preterm, those born before 32 weeks as very preterm (VLGA; very low gestational age) and those born before 28 weeks as extremely preterm (ELGA; extremely low gestational age) infants. For evaluating the influence of very preterm birth, a study population should be defined by GA that is confirmed by a high quality ultrasound before 20 gestational weeks. Previously, when estimations for GA were less accurate, prematurity was more often defined by birth weight (BW) as follows: low BW (<2500 g), very low BW (VLBW, <1500 g) and extremely low BW (ELBW, <1000 g). In preterm groups classified by BW, those born small for gestational age (SGA) are overrepresented in comparison to all births.

Small birth size results from constitutional smallness or pathological growth retardation. Growth below the 10^{th} centile is the commonly used cut-off value for SGA. However, this definition has a rather high false-positive rate in the detection of infants with pathological fetal growth. Growth below two standard deviations from the mean gestation-adjusted BW is a more reliable cut-off value for SGA, and though more strict, it likely encompasses the majority of infants with retarded fetal growth (Lee *et al.* 2003). Intrauterine/fetal growth restriction (IUGR/FGR) refers to a pathological condition in which a fetus is unable to reach its genetically determined potential size excluding congenital malformations and chromosomal abnormalities. Further, it has been suggested that the definition of SGA is justified as a proxy for FGR among early preterm births, whereas the SGA infants delivered at term are more likely to be constitutionally small (Ananth & Vintzileos 2009).

2.1.2 *Epidemiology*

Globally, the preterm delivery rate is estimated to be approximately 11% of all live births (Blencowe *et al.* 2012). Further, about 60–70% of premature births are classified as late preterm, about 20% moderately preterm, about 15% very preterm and 5% as extremely preterm (Goldenberg *et al.* 2008). In Finland, about 6% of infants are born before 37 gestational weeks and premature deliveries prior to 32 gestational weeks occur in about 1% of the pregnancies (Vuori & Gissler 2013). The rate has risen in many developed countries, especially in the USA, because of increasing indicated preterm births and preterm delivery of artificially conceived multiple pregnancies (Goldenberg *et al.* 2008). However, in several European countries the prevalence of singleton preterm birth remained stable or decreased between 1996 and 2008 (Zeitlin *et al.* 2013). Besides a large variation in rates between countries, there exist disparities between races (in 2009, preterm birth rates were 18% in black Americans compared to 11% in white Americans) and age groups (rates are higher in women younger than 17 years or older than 40 years) (Blencowe *et al.* 2012).

Risk factors of premature birth

A common precursor for medically indicated preterm delivery is pre-eclampsia, which may be accompanied by placental insufficiency resulting in FGR (and eventually fetal distress). Spontaneous preterm birth can occur with intact membranes or with preterm premature rupture of membranes (Goldenberg *et al.* 2008). It is a syndrome with multiple causes, including intrauterine infection or inflammation, uteroplacental ischemia or hemorrhage, uterine over-distension mainly caused by multiple gestation, stress, and other immunologically mediated mechanisms. Previous preterm birth, short interval between pregnancies, low socioeconomic status, periodontal disease, smoking, obesity or low maternal body-mass index are also shown to be risk factors for preterm birth (Goldenberg *et al.* 2008, Menon 2008, Muglia & Katz 2010, Raisanen *et al.* 2013). Moreover, many studies have indicated that genetic factors may contribute to preterm birth (Bezold *et al.* 2013, Muglia & Katz 2010). Susceptibility to preterm birth and diseases in premature infants may also reflect a shared common genetic background (Hallman 2012). However, the exact mechanisms underlying prematurity are poorly understood.

Survival and morbidity

In developed countries, the survival rate of extremely/very preterm infants has strikingly increased over the past decades mainly as the result of major advances in ante- and neonatal treatment practices. However, almost all high-income and middle-income countries report preterm birth as the leading cause of childhood mortality (Blencowe *et al.* 2012). Among liveborn VLGA infants, the average survival rate until hospital discharge was 86% in ten European countries in 2003 (Zeitlin *et al.* 2008). In a Finnish study, a one-year survival among liveborn VLGA infants in 2000–2003 was 22% at 22–23 weeks, 35% at 24–25 weeks, 85% at 26–27 weeks, 95% at 27–28 weeks and 97% at 30–31 weeks (Rautava *et al.* 2007). During the 21st century, the increase in survival has been only modest: the survival by one year of age for the VLGA/VLBW infants was 89% in 2000–2003 (Rautava *et al.* 2007) and 91% in 2011–2012 (Vuori & Gissler 2013). Besides the degree of prematurity, survival of the VLGA infant is affected by gender, fetal growth, and the level of the peri- and neonatal care (Saigal & Doyle 2008). Being born in a higher-level hospital has been related to increased survival of VLGA infants (Rautava *et al.* 2007).

Although the survival rates have improved, infants born at VLGA are still prone to multifold morbidities due to their fragile organs. The most common brain injuries among VLGA infants are white matter injury (WMI) and intraventricular hemorrhage (IVH). Acute pulmonary problems are manifested in respiratory distress syndrome (RDS) with the incidence highest among extremely preterm infants (80–93%) (Stoll *et al.* 2010, Tommiska *et al.* 2007). A chronic lung disease may develop later on. Bronchopulmonary dysplasia (BPD) affects 10–30% of all infants born very preterm (Bhandari & McGrath-Morrow 2013, Zeitlin *et al.* 2008) and is defined as a requirement for supplemental oxygen or any form of ventilation therapy that causes distension of the airways at the age of 36 postmenstrual weeks (Walsh *et al.* 2004). Gastrointestinal immaturity commonly causes feeding problems and malnutrition, which are related to postnatal growth failure. Necrotizing enterocolitis is relatively uncommon (incidence reported to be 5–10%) (Schulzke *et al.* 2007), but can potentially threaten gut vitality or even viability of a VLGA infant. Patent ductus arteriosus, a condition where a fetal connection between the pulmonary artery and aortic arch fails to close after birth, is common in VLGA infants (Hamrick & Hansmann 2010). Preterm infants are also vulnerable to postnatal infections, such as sepsis,

due to an immature immune system and invasive procedures during neonatal intensive care.

Besides its contribution to death rates and short-term morbidity, prematurity has long-term effects on neurodevelopmental functioning, health and quality of life (Saigal & Doyle 2008). Very preterm birth and neonatal diseases have been associated with an elevated risk of cerebral palsy (CP), cognitive disabilities and academic underachievement (Anderson 2014, Saigal & Doyle 2008) as well as with chronic diseases, such as cardiovascular, metabolic, pulmonary, and psychiatric diseases, in adulthood (Hack 2009).

2.2 Development of the brain – emphasis on the premature period

Preterm birth occurs at the time of rapid growth and development of the central nervous system (CNS). From mid-gestation to term, the weight of the brain grows spectacularly by 90% and the cerebral cortex alters from a smooth pattern to a complex and extremely organized structure (Kinney & Volpe 2012). Between 24 and 40 gestational weeks, several critical developmental processes take place, including neurogenesis, synaptogenesis, vascularization, brain folding, and myelination (Volpe 2009).

The neocortex arises at the outer surface of the embryonic cerebral vesicle, at the rostral end of the neural tube, as the neurons migrate from proliferative areas near the cerebral ventricle. The arrival of migrating neurons establishes laminar structures (Figure 1), some of which alter or even vanish during development (Bystron *et al.* 2008).

After the initiation of neurogenesis (approximately embryonic day 33), clusters of intermediate progenitors give rise to a new layer above the ventricular zone, called subventricular zone (SVZ). Early SVZ progenitors are mainly neurogenic. After 20 gestational weeks, the SVZ is split into inner and outer sublayers. By 25–27 gestational weeks the SVZ is still proliferating, while the ventricular zone has decreased in size. At that time, the SVZ becomes the main source of cortical neurons, which are largely gamma-aminobutyric acid (GABA)ergic (Bystron *et al.* 2008). The period after 22 gestational weeks is the most important time for regional, laminar and cytological differentiation of the cortical plate. By the seventh month, the cortex is clearly composed of six layers (Bystron *et al.* 2008).

Subplate neurons play a crucial role at 24–32 gestational weeks, during corticogenesis. The human subplate appears to act as a ‘waiting site’ for

thalamocortical and corticocortical axons. Later, these axons invade and track their ultimate destinations. Glutamatergic, GABAergic, and other transmitter specific neurons, transporters, and receptors have been detected in the subplate area (Kinney & Volpe 2012). GABAergic interneurons contribute substantially to cortical specification, output and synaptic plasticity. From 28 to 40 gestational weeks, a four-fold growth in cerebral volume, the increase in cortical surface area, and accelerated gyrification are demonstrated by volumetric magnetic resonance imaging (MRI) (Volpe 2009).

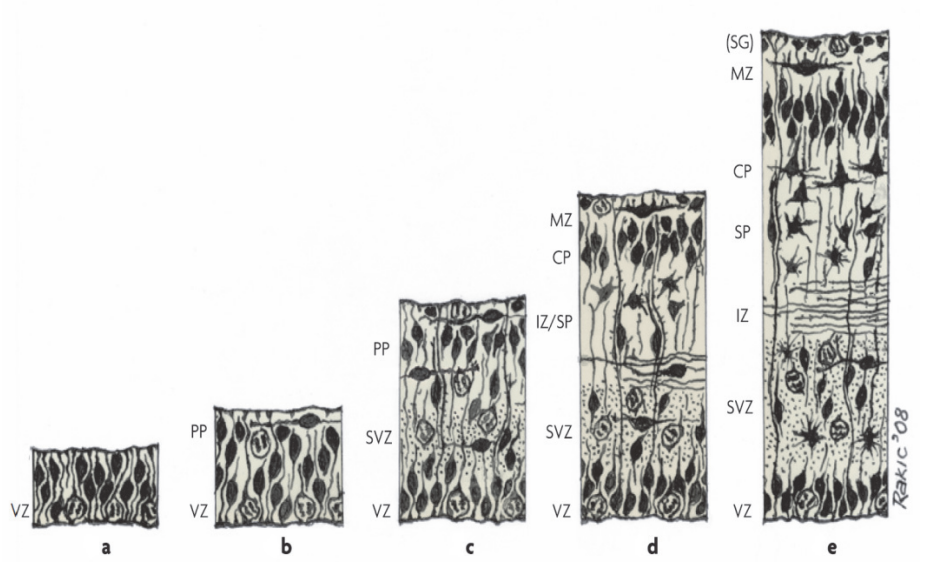

Fig. 1. Illustration of neocortical development by Rakic (modified from Bystron *et al.* (2008)). This figure summarizes the sequence of events and the formation of transient structures. The panels refer to the following approximate ages (for the lateral part of the dorsal telencephalon): a: embryonic day (E) 30; b: E31–E32; c: E45; d: E55; e: gestational week 14. CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; PP, preplate; SP, subplate; SVZ, subventricular zone; (SG), subpial granular layer (part of the MZ); VZ, ventricular zone. Reprinted with permission from Nature Publishing Group.

2.2.1 *Developmental events in the white matter*

Oligodendrocyte (OL) precursors are generated within the neuroepithelium at around 13 gestational weeks. OL precursors proliferate and migrate into the WM, and then differentiate into pre-myelinating OLs (pre-OLs) around 20 gestational weeks. Pre-OLs predominate during the preterm period and comprise 90% of the total OL population until about 28 gestational weeks. At 28–40 gestational weeks, pre-OLs start the differentiation to immature OLs, which reach 50% of the total OL population by term (Kinney & Volpe 2012). The mature, myelin basic protein expressing OLs are detected in WM from 30 gestational weeks, but myelin producing OLs appear abundant in the WM only after term (Volpe 2009).

At around 16–22 gestational weeks, the forebrain is rich in microglia, which play roles in several processes, including apoptosis, axonal development, angiogenesis, synaptic pruning and myelination. As microglial density decreases in WM, it elevates in the cortex, possibly reflecting continuing migration of microglia. Radial glial cells have their origins in the ventricular/subventricular zone and are able to produce neurons and astrocytes. From 19 to 30 gestational weeks, radial glial fibers are abundant and neurons and astrocytes use them as guides for migrating. Around 30–31 gestational weeks, radial glia start converting into fibrous astrocytes, and after that, they progressively vanish (Kinney & Volpe 2012). Beginning at 14 gestational weeks, associative axons are present in the periventricular area. The peak growth of long axonal pathways connecting the cortex with subcortical centers occurs at 22–34 gestational weeks (Vasung *et al.* 2010).

Arterial ingrowth from the pial surface into the developing brain parenchyma proceeds through long penetrating arteries that are directed into the deep periventricular WM and short penetrators that supply the more superficial WM (du Plessis 2009). The deep and periventricular regions of WM are vulnerable to low basal blood flow, since the amount of vascular perforators from leptomeninges in the WM is sparse and the vessels are poorly connected. As a result of gradual muscular layer development in premature period, the vasoregulation takes place in the superficial parenchyma, the deeper vessels appearing more passive. Cerebral vascular responsiveness to various stimuli starts to develop during the latter half of gestation. In the preterm infant, functional vascular autoregulation is compromised, with a tendency for pressure-passive cerebral circulation (Kinney & Volpe 2012).

2.3 Intraventricular hemorrhage (IVH)

2.3.1 *Definition and incidence*

In very preterm infants, IVH is a significant brain injury. Cranial ultrasound (US) has remained the main clinical imaging method of the preterm infant brain. Indeed, US has good sensitivity and specificity in detecting hemorrhages (Inder *et al.* 2003, Maalouf *et al.* 2001). Papile classification is generally used to grade the severity of IVH detected by US (Papile *et al.* 1978). Grade I hemorrhage is confined to the subependymal germinal matrix (GM). Grade II refers to IVH without distension of the ventricular system and grade III refers to IVH with ventricular distension. Grade IV refers to IVH with parenchymal involvement (Papile *et al.* 1978), also known as periventricular hemorrhagic venous infarction (PHI) (Volpe 2008). Post-hemorrhagic ventricular dilatation refers to progressive enlargement of the ventricular system and is a major complication of IVH (Volpe 2008). In most cases, hemorrhages start within 24 hours after the birth, IVH is evident by 72 hours after birth and the extension of the injury is detected within the first postnatal week (Paneth *et al.* 1993, Volpe 2008). The onset of progressive ventricular dilatation takes place 1–3 weeks after hemorrhage (Volpe 2008).

The overall occurrence of IVH in VLBW neonates has decreased from 40–50% in the early 80s to 15–25% in the late 80s. Since then, there has been no consistent reduction in IVH rates (Ballabh 2010). In a Finnish ELBW-study, the overall incidence of IVH increased (29% vs. 37%), but the incidence of IVH grades III–IV did not (16% vs. 17%) from 1996–1997 to 1999–2000 (Tommiska *et al.* 2007). In another Finnish study comprising VLBW infants born in 2001–2006, the incidence of IVH grades I–IV was 26% and the incidence of IVH grades III–IV was 7% (Maunu *et al.* 2011). Recent studies from the USA and Australia reported, that severe IVH (grades III–IV) occurred in 13–16% of infants born before 29 gestational weeks (Bolisetty *et al.* 2014, Stoll *et al.* 2010). The incidence of PHI is approximately 5% in VLBW infants (Volpe 2009). About one fifth of IVH patients have been reported to develop post-hemorrhagic ventricular dilatation (Vassilyadi *et al.* 2009). Mortality is substantial in infants suffering from severe IVH, and 25–50% of survivors develop CP (Beaino *et al.* 2010) and/or cognitive disability, whereas follow-up results of lower grades of IVH are inconclusive (Bolisetty *et al.* 2014, Luu *et al.* 2009, O'Shea *et al.* 2012, Payne *et al.* 2013, Sherlock *et al.* 2005).

2.3.2 *Risk factors*

A variety of risk factors for IVH have been proposed, including VLGA, male gender, vaginal birth, and intrauterine infection/inflammation (McCrea & Ment 2008). Extension of chorioamnionitis (CA) to the fetal compartment may be detrimental to the fetus. According to several studies fetal inflammatory response syndrome, defined by umbilical vasculitis (Leviton *et al.* 1999) or increased cord serum proinflammatory cytokines (Gomez *et al.* 1998), predicted the risk of IVH (Heep *et al.* 2003, Hofer *et al.* 2013, Tauscher *et al.* 2003). However, the association between CA/fetal inflammation and IVH has not been confirmed in all studies (Babnik *et al.* 2006, Bhandari *et al.* 2011, Sarkar *et al.* 2005, Ylijoki *et al.* 2012). According to a twin study, familial susceptibility significantly contributes to the risk of IVH (Bhandari *et al.* 2006). Platelet and coagulation disorders are not described as major contributors of IVH in general but may be important in some cases (Whitelaw 2001). In turn, IUGR and antenatal corticosteroids have been identified to be protective against IVH (Harding *et al.* 2001, Heuchan *et al.* 2002, Kari *et al.* 1994).

Among postnatal factors, severity of clinical illness, systemic hypotension, fluctuation of arterial blood pressure, hypercapnia, RDS, positive pressure ventilation, and pneumothorax, as well as systemic inflammation after birth have been associated with IVH (Ballabh 2010, Leviton *et al.* 2013, McCrea & Ment 2008, Whitelaw 2001). In recent studies, no significant association was found between hypotension or blood pressure variability and IVH (du Plessis 2009, Limperopoulos *et al.* 2007). Early postnatal hypotension is probably not a reliable indicator of poor cerebral perfusion.

2.3.3 *Pathogenesis of IVH*

The pathogenesis of IVH is complex. The origin of IVH in the preterm neonate is usually the GM, which is a site of active angiogenesis. Fragility of the GM vascular bed is potentially attributed to weakness in any component of the blood brain barrier (BBB); endothelial tight junctions, basement membrane, pericytes or astrocyte endfeet covering the blood vessels (Ballabh 2010). In addition to the vulnerability of the GM vasculature, deficient autoregulation of cerebral blood flow is also among the proposed factors leading to GM hemorrhage (Ballabh 2010). Cerebral hypoxia–ischemia/reperfusion can additionally perturb cerebral blood flow injuring GM vessels (Ballabh 2010, du Plessis 2008). Despite

convincing data from animal studies, the direct role of hemodynamic insults in the pathogenesis of IVH in preterm infants remains unsolved (du Plessis 2009). When the hemorrhage in GM (grade I) is substantial, it may rupture through the ependyma into one or both lateral ventricles (IVH grade II) and cause ventricular dilatation (IVH grade III). Finally, IVH may obstruct the blood flow in the terminal veins coursing through the GM, resulting in hemorrhagic venous infarction (PHI/IVH grade IV), which involves the destruction of periventricular WM (Papile *et al.* 1978, Volpe 2008).

Inflammatory conditions may also contribute to the pathogenesis of IVH. High levels of cord blood interleukin-1β (IL-1β), IL-6, and IL-8 have been found to associate with IVH in preterm infants (Heep *et al.* 2003, Kassal *et al.* 2005, Poralla *et al.* 2012, Tauscher *et al.* 2003). In preclinical studies, an inflammatory response has been shown to be carried through the BBB to the immature CNS by proinflammatory cytokines, prostaglandins, or by lipopolysaccharides (Hagberg *et al.* 2012). Hypoxia may also lead to the loss of BBB function and defective tight junction protein synthesis allowing cytokines from the peripheral blood access to the immature brain (Chen *et al.* 2012). However, evidence is lacking of how inflammatory mediators enter the CNS of the human fetus/infant (McAdams & Juul 2012). Besides inflammation related proteins growth factors and hormones have been suggested to act as biomarkers of IVH (Table 1) (Andrikopoulou *et al.* 2014, Douglas-Escobar & Weiss 2012).

Moreover, genetic susceptibility may have a role in the pathogenesis of IVH. In preterm infants, variations in genes connected to inflammation/infection, coagulation or vascular pathways have been suggested as potential candidates for IVH. These include polymorphisms in genes encoding IL-1β, IL-4, IL-6, tumor necrosis factor (TNF), coagulation factor V Leiden mutation, coagulation factor II polymorphism, and prothrombin polymorphism (Baier 2006, Ryckman *et al.* 2011). Nevertheless, many of the reported associations have not been studied in various independent populations or have not proved to be replicable. In a recent study, only the methylenetetrahydrofolate reductase polymorphism was associated with IVH (Aden *et al.* 2013). Furthermore, gene–environment interactions have been suggested to contribute to the pathogenesis of IVH (Ment *et al.* 2014).

Table 1. Biomarkers of brain injury in preterm infants.

Biomarker, Location	Function	Change	Association	Sensitivity, specificity, AUC
S100β, Neonatal urine, blood	Calcium binding protein, neurotrophic/ neurotoxic properties	↑	IVH (Gazzolo *et al.* 1999, Gazzolo *et al.* 2001)	Sensitivity and specificity 100% at a urine cut-off 0.70 µg/l.
Activin, Neonatal blood, urine	Growth factor	↑	IVH (Florio *et al.* 2006)	Sensitivity 100% and specificity 93% at an early neonatal blood cut-off 0.8 µg/l. AUC 0.98.
			IVH (Sannia *et al.* 2013)	Sensitivity 69% and specificity 85% at a urine cut-off 0.08 ng/l. AUC 0.79.
Erythropoietin, Cord blood	Hormone that controls erythropoiesis	↑	IVH (Bhandari *et al.* 2011)	N/A
IL-6, Cord, neonatal blood	Cytokine	↑	IVH (Heep *et al.* 2003, Kassal *et al.* 2005, Poralla *et al.* 2012)	N/A
			PVL (Yoon *et al.* 1996)	Sensitivity of 72% and specificity of 74% at a cord blood cut-off 400 pg/ml.
GFAP, Neonatal blood	Specific marker of differentiated astrocytes	↑	PVL (Stewart *et al.* 2013)	N/A
IL-6, IL-10, TNF Neonatal CSF	Cytokines	↑	PVL (Ellison *et al.* 2005)	N/A
TNF, IL-1β, IL-6 and their soluble receptors (sTNF-RI, sTNF-RII, sIL-1RA, sIL-6R), Neonatal blood	Cytokines, cytokine receptors	↑	PVL (Bass *et al.* 2008)	IL-6/sIL-6R interaction model: AUC: 0.72, Combined modeling: AUC 0.84.

This table is partly modified from two recent reviews (Andrikopoulou *et al.* 2014, Douglas-Escobar & Weiss 2012).

AUC, area under the curve; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; IL, interleukin; IVH, intraventricular hemorrhage; N/A, not available; PVL, periventricular leukomalacia; R, receptor; s, soluble; TNF, tumor necrosis factor.

2.4 White matter injury (WMI) and neuronal/axonal disease

2.4.1 *Definition and incidence*

The most significant complication of prematurity is probably WMI that is accompanied by neuronal and axonal abnormalities affecting several regions of the premature brain. Cystic periventricular leukomalacia (cPVL) results in focal necrosis with loss of cellular elements leading to cyst formation deep in the WM. The other component of WMI is more diffuse involving death or functional abnormality of pre-OLs, astrogliosis and microglial infiltration in central WM with subsequent hypomyelination and ventricular dilatation (Volpe 2009). PVL has been graded on cranial US images by de Vries *et al.* (de Vries *et al.* 1992). Several studies have shown that cranial US is reliable in detecting severe WMI/cPVL, but has significant limitations in the demonstration of the much more common, noncystic, diffuse WM lesions (Inder *et al.* 2003, Leijser *et al.* 2010, Maalouf *et al.* 2001). MRI at term is recommended for accurate diagnosis of WMI (Inder *et al.* 2003, Leijser *et al.* 2010).

Previously, cPVL was found in 3–15% of VLBW infants based on pooled US studies (Blumenthal 2004). More recently, the incidence of severe, cPVL has declined in preterm infants. In a large cohort of infants born at 25–34 weeks of gestation, a significant decrease was found in the occurrence for cPVL from 3.3% to 1.3% between birth periods 1990–1993 and 2002–2005 (van Haastert *et al.* 2011). In infants born at lower GA, the incidence is higher. In Sweden, almost 6% of infants born before 27 weeks of gestation developed US evidence of cPVL (EXPRESS Group *et al.* 2009). The advent of MRI has led to the increased recognition of the diffuse, noncystic WM abnormalities. Inder *et al.* proposed a scoring system for white and gray matter abnormalities on MRI (Inder *et al.* 2003). Qualitative WM changes of a different grade have been reported in up to 50–75% of very preterm infants (Inder *et al.* 2003, Maalouf *et al.* 2001, Skiold *et al.* 2010). In an unselected population of infants born at 24–32 week of gestation, one fifth had moderate to severe WMI. Diffuse WMI clustered in the tiniest infants born before 26 weeks of gestation, whereas the injury seemed to be mostly focal with cyst formation beyond 26 weeks (Inder *et al.* 2003).

Preclinical studies as well as histopathological and MRI studies in humans have revealed that WMI is often observed in conjunction with neuronal and axonal disease in thalamus, basal ganglia, cerebral cortex, brainstem, and cerebellum. Indeed, one suggestion is to name the combination of brain

abnormalities among preterm infants "encephalopathy of prematurity" (Volpe 2009). When compared to term infants, preterm ones have global and regional reductions in cortical and deep gray matter and cerebellum (Inder *et al.* 2005, Ment *et al.* 2009, Peterson *et al.* 2000).

In addition to IVH and WMI other types of brain injuries have been recognized among preterm infants in the era of increased use of MRI. In a prospective hospital-based population, 42% of the infants with perinatal arterial ischemic stroke were born before 36 gestational weeks (Benders *et al.* 2009).

2.4.2 *Risk factors for WMI and abnormal cortical development*

The risk of PVL has been strongly related to low GA (Larroque *et al.* 2003). In addition, other perinatal risk factors for PVL have been reported in very preterm infants, including intrauterine inflammation (Wu 2002), disturbances in placental circulation (Kumazaki *et al.* 2002), and preterm premature rupture of membranes (Bauer *et al.* 2009). The relationship between intrauterine inflammation and WMI has not been confirmed in all studies (Ylijoki *et al.* 2012), especially in those regarding diffuse WM abnormalities detected by MRI (Dyet *et al.* 2006, Inder *et al.* 2003). It has been suggested that inflammation alone is not sufficient to contribute to brain injury but could have an important role if combined with other exposures such as placental perfusion defect or hemodynamic insults (Hagberg *et al.* 2012, Kaukola *et al.* 2006). The introduction of antenatal corticosteroids as standard care of preterm birth may have reduced the impact of prenatal inflammation on the immature brain (Ylijoki *et al.* 2012). Antenatal betamethasone but not dexamethasone was associated with a reduced risk of cPVL in preterm infants (Baud *et al.* 1999). The beneficial effect of betamethasone has been confirmed in other studies (Canterino *et al.* 2001). In a recent study, the benefits of antenatal corticosteroids were also evident in the tiniest infants born at 23–25 weeks of gestation (Carlo *et al.* 2011a).

Neonatal factors that have been found to associate with PVL include systemic hypotension (Inder *et al.* 2003, Martens *et al.* 2003) and hypocarbia (Shankaran *et al.* 2006). However, there are controversies regarding the role of these factors. Isolated early postnatal hypotension or single blood gas abnormalities might not directly predict the risk of brain injury, but might rather be indicators of immaturity and illness severity (Leviton *et al.* 2010, Logan *et al.* 2011). Neonatal diseases, such as sepsis and IVH, have been linked to WMI (Inder *et al.* 2003, Shah *et al.* 2008).

In some studies, GA at birth adversely affected cortical surface area and cortical gray matter growth (Inder *et al.* 2005, Kapellou *et al.* 2006). However, Boardman *et al.* observed that cerebral volume was preserved in the majority of preterm infants, whereas a prolonged requirement for supplemental oxygen was associated with reduced brain growth (Boardman *et al.* 2007). Kaukola *et al.* demonstrated that perinatal illness severity was related to the decrease in cortical surface area growth (Kaukola *et al.* 2009). In addition, uncomplicated IVH was found to correlate with impaired cortical development in VLBW infants (Vasileiadis *et al.* 2004).

Thompson *et al.* found that IUGR and WMI were associated with a reduction in posterior brain volumes, whereas BPD had more global effects on brain volumes. In this study, degree of prematurity had no effect on regional brain growth (Thompson *et al.* 2007). Others have also shown that placental insufficiency with IUGR has specific structural and functional influence on cerebral cortical brain development (Tolsa *et al.* 2004), and that the effect of IUGR on brain structure and development persists at one year of age (Padilla *et al.* 2011). In addition, antenatal smoking exposure was related to smaller volumes of frontal lobe and cerebellum in preterm infants (Ekblad *et al.* 2010). Delayed microstructural development of the cortical gray matter was also found in preterm infants with poor growth after birth (Vinall *et al.* 2013).

2.4.3 *Pathogenesis of WMI and neuronal/axonal disease*

In VLGA infants, the most prevalent pathogenetic processes that are thought to underlie WMI comprise infection/inflammation and ischemia, which can coexist and potentiate each other (Khwaja & Volpe 2008) (Figure 2). The inflammatory responses are partly mediated by pattern recognition receptors (PRRs) that recognize pathogen-associated and damage-associated molecular pattern molecules (DAMPs). Toll-like receptors are among the PRRs, whereas several intracellular proteins including S100 and heat-shock proteins are DAMPs. The activation of either PRRs or DAMPs induces inflammatory mediators such as cytokines and chemokines, which further modulate the immune responses by inducing oxidants and apoptotic factors. Moreover, sterile exposures, such as hypoxia–ischemia can initiate an inflammatory reaction in the immature CNS (Hagberg *et al.* 2012). The upstream factors, infection/inflammation and ischemia, can activate the principal downstream mechanisms: excitotoxicity and free radical attack leading to injury of pre-OLs. Further, activation of microglia

serves as an interface for these upstream and downstream mechanisms (Khwaja & Volpe 2008). Microglia can generate reactive oxygen and nitrogen species, enhance excitotoxicity, and secrete potentially detrimental cytokines, such as TNF (Volpe *et al.* 2011). TNF can further potentiate the maturation-dependent toxicity by IFN-γ (Buntinx *et al.* 2004). Excitotoxicity involves increased expression of calcium-permeable glutamate receptors and the main glutamate transporter, which can serve as a source of damaging glutamate. Due to delayed development of antioxidant enzymes, the pre-OLs also have maturation-dependent vulnerability to free radical attack, characterized by the massive generation of reactive oxygen and nitrogen species (Volpe *et al.* 2011).

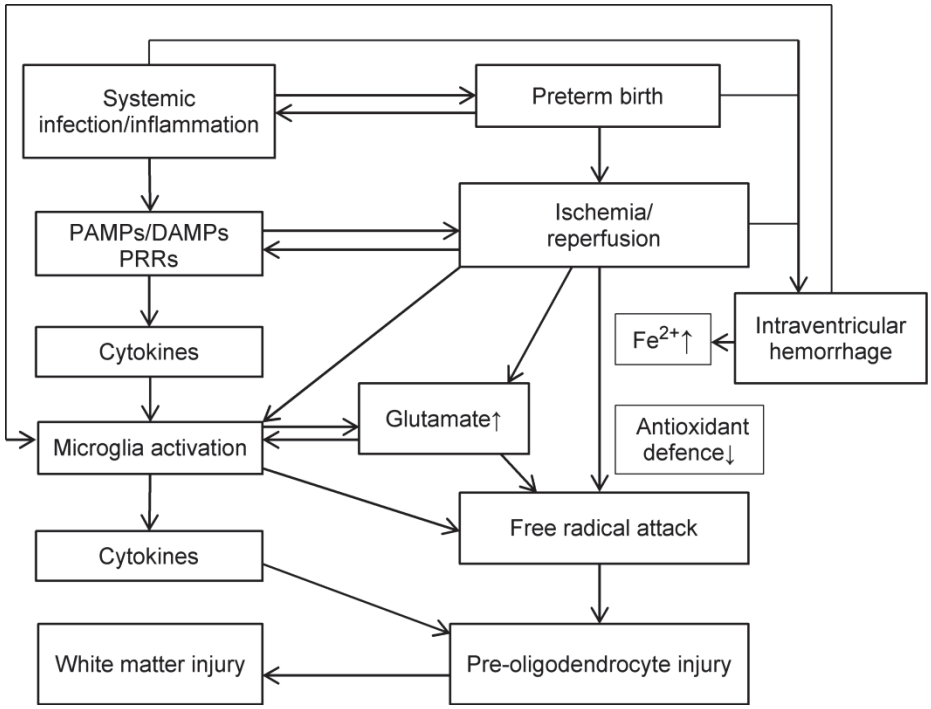

Fig. 2. Pathogenetic mechanisms in white matter injury (modified from reviews (Hagberg *et al.* 2012, Khwaja & Volpe 2008, Volpe *et al.* 2011)). DAMP, damage-associated molecular pattern molecule; PAMP, pathogen-associated molecular pattern molecule; PRR, pattern recognition receptor. See text for details.

Pre-OL injury causes pre-OL death or loss of pre-OL processes with arrested maturation of the OL lineage (Volpe *et al.* 2011). In addition, pre-OL progenitors may be replenished by proliferation and/or migration especially in chronic lesions, but again differentiation is disrupted (Back & Rosenberg 2014). These disturbances lead to impaired myelination. The pre-OL damage may also result in impaired axonal development and finally axonal degeneration (Volpe 2009). A recent study showed, that neurogenesis continued until the 28th gestational weeks in humans and was suppressed by premature birth in rabbit pups (Malik *et al.* 2013). It has been suggested that neurons are injured as they migrate through WM undergoing damage, from the GM to the suplate and cortex (Leviton & Gressens 2007).

Inflammatory cells and extracellular cytokines may have both beneficial and deleterious effects depending on the context and time (Hagberg *et al.* 2012). Cytokines have important roles not only in the immune response to inflammatory pathogens, but also in generating neurotrophic functions in the developing brain. Neuronal cells, such as astrocytes and OLs, can act as providers of growth factors to neurons (Du & Dreyfus 2002). Despite participating in pathological processes, microglia have role in developmental, anti-inflammatory and immunosuppressive processes and repair in immature brain (Hagberg *et al.* 2012).

Potential biomarkers have been investigated in predicting WMI (Table 1) (Andrikopoulou *et al.* 2014). High levels of proinflammatory cytokines in amniotic fluid, especially TNF, IL-1β, IL-6, and IL-8, reflect immunological activation in utero and were associated with WM lesions (Yoon *et al.* 1997). In addition, preterm infants with WMI detected by MRI had higher concentrations of IL-1β, IL-6, IL-10, and TNF and CD45RO+ T lymphocytes in cord blood (Duggan *et al.* 2001). In contrast, no association was found between individual cytokine or soluble receptor concentrations and the development of WMI identified by US in another study (Bass *et al.* 2008). Nevertheless, modeling cytokines with their receptors significantly enhanced the predictability of WMI in this study of preterm infants highlighting the importance of cytokine–receptor interactions (Bass *et al.* 2008). Hormonal and nutritional factors may also affect brain development. In VLGA infants, reduced brain volumes were associated with low postnatal concentrations of IGF-I and IGFBP-3 (Hansen-Pupp *et al.* 2011).

Severe IVH may be involved in the pathogenesis of WMI; blood in the ventricles stretch and thus disrupt the ependymal barrier allowing potentially damaging intraventricular components to enter adjacent parenchyma (Adler *et al.* 2010). These molecules comprise inflammatory cytokines and blood components,

such as extracellular hemoglobin (Hb) and free iron (Fe^{2+}). Accumulation of met-Hb in the intraventricular space induces expression of pro-inflammatory cytokines that might be essential in the initiation of brain injury following IVH (Gram *et al.* 2013). Interestingly, a recent study suggested that besides severe IVH, low grade IVH may also influence brain development of premature infants, with an important mediating effect by microglial activation (Supramaniam *et al.* 2013). Moreover, destruction of glial precursors in the GM and the subsequent damaging effect on myelination could be a link between IVH and WMI (Volpe 2008).

Genetic factors can also affect pathogenetic processes of WMI (Baier 2006). A recent study suggested that polymorphism in genes associating with schizophrenia and lipid metabolism modulated the risk for WM abnormality measured by diffusion tensor imaging in preterm infants (Boardman *et al.* 2014). Interplay between genes and environment is most likely an essential contributor of brain development and susceptibility to WMI. Firstly, the genetic polymorphism or copy number variant may increase the susceptibility to injury after an environmental insult. Secondly, an environmental insult can produce epigenetic alterations that could cause long-term modifications of gene function (Stolp *et al.* 2012).

Finally, it has been suggested that injury processes may persist for weeks, months, or even years after the initial, perinatal insult to the immature brain. These late mechanisms of injury have been thought to include persistent inflammation, astrocyte cytotoxicity, and epigenetic alterations, which could arrest OL maturation, disrupt neurogenesis, inhibit axonal growth, or impair synaptogenesis. These processes can lead to myelin deficits, impaired plasticity, and altered cell count (Fleiss & Gressens 2012).

2.5 Neurodevelopmental outcomes after preterm birth

2.5.1 *Cerebral palsy (CP)*

Definition, clinical features and prevalence

CP is a collective term for disorders of the development of movement and posture causing activity limitation that are attributed to nonprogressive disturbances occurring in the developing fetal or infant brain (Rosenbaum *et al.* 2007).

Neurocognitive and sensory impairments commonly co-occur with motor disability, which together affect quality of life throughout the patient's lifespan. Indeed, CP is a heterogeneous condition considering the etiology as well as type and severity of impairments. The disorder of movement and posture is categorized as bilateral spasticity, unilateral spasticity, dyskinesia, ataxia, and mixed (Surveillance of Cerebral Palsy in Europe 2000). According to the definition, CP must be of sufficient severity to cause activity limitation that is reliably categorized using Gross Motor Function Classification System (Smithers-Sheedy *et al.* 2014).

While the disturbance in the brain causing CP is non-progressive, the clinical presentation of CP may alter over time. In studies comprising ELBW children, a CP diagnosis could be confirmed with sufficient reliability at 18 months of adjusted age (Peralta-Carcelen *et al.* 2009) or at 2 years of adjusted age (Voss *et al.* 2007). However, it is advisable to perform the final ascertainment of the diagnosis at or around 5 years of age to confirm that the condition is nonprogressive (Smithers-Sheedy *et al.* 2014). The occurrence of comorbidities is dependent on the subgroup and severity of CP. Intellectual disabilities are reported in 30–65%, speech and language problems in 40%, epilepsy in 30–50%, visual impairments in 40%, and hearing deficits in 5–15% of cases (Moreno-De-Luca *et al.* 2012).

The prevalence of CP rose during the 1970s, remained stable during the 1980s, and since then, a significant decline in CP prevalence has been reported in both the preterm and term groups (Himmelmann *et al.* 2005). In a European multicenter study, the prevalence fell from 60.6 per 1000 liveborn VLBW infants in 1980 to 39.5 per 1000 liveborn VLBW infants in 1996 (Platt *et al.* 2007). In another study among children born before 34 weeks, CP incidence decreased from 6.5% in the period from 1990–1993 to 2.2% in the period from 2002–2005, simultaneously with reduced severity of CP (van Haastert *et al.* 2011). The worldwide prevalence of CP has remained constant during the last two decades; the prevalence was 2.11 per 1000 live births and revealed clustering to preterm infants. The prevalence of CP was 111.8 per 1000 live births in children born before 28 weeks of gestation (Oskoui *et al.* 2013). Moreover, a population-based study covering nearly 100 000 live births in western Sweden showed a stable overall prevalence of 2.18 per 1000 live births in 2003–2006. The GA-specific prevalence for 28–31 weeks was 39.6 per 1000 live births and for below 28 weeks 71.4 per 1000 live births (Himmelmann & Uvebrant 2014). Table 2 shows the CP rates in preterm cohorts.

Factors contributing to CP

The specific causal mechanism of CP remains elusive in many individuals (Moreno-De-Luca *et al.* 2012). However, recent studies suggest that 70–80% of the CP cases are probably accounted by acquired prenatal and genetic factors (Sukhov *et al.* 2012), whereas factors operating during labor and after birth have less influence on the development of CP. For instance birth asphyxia accounts for less than 10% of CP cases (Moreno-De-Luca *et al.* 2012, Sukhov *et al.* 2012). In a large epidemiologic study, prematurity, IUGR, perinatal infection, and multiple gestations presented the most significant risk factors for CP (O'Callaghan *et al.* 2011). The events promoting the risk of CP in preterm infants are considered to occur more often peri- or neonatally compared to term infants (Himmelmann *et al.* 2005, Stoknes *et al.* 2012). In a retrospective study of 2733 preterm infants with CP, delivery before 28 weeks had the greatest effect on CP development. Additional risk factors were male gender, birth asphyxia, birth defects, cord prolapse, and fetal distress as well as diseases in the neonatal period (Sukhov *et al.* 2012). In a prospective study of 1812 very preterm infants, cerebral lesions on US were the most important predictors of CP; the infants with cPVL or PHI were 30 times more likely to develop CP. However, one third of the CP cases did not have lesions detectable on brain US (Beaino *et al.* 2010).

The more sensitive imaging modality for children with CP is MRI; it reveals brain abnormalities in over 80% of cases. WMI is the most common abnormality. Combined gray and white matter alterations are often found in children with hemiplegia; isolated WMI is prevalent with bilateral spasticity or athetosis, and with ataxia, while isolated gray matter abnormalities are the most uncommon findings (Korzeniewski *et al.* 2008). Still, conventional MRI fails to identify brain abnormalities in about 15% of CP cases (Korzeniewski *et al.* 2008), which may be due to the inability of conventional MRI to detect microstructural WM pathology (Scheck *et al.* 2012). Alterations in markers within descending corticomotor tracts obtained by diffusion MRI-based connectivity techniques seem to correlate with assessments of clinical severity of CP. Indeed, sophisticated MRI techniques may enhance the understanding of the structure–function relationships of neural networks in CP (Scheck *et al.* 2012).

According to a meta-analysis covering studies until 2000, clinical CA was significantly associated with CP, whereas histological CA (HCA) was not (Wu 2002). In a more recent meta-analysis encompassing publications between 2000 and 2009, a strong association was found between CP and both clinical CA and

HCA (Shatrov *et al.* 2010). Further, elevated concentrations of proinflammatory cytokines; IL-6, and IL-8, in amniotic fluid as well as funisitis were associated with development of CP in preterm infants (Yoon *et al.* 2000). Increased levels of cytokines and coagulation factors were found in neonatal blood in a population comprising mostly term-born CP children compared to controls (Nelson *et al.* 1998). Kaukola *et al.* demonstrated that a pattern of inflammatory mediators and growth factors in cord serum were higher in infants who developed CP, and that those immunoprotein patterns differed between preterm and term cases (Kaukola *et al.* 2004). Further, IL-8 levels on day 3 were significantly higher in ELBW infants who developed CP compared to controls (Carlo *et al.* 2011b). However, in some studies, inflammatory cytokines in cord serum or neonatal blood failed to associate with subsequent CP in preterm infants (Nelson *et al.* 2003, Varner *et al.* 2014). Interestingly, preterm-born children with PVL-induced CP had altered inflammatory responses, including elevated plasma levels of TNF and mRNA expression of Toll-like receptor-4 and TNF, at school-age. This invokes a hypothesis of the long-term programming influence of PVL or inflammation-related events during the perinatal period (Lin *et al.* 2010).

High familial risk for CP has been reported indicating partial heritability for CP (Hemminki *et al.* 2007). Indeed, genetic susceptibility is likely to have a role in the pathogenesis of CP (Moreno-De-Luca *et al.* 2012). Several genes, involved in inflammation and infection or coagulation (O'Callaghan *et al.* 2009, Wu *et al.* 2011) have been suggested as potential candidate genes for CP. Recent studies assessing multiple genes encoding TNF, IL-6, prothrombin, factor V Leiden, apolipoprotein-E, methylenetetrahydrofolate reductase, lymphotoxin-α, and others have yielded modest or no associations with CP (O'Callaghan *et al.* 2012, Wu *et al.* 2011). The heritable factors and gene–environment interactions in complex pathogenesis of CP require further investigation.

2.5.2 *Cognitive outcomes*

Follow-up data for cohorts born in the 1990s and 2000s continue to highlight the cognitive impairments among preterm populations (Table 2) (Johnson 2007, Kerr-Wilson *et al.* 2012). Although VLGA children typically have a group mean intelligence quotient (IQ) and other overall cognitive scores within normal range, they score significantly lower compared to term-born children (Bhutta *et al.* 2002, Johnson 2007). VLGA children have been reported to achieve a mean IQ score 12 points below that of controls (Kerr-Wilson *et al.* 2012, Lind *et al.* 2011).

Table 2. Neurodevelopmental outcomes in very or extremely preterm children.

Cohort (Reference) GA/BW, Age at assessment	CP n (%)	Cognitive impairment n (%)	Mean IQ (SD), test, (n) Mean difference in IQ (95% CI) vs. controls
Finland, national, 1996–1997 (Mikkola *et al.* 2005) <1000 g, 5 y	28/203 (14%)	19/203 (9%)	96 (19), WPPSI-R, (n = 172) —
Finland, regional, PIPARI, 2001–2006 (Munck *et al.* 2010, Munck *et al.* 2012) ≤1500 g, 2 y[1]/5 y	13/182 (7%)[1]	9/124 (7%), IQ <70	99 (18), WPPSI-R. (n = 124) –12 (–16 to –9)
UK and Ireland, EPICure, 1995 (Marlow *et al.* 2005) <26 wk, 6 y	49/241 (20%)	50/241 (21%), IQ <70 98/241 (41%), K-ABC < –2 SD, vs. classmates	82 (19), K-ABC, (n = 241) –24 (–27 to –20)
Australia, regional, 1997 (Hutchinson *et al.* 2013) <28 wk/1000 g, 8 y	24/189 (13%)	12/189 (6%), IQ <70 28/189 (15%), WISC-IV < –2 SD, vs. term controls	93 (16), WISC-IV, (n = 189) –13 (–16 to –10)
USA, regional, 1992–1995 (Hack *et al.* 2005) <1000 g, 8 y	31/200 (16%)	32/200 (16%), IQ <70	88 (19), K-ABC, (n = 200) –12 (–15 to –9)
France, EPIPAGE, 1997 (Larroque *et al.* 2008) <33 wk, 5 y	159/1817 (9%)	182/1534 (12%), IQ <70	94 (19), K-ABC, (n = 1534) –13 (–15 to –11)
Sweden, EXPRESS, 2004–2007 (Serenius *et al.* 2013) <27 wk, 2.5 y (corrected)	32/456 (7%)	45/399 (11%), cognitive score < –2 SD	94* (12), Bailey-III, (n = 399) –9* (–12 to –7)
England, EPICure 2, 2006 (Moore *et al.* 2012) <27 wk, 3 y (corrected)	83/576 (14%)	94/576 (16%), cognitive score < –2 SD	89* (19), Bailey-III, (n = 501) —

BW, birth weight; CI, confidence interval; CP, cerebral palsy; EPIPAGE, Etude EPIdémiologique sur les Petits Ages GEstationnels; EXPRESS, Extremely Preterm Infants in Sweden Study; GA, gestational age; K-ABC, Kaufman Assessment Battery for Children; IQ, intelligence quotient; PIPARI, Pienipainoiset riskilapset; WISC-IV, Wechsler Intelligence Scale for Children, Fourth Edition; WPPSI-R, Wechsler Preschool and Primary scale of intelligence, Revised. *Cognitive score.

In addition, the cognitive deficits are generally more substantial in ELGA cohorts (Hutchinson *et al.* 2013, Marlow *et al.* 2005, Mikkola *et al.* 2005, Moore *et al.* 2012, Serenius *et al.* 2013). Some of the disabilities become evident only later in childhood and during school years. In most studies, the gap between VLGA and their term peers remains or increases with increasing age (Anderson 2014) and the cognitive deficits are still observed in adolescence and young adulthood (Hack 2009, Pyhala *et al.* 2011).

Over the past decade, there has been mounting concern regarding the more subtle deficits in sensorimotor–visuospatial functions, language, memory, attention–executive processes, and behavioral skills among VLGA children (Aarnoudse-Moens *et al.* 2009, Barre *et al.* 2011, Geldof *et al.* 2012, Marlow *et al.* 2007, Mulder *et al.* 2009, Reidy *et al.* 2013).

Neurocognitive skills

Visuospatial processes, perceptual and sensorimotor functions are important for academic competence, social relationships and everyday functions. In preterm-born children, performance IQ (representing difficulties for example in visual perception and eye-hand coordination), as well as sensorimotor/visuomotor and visuospatial processes seem to be more adversely affected than verbal skills (Lind *et al.* 2011, Mikkola *et al.* 2005). In the EPICure cohort, children born before 26 weeks of gestation without CP had impairments in visuospatial, perceptuomotor and attention–executive functions. These deficits independently contributed to weak school performance at six years of age (Marlow *et al.* 2007). A meta-analysis provided evidence for deficits in visual perceptive skills in VLBW children and adolescents. Impairments in visuomotor integration were observed to persist from early childhood into adolescence (Geldof *et al.* 2012).

Language skills are crucial for communication and social interactions and are associated with school performance. According to a meta-analysis, children born preterm scored significantly poorer in both simple and complex language tests. While growing up, group differences increased for complex language processes (van Noort-van der Spek *et al.* 2012). Another meta-analysis revealed that VLGA children performed poorer in expressive language, receptive language and semantics (meaning of word and sentences) (Barre *et al.* 2011). Further, a recent study of language skills reported significant deficits in phonological awareness (speech sounds), semantics, grammar (sentence structure), discourse

(understanding of passages of text or conversation) and pragmatics (use of language in social context) in VLGA children (Reidy *et al.* 2013).

Besides working memory, other memory functions and learning processes are less well evaluated among preterm populations. In a recent study, VLGA children had deficits in immediate memory, working memory, long-term memory, and learning compared to term controls (Omizzolo *et al.* 2013). A few other studies have reported that preterm-born children perform significantly below term controls in memory modalities and verbal learning tasks (Anderson 2014). It has been proposed that severity of memory impairments may lessen in adolescence (Anderson 2014). However, one study reported that VLBW adolescents performed poorer in everyday memory tasks compared to term controls (Narberhaus *et al.* 2007).

Attention is a complex construct that consists of the ability to selectively focus, sustain, encode, shift, and divide attention. Attention skills are closely connected with executive processes that are required for purposeful and goal-directed behavior (Anderson 2014). According to several studies, (Aarnoudse-Moens *et al.* 2009, Mulder *et al.* 2009) preterm-born children have difficulties in several domains of attention and executive functioning. In a meta-analysis, deficits in selective and sustained attention as well as in verbal fluency were observed whereas results concerning shifting attention and inhibition tasks were more inconsistent (Mulder *et al.* 2009). In another meta-analysis, very preterm children had adverse outcomes in verbal fluency, working memory and cognitive flexibility compared to term controls (Aarnoudse-Moens *et al.* 2009). Impairments in executive functions are still present in adolescence (Burnett *et al.* 2013) and adulthood (Nosarti *et al.* 2007, Pyhala *et al.* 2011), and adjustment for IQ estimate seem to have only a minor influence on the outcome (Pyhala *et al.* 2011). Executive dysfunction appears to associate with poor academic achievement and social–emotional competence (Burnett *et al.* 2013).

School achievement

Prematurity is linked with compromised school performance. Children born very preterm were reported to have more difficulties in school readiness compared to term-born children at 5 years of age (Roberts *et al.* 2011). In another study, VLGA children performed comparably with term-born children in early linguistics, but showed deficits in numerical reasoning skills at preschool-age (Aarnoudse-Moens *et al.* 2011). A meta-analysis showed that academic areas of

weakness in very preterm children were reading, mathematics and spelling (Aarnoudse-Moens *et al.* 2009). Both psycho-educational and teacher-rated evaluations have shown poorer academic attainment in VLGA children (Mulder *et al.* 2010) with more pronounced difficulties in mathematics than in reading (Simms *et al.* 2013). VLGA children also require special education intervention and repeat a class more frequently (Anderson 2014). The adverse effect of prematurity on academic achievement has been shown to persist into adolescence (Grunau *et al.* 2004) and young adulthood (Hack 2009). In Sweden, 26% of young adults born at 24–28 weeks of gestation had a university degree compared to 38% of those born at term (Hack 2009).

Behavioral, social and emotional issues

According to several studies, VLGA children are also prone to behavioral, social and emotional difficulties (Aarnoudse-Moens *et al.* 2009, Johnson & Marlow 2011). Conflicting results have been reported regarding the risk for internalizing (withdrawn behavior and symptoms of depression) and externalizing (delinquent and risk-taking behavior) problems. While an earlier meta-analysis reported an increase in both internalizing and externalizing symptoms (Bhutta *et al.* 2002), in a more contemporary meta-analysis, VLGA children had more internalizing problems than term peers, but no differences were found in externalizing problems between the gestational groups (Aarnoudse-Moens *et al.* 2009). Studies have reported a two- to three-fold increased risk for attention deficit hyperactivity disorder in VLGA children and a four-fold increased risk in those born at ELGA (Johnson & Marlow 2011). VLGA children appear to have more symptoms of inattention than hyperactivity/impulsivity. Indeed, it has been suggested that attention deficit disorder can be a part of childhood-onset dysexecutive syndrome comprising social withdrawal, internalizing problems, an absence of aggression, academic underachievement, and deficits in working memory and processing speed. These characteristics are often observed in VLGA children (Johnson & Marlow 2011).

A few studies have also revealed a higher prevalence of autism spectrum disorders in ELGA populations. In these studies, autism spectrum disorder symptoms correlated strongly with cognitive impairment (Johnson & Marlow 2011). Children, adolescents and young adults born very preterm are about 3.5 times more likely to receive a diagnosis of any psychiatric condition and almost three times more likely to receive a diagnosis of an anxiety or depressive disorder

than controls (Burnett *et al.* 2011). In a recent study, preterm-born adolescents had more socialization problems compared to term controls. These problems were not due to impairments in general cognition. The authors also suggested that atypical social development is related to increased vulnerability to later psychiatric disorder (Healy *et al.* 2013).

Factors contributing to neurocognitive outcome

Several studies have shown that neurocognitive problems increase with decreasing GA in children born preterm (Anderson 2014, Bhutta *et al.* 2002, Marlow *et al.* 2005, Serenius *et al.* 2013), whereas other studies have suggested that the poor cognitive outcome is not necessarily explained by the degree of prematurity, but rather by ante- or perinatal complications, postnatal diseases, or factors that operate beyond the first hospitalization (Charkaluk *et al.* 2010, Morsing *et al.* 2011, Padilla *et al.* 2011). Previous studies have found correlations between cognitive deficits and various antenatal and neonatal factors, including male gender (Skiold *et al.* 2014), maternal obesity, thrombosis of fetal vessels in the placenta (Helderman *et al.* 2012), compound placental defect (Kaukola *et al.* 2006), CA (Pappas *et al.* 2014), IUGR/SGA (De Jesus *et al.* 2013, Guellec *et al.* 2011, Lohaugen *et al.* 2013, Morsing *et al.* 2011, Padilla *et al.* 2011), BPD (Brevaut-Malaty *et al.* 2010, Potharst *et al.* 2013), brain injury (Brevaut-Malaty *et al.* 2010, Luu *et al.* 2009, Reidy *et al.* 2013), and necrotizing enterocolitis (Schulzke *et al.* 2007). In addition, environmental factors during intensive care, including light, sound, and other sensory stimuli, or exposure to procedural stressors (Smith *et al.* 2011), may alter brain development. Early parent–infant closeness (Flacking *et al.* 2012) or socioeconomic factors (Charkaluk *et al.* 2010, Wong & Edwards 2013) may also affect the outcome.

MRI studies have brought to light neuroanatomical connections between prematurity and neurodevelopmental deficits (Ment *et al.* 2009). Advanced imaging techniques include diffusion tensor imaging and functional connectivity MRI as well as sophisticated image analysis methods, such as voxel-based morphometry, mathematical morphology-based cortical folding designs, and tract-based spatial statistics (Ment *et al.* 2009). Brain abnormalities related to prematurity persist through childhood and adolescence into adulthood (Allin *et al.* 2011, Counsell *et al.* 2008, de Kieviet *et al.* 2012, Skranes *et al.* 2007). Further emphasis is laid on investigating the long-term neurodevelopmental predictive

value of MRI findings (Counsell *et al.* 2008, Peterson *et al.* 2000, Skranes *et al.* 2007, Woodward *et al.* 2012).

Neonatal WM abnormalities on MRI predicted deficits in language abilities at 7 years of age in very preterm children (Reidy *et al.* 2013). In the same cohort, neonatal brain abnormalities, deep gray matter alterations in particular, were associated with poorer memory and learning outcomes (Omizzolo *et al.* 2014). Rathbone *et al.* showed that after preterm birth, reduced cortical surface growth was related to adverse neurocognitive outcomes at 2 and 6 years of age (Rathbone *et al.* 2011). Other studies also showed that very preterm-born children had reductions in brain volumes and that these findings were associated with poorer cognitive outcome (Peterson *et al.* 2000). Further, specific neurodevelopmental impairments, like deficits in eye–hand coordination, were linked to microstructural abnormalities in particular regions of WM (Counsell *et al.* 2008). Deficits in more complex functioning like visual motor integration, arithmetic and attention were associated with more extensive aberrations in WM microstructure among adolescents born with VLBW (Skranes *et al.* 2007). Socialization difficulties in preterm-born adolescents were associated with volumetric changes in an emotion-processing brain network in a recent study (Healy *et al.* 2013).

In ELGA infants, abnormal somatosensory responses assessed using magnetoencephalography at term were associated with adverse neuromotor development at 2 years of corrected age (Rahkonen *et al.* 2013). These responses were also detectable in electroencephalogram accompanied with median nerve stimulation that is routinely available at bedside (Nevalainen *et al.* 2014).

2.5.3 *Interventions to prevent or alleviate neurodevelopmental sequelae*

Prevention of life-long disabilities is a significant challenge in preterm-born children. Antenatal steroids have been associated with a significant decrease in the occurrence of brain injuries in preterm infants. However, the long-term effects of antenatal steroids on neurodevelopmental outcome are less clear (Favrais *et al.* 2014). According to a Cochrane review including 6145 very preterm infants, administration of antenatal magnesium sulfate was associated with a significantly lower rate of CP (risk ratio 0.68; 95% CI 0.54–0.87) (Doyle *et al.* 2009). In the randomized, placebo-controlled Caffeine for Apnea of Prematurity trial, caffeine treated infants had significantly lower rates of CP (4.4% vs. 7.3%) and cognitive delay (33.8% vs. 38.3%) than the controls at 18 months of age. However, at 5

years of age, caffeine was no longer associated with a significantly improved rate of survival without disability in children with VLBW (Favrais *et al.* 2014). Recombinant human erythropoietin treatment may have beneficial neurodevelopmental effects in preterm infants, but this needs to be further investigated (Favrais *et al.* 2014). Melatonin serves as a neuroprotectant in experimental models of brain injury. Clinical trials to test the neuroprotective properties in preterm infants are currently underway. Future randomized trials should perhaps combine melatonin with other protective therapies, including magnesium sulfate or antenatal steroids (Biran *et al.* 2014).

Other potential treatment methods for brain injury have been proposed, including the modification of microglia and lymphocyte activation, preventing astrocyte over-activation, modification of epigenetic conditions, and using cell therapies to promote repair and regeneration (Fleiss & Gressens 2012). However, converting the translational treatment methods into safe, clinical applications will be a complex task.

According to a review, early intervention programs for preterm infants have a positive effect on cognitive and motor outcomes during infancy, with the cognitive benefits persisting into pre-school age. However, further research is required to evaluate which early developmental and psychosocial interventions are the most effective at improving cognitive and motor outcomes in VLGA children. In addition, the long-term effects of these programs should be studied (Spittle *et al.* 2012).

3 Aims of the study

Our purpose was to provide new information about the role of ante- and postnatal factors and their interactions in brain damage and neurologic and neurocognitive outcomes in very preterm children. The specific aims of the study were:

1. To determine whether specific immunoproteins at birth predict the risk of IVH and whether their receptors are localized at the bleeding site (I).
2. To investigate whether a group of blood cytokines during the perinatal period predicts the risk of CP (II).
3. To investigate whether common polymorphisms in the *CCL18* gene associate with the susceptibility to CP in children born at VLGA (III).
4. To evaluate the role of antenatal factors and neonatal diseases on the neurocognitive outcomes in schoolchildren born very preterm without impairments (IV).

4 Subjects and methods

4.1 Study populations

All VLGA children born alive in Oulu University Hospital between November 1998 and November 2002 were considered to be included in the prospective cohort study (I–II). The following inclusion criteria were used; signed informed consent by the parents, availability of umbilical cord blood samples for biomarker studies, no defined life-threatening congenital malformation, no chromosomal abnormality, and no congenital metabolic disease or generalized viral or protozoal infection and survival during the first hospitalization. Altogether, 232 VLGA infants were born alive during the time frame. Sixty-six of these infants could not be included in the cohort (refusal of consent [n = 6]; unavailability of umbilical cord sample [n = 42]; death in delivery room [n = 3]; death after birth [n = 15]). Additionally, no follow-up information could be obtained for three children. The remaining 163 children comprised the original VLGA cohort (I). In addition, cord blood was analyzed from 33 term-born infants (I). The sera from these low-risk infants were obtained without identification of the individuals or obtaining consent. In addition, seven VLGA infants, who fulfilled the inclusion criteria, were born in Tampere University Hospital between March 2000 and January 2001 (II).

We further recruited the original VLGA population at 9 years of age (IV). Two of the 163 children died after the first hospitalization before school age. Seven children were further excluded from the follow-up, because they would not have been able to participate in psychological assessments. Of the excluded children, four had severe cognitive impairment (IQ <50), two were both blind and had severe cognitive impairment, and one did not speak Finnish. An informational letter was sent to the families of the 154 eligible VLGA children and all parents were contacted also by phone multiple times if needed. A comparison group of 90 birth date- and gender-matched children born at term in Oulu University Hospital were recruited similar to the VLGA children. The recruitment and inclusive follow-up assessments were done during a four-year period, between November 2007 and November 2011, in Oulu University Hospital. All participants were studied face-to-face at 9 years of age (range 8.7–9.4 years).

DNA samples and data regarding CP diagnosis (yes/no) were available for a total of 220 VLGA children born between 1997 and 2008 in the following three

hospitals in northern and central Finland: Oulu (24 cases and 172 controls), Tampere (one case and 20 controls), and Seinäjoki (no cases and three controls) (III). Additional populations of 271 VLGA children born in two hospitals in southern Finland, Turku (seven cases and 159 controls) and Helsinki (eight cases and 56 controls), between 1997 and 2006, as well as in Vancouver, Canada (eight cases and 33 controls) between 2006 and 2008, were included in the replication study (III). Due to the small number of cases, the replication populations were combined rather than reported separately. Only children of European origin and only one member of a pair of identical twins were included. Both DNA and serum from the cord blood were available for a subpopulation of 99 VLGA infants born in Oulu University Hospital between 1998 and 2002. This enabled us to study the association between *CCL18* polymorphisms and cord blood levels of the chemokine CCL18 (III).

4.2 Ethical considerations

The study protocols were approved by the appropriate ethics committees. Written informed consent was obtained from the parents (I–IV) and from the children (IV).

4.3 Methods

4.3.1 *Clinical characteristics*

Table 3 lists the prospectively collected antenatal and neonatal data selected for the analyses. GA was confirmed by ultrasound before 20 weeks of gestation. Infants with BW below two standard deviations from the mean of gestation-adjusted BW were classified as SGA (Pihkala *et al.* 1989).The definition of FGR in the present study was based on BW below two standard deviations from the mean of gestation-adjusted BW, documented evidence of growth restriction due to placental insufficiency defined by Doppler US and histological examination, as well as lack of congenital infections or malformations. Preeclampsia was defined according to ACOG guidelines (American College of Obstetricians and Gynecologists 1996). The Clinical Risk Index for Babies score I assesses initial neonatal risk for mortality and morbidity (The International Neonatal Network 1993). In addition to constitutional and clinical risk factors, maternal educational

level (low, <12 years of education or high, ≥12 years of education) was included in the analyses (IV).

Table 3. Clinical data.

Maternal factors	Intrapartum and birth characteristics	Neonatal factors
Age at birth (I)	Presence of active labor (I)	Oxygenation Index in the 1st 24 h (I)
History of previous preterm births (I)	Mode of delivery (I)	Lowest and highest $PaCO_2$ in the 1st 24 h (I)
Parity (I–II)	Gestational age at birth (I–IV)	Lowest mean blood pressure in the 1st 24 h (I–II)
Diabetes/other medical conditions (I)	Birth weight (I–III)	Highest mean blood pressure in the 1st 24 h (I)
Multiple pregnancy (I–IV)	Small for gestational age (I–III)	Patent ductus arteriosus (I)
Preeclampsia (I–II)	Fetal growth restriction (IV)	Respiratory distress syndrome (I, IV)
Preterm premature rupture of membranes (I)	Gender (I–IV)	Duration of mechanical ventilation (II)
Administration of antenatal steroids (I, IV)	Umbilical cord pH (I)	Intraventricular hemorrhage (I–IV)
Fever ≥37.8 C (I–II)	Apgar score at 5 min (I–II, V)	Cystic periventricular leukomalacia (II)
Histologic chorioamnionitis (I–II, IV)	Age when arriving to the NICU (I)	Blood culture–positive sepsis (II, IV)
	First body temperature (I)	Bronchopulmonary dysplasia (II–IV)
	Clinical Risk Index for Babies score I (I–II)	Postmenstrual age at discharge (IV)

Roman numerals in parentheses refer to the publications included in this thesis.

4.3.2 *Brain imaging*

Cranial US screening was performed using an HDI 5000 (Advanced Technology Laboratories Ultrasound, Botwell, WA) with a curved-array 5–8 MHz transducer. According to the protocol, the infants underwent serial brain ultrasound assessments at the following ages: 1 to 3 days, 1, 2 and 4 weeks, and thereafter every four weeks until discharge or at term. Examinations were performed by a pediatric radiologist and the results were reviewed afterwards by a research

radiologist without knowledge of the clinical data. IVH was classified using standard criteria (Papile *et al.* 1978) and the most severe grade was included in the present analysis. cPVL was classified according to de Vries *et al.* (1992).

4.3.3 *Analysis of immunoproteins (I–III)*

Cord blood

After double clamping the umbilical cord at birth, arterial blood was drawn into a sterile tube. Cord blood serum was separated at 3,000 rpm for 15 minutes and the samples were stored frozen at −70°C. Cord blood cytokines were analyzed using antibody-based protein microarrays with DNA amplification (Schweitzer *et al.* 2002). Monoclonal antibodies (R&D Systems, Minneapolis, MN, USA; PharMingen, San Diego, CA, USA) were dispensed onto the microarrays. After incubation, the captured proteins were detected by secondary antibodies containing an oligonucleotide DNA primer to generate a fluorescent signal (GenePix; Axon Instruments, Foster City, CA, USA), which was used to quantify specific proteins. Altogether 109 different cytokines were analyzed (study II, Table 4) and 107 of these cytokines were available for study I. The concentrations were reported as fluorescence units (FU).

Blood sample after birth

Two peripheral samples of arterial blood were collected on day 1 and 7 after birth (n = 125 infants). EDTA plasma specimens were separated and stored at –70°C. The analysis of the cytokines (Table 4) was performed using the Cytometric Bead Array Kit (BD Biosciences, San Diego, California). Bead populations with distinct fluorescence intensities for specific soluble proteins were measured with flow cytometry together with the BD™ CBA Software. The concentrations are reported as pg/ml.

Quantitative analysis

Concentrations of chemokine (C-C motif) ligand 18 (CCL18) in cord blood and in plasma on day 1 and 7 after very preterm birth (23 infants with all available specimens) and in cord blood at term (4 specimens) were measured using Duoset

ELISA (R&D Systems Inc, Minneapolis, MN) according to the manufacturer's instructions. The concentrations measured by enzyme-linked immunosorbent assay (ELISA) correlated with the values obtained by antibody-based protein microarray with DNA amplification (r = 0.704).

Table 4. The cytokines analyzed from umbilical cord blood obtained at time of birth.

1. Chemokines (n = 32)	2. Growth factors (n = 28)	3. TNF family (n = 12)	4. Hematopoietin family (n = 20)	5. IL-1 family (n = 5)	6. Others (n = 12)
CCL1–5	AR	CD27	FLT3lig	IL-1ra	ALCAM
CCL7, 8	ANG	CD30	GM-CSF[1]	IL-1α, -1β[1]	FST
CCL11[1]	βNGF	DR-6	IL-2	IL-1sr2	ICAM-1, -3
CCL13–20	BDNF	FAS	IL-2sRα	ST2/IL-1r4	IL-10rβ
CCL23, 24	BTC	FASL	IL-2rβ, γ		IL-17
CCL26–28	EGF	HVEM	IL-3[1], -4[1], -5		L-selectin
CXCL2, 3, 5	FGF1, 2, 4	RANK	IL-5Rα		MMP-7, -9
CXCL6, 8[1], 9	FGF7, 9	TNF-R1	IL-6[1], -7, -9		PECAM-1
CXCL11–13	GDNF	TNF[1], -β	IL-13, -15		TIMP-1, -2
CX3CL1	HGF	TRAIL-R1, 4	Leptin		
XCL1	IGF-1R		OSM		
	IGF-2		SCF, -R		
	IGFBP1,4		spg130		
	M-CSF				
	M-CSF-R				
	NT3, 4				
	PLGF				
	PDGF-Rα				
	TGF-α				
	VEGF, -R2				

[1]The indicated cytokines as well as IL-10, IL-12p70, and G-CSF were additionally analyzed from cord blood and peripheral blood collected 1 d and 7 d after birth using an independent immunoassay.

4.3.4 *Placental pathology (I–II, IV)*

Placentas (n = 159) were fixed in 10% neutral buffered formalin immediately after birth. The rim of the membranes was taken from the site of membrane rupture. The umbilical cord specimens were obtained from the fetal and placental sides and from midway between the sides of insertion. A full-thickness specimen of placental parenchyma was taken from midway between the umbilical cord insertion and the placental margin. For histologic examination, paraffin blocks were cut into 5-μm slices and stained with hematoxylin–eosin. HCA was defined according to the criteria of Salafia *et al.* (1989). Placental perfusion defect was defined as poorly vascularized villi, multiple capillary lumina in some villi, increased intervillous volume, and reduced total villous capillary bed (Benirschke & Kaufmann 2000). The placentas were evaluated by a single pathologist without any knowledge of clinical data.

4.3.5 *Immunohistochemistry of the brain (I)*

Nine preterm and five term infants who died between 1998 and 2008 were included in the study to detect the cellular localization of chemokine (C-C motif) receptor 3 (CCR3). Seven of the preterm infants were born between 23 and 27 weeks of gestation and two infants between 31 and 32 weeks. The primary causes of death were IVH (n = 3), RDS (n = 2), septicemia (n = 2), severe asphyxia due to placental abruption (n = 1), and pericardium tamponade caused by hydrops fetalis (n = 1). Causes of death of the full-term infants included septicemia (n = 1) and perinatal asphyxia (n = 4).

The formalin-fixed, paraffin-embedded blocks of representative brain tissue were cut into 4-μm slices, deparaffinized, and rehydrated. The antigen retrieval was done in a microwave oven in Tris-EDTA buffer, pH 9. Endogenous peroxidase activity was inhibited with peroxidase blocking solution (DAKO, Glostrup, Denmark). The sections were incubated with 1:100 dilution of rabbit anti-human CCR3 polyclonal antibody (AbD Serotec, AHP1010). CCR3 detection was done using the DakoCytomation kit (DAKO) and the samples were stained with DAB substrate (DAKO). The specificity of the antibody was confirmed by optimization of the immunohistochemical staining conditions on positive control tissues (liver and spleen).

4.3.6 *DNA sample preparation, SNP selection and genotyping (III)*

Genomic DNA was extracted from buccal cells, umbilical cord blood, or paper blood samples. Extraction methods used were Chelex 100 (Bio-Rad, Hercules, CA, USA) for buccal cells, UltraClean DNA Blood Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) for umbilical cord blood, and MO BIO UltraClean BloodSpin DNA Isolation Kit (MO BIO Laboratories) for dried paper blood spots on Guthrie cards. A whole-genome amplification was performed for buccal cell and paper blood DNA samples, as described previously (Karjalainen *et al.* 2012).

A total of five common CCL18 single nucleotide polymorphisms (SNPs) that occur with a minor allele frequency of >0.1 in the CEU population (CEPH; Utah residents with ancestry from northern and western Europe) and/or were previously reported (Modi *et al.* 2006), were selected for the study. For children in the original study population, SNPs with accession numbers rs1102934, rs2015086, rs2015070, rs2735835, and rs712044 in the dbSNP database (http://ncbi.nih.gov/SNP) were genotyped using Sequenom iPLEX Gold chemistry. SNPs rs1102934 and rs2015086 were located upstream of the gene, and the other SNPs were intronic (SNP locations within the CCL18 gene are shown in Figure 3).

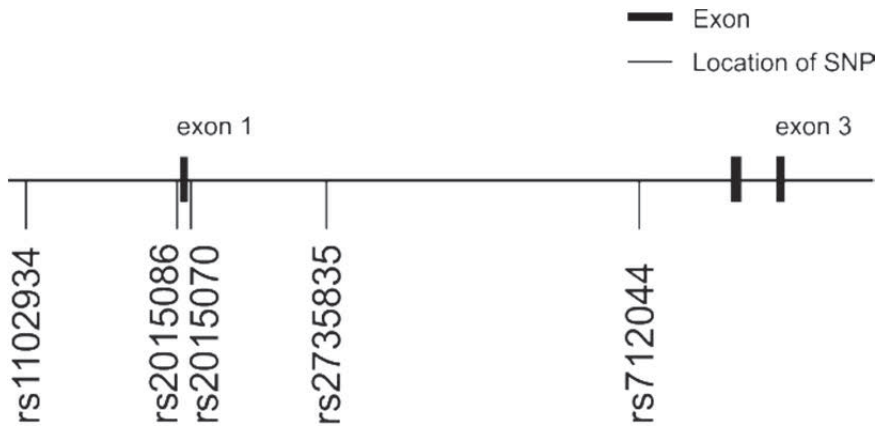

Fig. 3. Locations of the genotyped polymorphisms within the *CCL18* gene. The positions of the three exons (black bars) and the five single nucleotide polymorphisms (narrow lines) within the *CCL18* gene are shown.

The SNP rs2735835 was selected for the replication study. The genotyping of rs2735835 was performed by polymerase chain reaction and restriction fragment length polymorphism analysis with the following primer pair: 5′-TGGCCTTTGT TCCATCTCTT-3′ (forward) and 5′-AGAGGGCTAGGACCCATTGT-3′ (reverse) and by restriction enzyme digestion with PshAI (New England Biolabs, Ipswich, MA, USA).

4.3.7 *Diagnosis of CP (II–IV) and cognitive impairment (IV)*

In Finland, the diagnosis and classification of CP was confirmed at the age of 5 years by a child neurologist, based on the standardized criteria (Surveillance of Cerebral Palsy in Europe 2000) (II–IV). In Canada, CP was diagnosed using criteria based on the Bax definition (Bax *et al.* 2005) or by the standardized assessment method of The Canadian Neonatal Follow-Up Network (www.cnfun.ca/Resources.aspx) at 18 months (III). The diagnosis of cognitive impairment (IQ <70) was based on previous psychological evaluations. Data concerning cognitive impairment were obtained from medical records and from the parents (IV).

4.3.8 *Neurocognitive assessments (IV)*

Neurodevelopmental and behavioral assessments were performed by a child psychologist. Fourteen subtests from the second edition of the neuropsychological test of development for children (NEPSY-II) (Korkman *et al.* 2007) and six subtests from the third version of the Wechsler Intelligence Scale for Children (WISC-III) (Wechsler 1999) were used (Table 5). The subtest scores have a normed mean of 10 and a standard deviation of 3 (range 1–19). Standardized scores were calculated and analyzed for each of the 20 subtests. In addition, mean scores were calculated and analyzed for each of the five domains: visuospatial–sensorimotor processing, attention–executive functions, language, memory–learning, and social perception.

4.3.9 *Questionnaires (IV)*

Parents completed a questionnaire concerning socio-demographic status, children's health, illnesses, impairments and school attendance. They also completed a questionnaire comprising 82 questions of the Five to Fifteen (FTF).

The FTF is a validated questionnaire with 181 questions that cover different aspects of the development and behavior of children (Kadesjo *et al.* 2004). The domains included in the present study were attention–executive functions (25 questions), perception (five questions), social skills (27 questions) and emotional–behavioral problems (25 questions). For each domain, a standardized mean score ranging from 0 to 2 was calculated and used for the analyses. In addition, teachers completed a questionnaire concerning the children's educational attainments graded on a five-point scale from 1 (excellent) to 5 (poor).

Table 5. Domains and subtests of neurocognitive assessment.

Domains	Subtests	
	NEPSY-II	WISC-III
Visuospatial–sensorimotor processing	Design copy	Picture completion
	Visuomotor precision	Coding
	Imitating hand positions	Block design
Attention–executive functions	Auditory attention	
	Response set	
	Inhibition/naming	
	Inhibition/inhibition	
	Inhibition/switching	
Language	Phonological processing	Information
	Comprehension of instructions	Similarities
Memory–learning	Narrative memory	Digit span
	Word list interference	
Social perception	Theory of mind	
	Affect recognition	

NEPSY-II, The second edition of the neuropsychological test of development for children; WISC-III, The third version of the Wechsler Intelligence Scale for Children.

4.4 Statistics

Classification and regression trees (CART) analysis was used as a primary evaluation to identify the major risk factor(s) for IVH grade II–IV (I). CART is a data mining method that can model and detect complex relationships between dependent and independent variables. The analysis goes through all the available independent variables and splits the tree into 2 according to a certain value that will provide the highest accuracy of prediction of the disease. Typically, a large tree that predicts the outcome extremely well is grown. The tree is then pruned to a smaller tree with the use of the complexity parameter. We used a conservative application of CART that is not affected by problems of multiple testing. R software (version 2.6.1; R Core Development Team, Vienna, Austria) and its extension package rpart (version 3.1) were used for the CART analysis by a biostatistician (Tuimala). Regression models are typically well suited for the description of additive dependencies between variables.

Most of the statistical analyses were performed using the SPSS program (SPSS Inc, Chicago, IL). We used factor analysis as the primary statistical method to investigate the interdependencies in the variation of the complete set of altogether 109 cord blood cytokines (II). This analysis considers the variation of the individual cytokines and does not consider the differences in the absolute values of individual cytokines. Factor analysis combines the pattern of variation of individual cytokine variables that correlate with each other into a single factor. This factor is a linear combination of variables in a (multi-dimensional) scatter plot into which an orthogonal regression line (or a plane) has been fitted. Varimax rotation was then used to maximize the variability of a factor. The factor extracting was halted after 60% of the variability of the cytokines was explained. Altogether five factors were identified. The cut-off values for the maximum accuracy of cytokines in predicting the risk of IVH grade II–IV and CP were derived from the receiver operating characteristic curve (I–II). Categorical variables were assessed using the χ^2 test or Fisher's exact test. Differences in continuous variables between two outcome groups were studied using the Mann–Whitney U test (I–II), Kruskal–Wallis test (II) and Student's t-test (I–II, IV). Multiple stepwise logistic and linear regression models were used as secondary analyses to evaluate covariates as independent predictors for dependent variables. All tests were 2-tailed. Statistical significance was set at $P < 0.05$.

Haploview v.4.2 (Barrett *et al.* 2005) was used to test Hardy–Weinberg equilibrium, to obtain pairwise linkage disequilibrium (LD; D' and r^2) values, and

for case–control comparisons of the allele frequencies (III). Haploview uses the χ^2 test for case–control analysis. SNP associations were corrected for multiple testing using SNPSpD (Nyholt 2004); this method takes into account the LD between SNPs. According to this correction, a P value of ≤0.014 was determined as the significance threshold for multiple comparisons (III). PLINK 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell *et al.* 2007) was used for logistic regression analyses, and for the Cochran–Mantel–Haenszel and Breslow–Day tests (that are used to control for the possible effect of clustering in case–control analyses with different combined populations, and to test for heterogeneity of the disease–gene association between clusters, respectively) (III).

The results were controlled for multiple comparisons using the method developed by Benjamini and Hochberg (Benjamini & Hochberg 1995) (IV). Effect sizes were calculated in terms of Cohen's d. Cohen's guidelines were used to classify the magnitude of the effect sizes, with 0.20, 0.50 and 0.80 referring to small, medium and large effect size, respectively (Cohen 1988) (IV).

5 Results

5.1 Perinatal immunoproteins and IVH (I)

Among the VLGA cohort (n = 163), twenty-three (14%) infants had IVH grade II–IV. IVH grade II–IV was more common in ELGA infants (14/49; 29%) compared to infants born ≥28 weeks of gestation (9/114; 8%) (P = 0.001). Differences in factors between infants with IVH grade II–IV and infants with no IVH or IVH grade I are shown in Table 6.

Table 6. Differences of factors in univariate analyses between 2 outcome groups.

Factor	IVH grade II–IV (n = 23)	No IVH or IVH grade I (n = 140)	P
HCA[1]	13 (57)	51 (38)	0.085[2]
Funisitis[1]	6 (26)	27 (20)	0.495[2]
Preeclampsia[1]	5 (22)	37 (26)	0.634[2]
Antenatal steroid[1]	18 (78)	122 (87)	0.257[2]
Vaginal delivery[1]	9 (39)	38 (27)	0.240[2]
GA (wks+days)[3]	27+5 (25+1 – 28+6)	29+4 (27+6 – 30+6)	<0.0001[4]
SGA[1]	4 (17)	34 (24)	0.600[5]
5 min Apgar score[3]	6 (5–7)	7 (6–8)	0.023[4]
1st 12 h, CRIB score I[3]	9 (3–11)	2 (1–5)	<0.0001[4]
1st 24 h, lowest mean BP (mmHg)[3]	21 (16–24)	27 (23–31)	<0.0001[4]
1st 24 h, highest paCO2 (kPa)[3]	6.5 (5.7–7.6)	6.1 (5.2–7.1)	0.117[4]
RDS[1]	23 (100)	82 (59)	<0.0001[5]
PDA[1]	15 (65)	29 (21)	<0.0001[2]
CCL18 (FU)[3]	4060 (3078–5866)	6471 (4796–7670)	0.002[4]

[1] Data are given as frequency; n (%), [2] χ^2 test, [3] Data are given as median (25th and 75th percentiles), [4] Mann-Whitney U test, [5] Fisher's exact test.

BP, blood pressure; CCL18, chemokine (C-C motif) ligand 18; CRIB, clinical risk index for babies; FU, fluorescence unit; GA, gestational age; HCA, histologic chorioamnionitis; IVH, intraventricular hemorrhage; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome.

5.1.1 *Chemokine CCL18 was associated with IVH*

Of the 107 cord serum proteins, chemokine CCL18 emerged as the only cytokine associating with IVH grade II–IV in CART analysis. Infants with IVH grade II–IV had a lower median level of CCL18 compared to infants without IVH or with IVH grade I (Table 6). CCL18 predicted the risk of IVH grade II–IV with a specificity of 73% and a sensitivity of 74% at a cut-off value of 4969 FU (area under receiver operating characteristic curve 0.72, P = 0.002) (Figure 4). In the logistic regression model (covariates: CCL18 <4969 FU and GA <28 wk), CCL18 independently predicted IVH grade II–IV (odds ratio [OR] 7.6; 95% confidence interval [CI] 2.5–23.0, P <0.0001). GA had no significant effect. Further, low CCL18 concentration remained an independent risk factor for IVH grade II–IV when factors associating with IVH grade II–IV in univariate analyses (shown in Table 6) were considered (OR 7.7; 95% CI 2.2–27.0, P = 0.001).

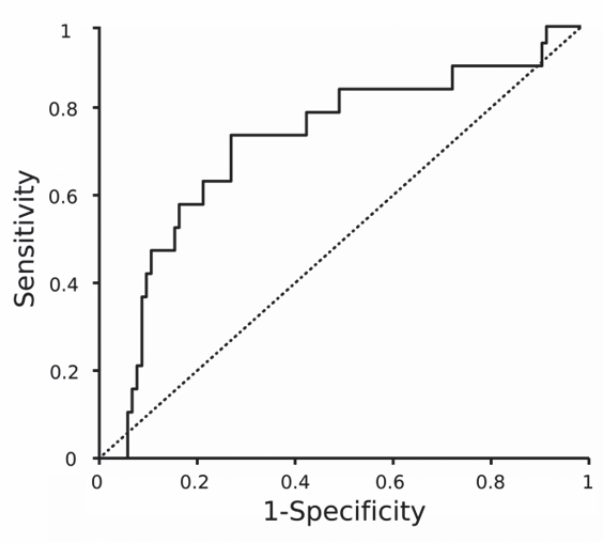

Fig. 4. Receiver operating characteristic curve showing the cord blood concentration of CCL18 in prediction of the risk of intraventricular hemorrhage grade II–IV.

None of the other proteins analyzed showed an association with IVH grade II–IV (data not shown). Term-born infants had a higher median level of CCL18 compared to very preterm infants (10097 FU vs. 6053 FU, P <0.0001). However,

GA did not associate with CCL18 in VLGA infants (P = 0.667). Further, no detectable association was found between CCL18 and HCA or funisitis.

Cytokine analysis after birth

The cytokines analyzed from arterial blood of 125 VLGA infants taken on day 1 or on day 7 did not associate significantly with IVH grade II–IV. However, IL-6 concentrations tended to be higher in infants with IVH grade II–IV compared to infants with no IVH or IVH grade I (53.9 FU vs. 24.9 FU, P = 0.05) on day 1.

Quantitative analysis

To further evaluate the low CCL18 concentration as a risk factor of IVH, the available specimens from 23 infants were submitted to quantitative analysis (Table 7). Low cord blood CCL18 in VLGA infants developing IVH grade II–IV was confirmed. CCL18 significantly increased within the first 24 hours. The increase in CCL18 between day 1 and 7 was not significant. After birth CCL18 levels tended to be lower in infants with IVH grade II–IV than in those without IVH or with IVH grade I. However, these differences were not significant. By the age of 7 days, the levels of CCL18 in VLGA infants (median 9165 pg/ml) had approached the cord blood levels of CCL18 at term birth (median 14619 pg/ml).

Table 7. Analysis of plasma concentrations of CCL18 (*p*g/ml) from very preterm infants with IVH grade II–IV and no IVH or IVH grade I using enzyme-linked immunosorbent assays.

Postnatal age	IVH grade II–IV				No IVH or IVH grade I				P[1]
	Mean	(SD)	Median	n	Mean	(SD)	Median	n	
Day 0, cord blood	4177	(860)	4181	12	6011	(1866)	5684	11	0.01
Day 1	7957	(2160)	7670	12	9751	(2918)	10292	11	0.077
Day 7	10645	(10525)	7072	12	13176	(10525)	10964	11	0.123

[1]Student's t-test (equal variances not assumed). Both in the IVH grade II–IV group and no IVH or IVH grade I groups CCL18 concentrations increased from day 0 to day 1 (P <0.05, paired t-test).
CCL18, chemokine (C-C motif) ligand 18; IVH, intraventricular hemorrhage.

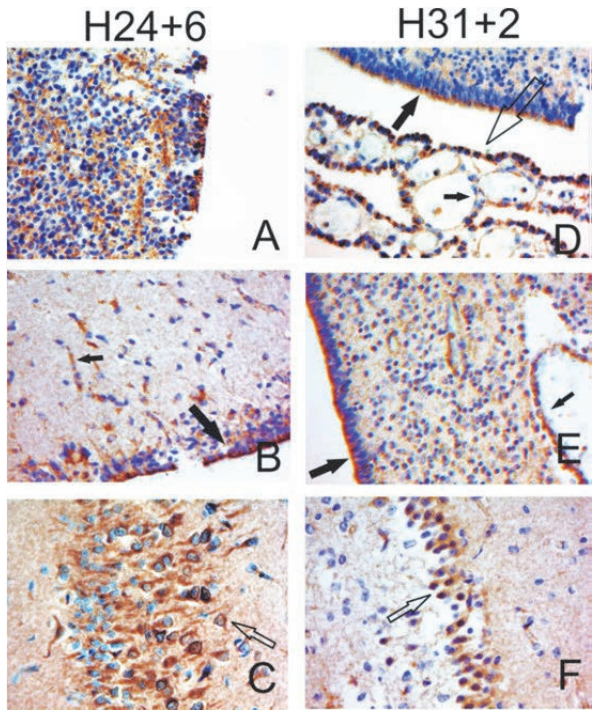

Fig. 5. Immunohistochemical localization of CCR3 in the brain. Representative brain samples from two preterm (A–F) infants from different gestational weeks as indicated were stained with anti-CCR3 antibodies. Panel A shows the positive staining in the germinal matrix. Big black arrows indicate the staining in the ependymal cells, small black arrows in the endothelium of the capillaries, small open arrows in the neurons of the hippocampus and big open arrow in the choroid plexus.

5.1.2 *Chemokine receptor CCR3 was detectable in the immature brain*

As CCR3 is the known CC-chemokine receptor for CCL18 (Nibbs *et al.* 2000), immunohistochemistry of the brain was performed to detect the cellular localization of CCR3. We found CCR3 to be detectable already at 23 weeks of gestation. Immunopositivity was detected in the ependymal cells, the choroid plexus, the GM, the endothelium of the capillaries, and in the neurons of the hippocampus of all seven infants born before 28 weeks of gestation. The localization of CCR3 in two infants born between 31 and 32 weeks of gestation

resembled those born between 23 and 27 weeks of gestation; however, immunostaining was no longer detectable in the GM (Figure 5).

5.2 Perinatal immunoproteins and CP (II)

The study group comprised 169 VLGA children. The clinical data of children with CP and without CP are shown in Table 8.

Table 8. Characteristics of VLGA children with and without CP.

Factor	CP children (n = 19)	Non CP-children (n = 150)	P
Gestational age at birth (wks+days)	28+0 (23+2 – 31+4)	29+4 (24+1 – 31+6)	0.090
ELGA	9 (47)	41 (27)	0.071
Male	13 (68)	77 (51)	0.160
Birth weight (g)	1110 (540–1970)	1211 (370–2295)	0.289
Maternal fever	10 (53)	48 (32)	0.603
Preeclampsia	5 (26)	52 (35)	0.457
Spontaneous premature birth	14 (74)	95 (63)	0.393
Antenatal steroid	16 (84)	124 (83)	1.0
Histologic chorioamnionitis	10 (53)	53 (35)	0.226
Small for gestational age	4 (21)	40 (27)	0.783
5-minute Apgar score	7 (3–8)	7 (0–10)	0.784
CRIB I score	5 (0–15)	2 (0–15)	0.047
Lowest mean arterial pressure (24 h)	22 (1 –36)	26 (10–55)	0.270
Duration of mechanical ventilation (d)	7.5 (0–79)	2.9 (0–96)	0.043
Additional O_2 at 36 wks of gestation	9 (47)	32 (21)	0.013
Neonatal sepsis	6 (32)	41 (27)	0.779
IVH grade II–IV	9 (47)	15 (10)	<0.001
cPVL	7 (37)	3 (2)	<0.001

Data on dichotomized factors are given as frequency; n (%) and data on continuous factors as median (range).

VLGA, Very low gestation age; CP, cerebral palsy; ELGA, extremely low gestational age; CRIB, Clinical Risk Index for Babies; IVH, intraventricular hemorrhage; cPVL, cystic periventricular leukomalacia.

5.2.1 *A cluster of cord blood cytokines was associated with CP*

To investigate several groups of interrelated cytokines and their relationship to a predicted outcome, CP, we performed factor analysis. Factor analysis sorts groups of variables with distinct factor loadings and eigenvalues describing correlations and variability inside factors. The cytokines that were loaded onto each of the five factors and explanations for the variations are presented in Table 9. The total variance explained by the five factors was 60.7%.

Table 9. Summary of factor analysis.

Factor 1 (27.7%)	Factor 2 (12.1%)	Factor 3 (9.1%)	Factor 4 (6.7%)	Factor 5 (5.1%)
Eot3/CCL26	IL-5Rα	NT3	IL-3	IL-1β
TRAIL-R1	IL-9	SGP130	GDNF	MIP-3α/CCL19
IGF-1R	IL-2rγ	SCF	Eot/CCL11	IL-1α
IL-2rβ	BTC	NT4	MCP-3/CCL7	MIP-1β/CCL4
FGF2	FASL	TNF-α	IL-7	GCP-2/CXCL6
CD30	ST2/IL-1r4	M-CSF	IL-13	IL-6
Lymphotactin/XCL1	VEGF-R2	TNF-β	IL-4	ENA78/CXCL5
TGF-α	CCL28	IL-17	PLGF	IL-8/CXCL8
FGF4	HCC1/CCL14	I-309/CCL1	GM-CSF	MCP-1/CCL2
PDGF-Rα	Fractalkine/CX3CL1	FLT3lig	IL-5	MCP-2/CCL8
SDF-1β/CXCL12	SCF-R	RANTES/CCL5	FGF7	
βNGF	MCP-4/CCL13	IL-2	FAS	
IGF-2	HVEM	VEGF		
IL-10rβ	RANK	MIP-1δ/CCL15		
CTACK/CCL27	TRAIL-R4	IGFBP3		
IL-1sr2		TNF-R1		
IGFBP4				
CD27				

All cytokines had a factor loading above 0.500 and are presented in descending order. The total variance of cytokines explained in each factor is presented in parentheses.

Next, we used a multiple logistic regression analysis to investigate the relationship between the factor scores and CP after adjustment for GA and HCA.

Scores from factors 1–5 were used as continuous variables presenting the weighted mean scores of cytokines for a given factor. Factor scores for factor 1 and 2 independently predicted CP (OR 2.8, 95% CI 1.5–5.5, P = 0.002 and OR 2.2, 95% CI 1.2–4.1, P = 0.014, respectively). The ORs were higher when considering, as an outcome, only the 14 CP children who were born after spontaneous onset of labor (OR 4.8, 95% CI 1.8–12.5, P = 0.001 and OR 2.8, 95% CI 1.2–6.4, P = 0.014, respectively). In contrast, GA, HCA, and scores for factors 3–5 did not explain the risk of CP. However, CP children tended to be born at a lower GA (Table 8), and most of the umbilical cord blood cytokines in factors 1 and 2 were higher in HCA compared to non-HCA pregnancies (data not shown). The levels of all of the cord blood cytokines in factors 1 and 2 tended to be higher in CP children compared to very preterm children without CP (data not shown).

5.2.2 *Inflammatory cytokines after birth were associated with CP*

None of the 11 inflammatory cytokines measured from cord blood (Table 4) differed between children with and without CP (data not shown). The concentration of IL-12p70 on day 1, but not on day 7, was higher among CP children compared to children without CP (median 4.8 pg/ml, range 1.6–8.5 vs. median 3.0 pg/ml, range 0.5–10.3, respectively; P = 0.023). The concentration of IL-8 on day 7 was higher among CP children compared to children without CP (77.4 pg/ml, range 12.8–312.5 vs. 27.9 pg/ml, range 1.4–422.4, respectively; P = 0.034); this difference was not detectable on day 1. None of the other cytokines from peripheral blood associated with CP (data not shown).

For IL-12p70 on day 1, the cut-off value of 4.2 pg/ml or higher, predicted CP with a sensitivity of 75% and a specificity of 73%. In a multiple logistic regression model, dichotomized IL-12p70 on day 1 predicted CP (OR 6.75, 95% CI 1.11–41.0; P = 0.038), whereas the clinical variables (GA <28 wk, Clinical Risk Index for Babies score I, duration of mechanical ventilation, and additional oxygen at 36 wk of GA) did not predict CP. IL-8 on day 7 was also modeled as a dichotomized variable (cut-off value 30.3 pg/ml or higher, predicting CP with a sensitivity of 71% and specificity of 53%) and put in to a multiple logistic regression model. In this analysis, only the duration of mechanical ventilation emerged as a significant factor predicting the risk of CP (OR 1.047, 95% CI 1.019–1.076; P = 0.001).

Infants with cPVL had a higher IL-12p70 concentration on day 1 (4.5 pg/ml, range 3.1–8.5) compared to infants without cPVL (3.0 pg/ml, range 0.5–10.3) (P = 0.039). Infants with cPVL had a higher IL-8 concentration on day 1 (327.9 pg/ml, range 47.1–532.8) compared to infants without cPVL (85.9 pg/ml, range 12.1–1,425.9) (P = 0.020). Other analyses of inflammatory cytokine levels in cord blood or in peripheral blood revealed no association with the risk of cPVL.

Finally, we conducted a multiple logistic regression analysis including continuous variables GA at birth, factor scores for factors 1 and 2; dichotomized IL-12p70 on day 1, and IL-8 on day 7, as independent predictors, whereas CP was an outcome variable. Factor scores for factor 1 and IL-12p70 on day 1, independently predicted CP (OR 7.07, 95% CI 1.47–33.96; P = 0.015 and OR 1.97, 95% CI 1.14–3.40; P = 0.015, respectively).

5.3 *CCL18* polymorphisms and CP (III)

Table 10 shows the clinical data of the original and replication populations. In the combined population, the 48 children with CP had a higher incidence of IVH grade II–IV and BPD grade 2–3, and a lower GA compared to the children without CP (data not shown).

Table 10. Clinical characteristics of the populations in study III.

Factor	Original population			Replication population		
	CP cases (n = 25)	Controls (n = 195)	P	CP cases (n = 23)	Controls (n = 248)	P
Multiple pregnancy[1]	2/25 (8)	42/195 (28)	0.18[2]	7/23 (30)	78/248 (32)	0.92[2]
Male gender[1]	17/25 (68)	88/195 (55)	0.21[3]	14/23 (61)	138/248 (56)	0.63[3]
GA (wk)[4]	28.4 (1.7)	28.8 (2.0)	0.39[5]	26.1 (1.4)	27.8 (2.2)	<0.0001[5]
BW (g)[4]	1140 (362)	1173 (352)	0.67[5]	873 (216)	998 (326)	0.017[5]
SGA[1]	6/25 (24)	49/195 (25)	0.90[3]	5/23 (22)	67/248 (27)	0.57[3]
IVH, grade II–IV[1]	7/25 (28)	14/195 (7)	0.001[3]	11/23 (48)	32/248 (13)	<0.0001[3]
BPD, grade 2–3[1]	7/25 (28)	44/191 (23)	0.58[3]	14/23 (61)	72/248 (29)	0.002[3]

[1]Data is given as frequency; n (%), [2]Fisher's exact test, [3]χ^2 test, [4]Data is given as mean (SD), [5]Student's t-test.

CP, cerebral palsy; GA, gestational age; BW, birth weight; SGA, small for gestational age; IVH, intraventricular hemorrhage; BPD, bronchopulmonary dysplasia.

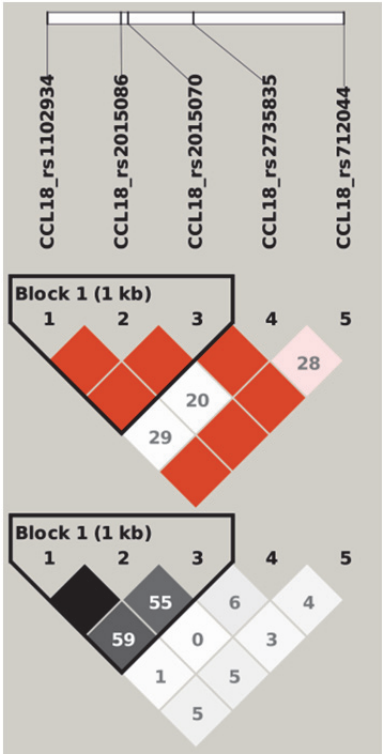

Fig. 6. Linkage disequilibrium (LD) plot of the *CCL18* single nucleotide polymorphisms genotyped for the original study population. Names and relative positions of the polymorphisms are shown on the top. Numbers in the squares are pairwise D′ values (upper panel) or r^2 values (lower panel) multiplied by 100; the darker the square, the higher the LD. Squares without numbers equals a D' or r^2 value of 1.0.

None of the *CCL18* SNPs deviated from Hardy–Weinberg equilibrium. There was a relatively strong LD among the SNPs (Figure 6). We analyzed the allele frequency distributions of the five *CCL18* SNPs in the CP cases and controls. In the original population, SNP rs2735835 was associated with CP; the minor allele A was underrepresented in CP cases compared to controls (Table 11). This association was significant at the multiple testing–corrected significance threshold. In a logistic regression model that controlled for GA and IVH grade II–IV, the SNP association remained significant (OR 0.45, 95% CI 0.22–0.88, P = 0.021). IVH grade II–IV additively predicted CP (OR 4.23, 95% CI 1.41–12.64, P = 0.01).

Table 11. Case–control analysis of association between five *CCL18* SNPs and CP in the original population.

CCL18 polymorphism	Minor allele	Minor allele frequency cases/controls	P	OR (95% CI)
rs1102934	G	0.17/0.15	0.73	1.15 (0.51–2.61)
rs2015086	C	0.16/0.15	0.84	1.08 (0.48–2.43)
rs2015070	A	0.10/0.09	0.76	1.16 (0.43–3.13)
rs2735835	A	0.24/0.43	0.01[1]	0.42 (0.21–0.83)
rs712044	G	0.17/0.26	0.15	0.56 (0.26–1.25)

[1]Significant at multiple testing–corrected significance threshold (P ≤0.014).

SNP, single nucleotide polymorphism; CP, cerebral palsy.

We further analyzed SNP rs2735835 in the replication population. In this population, the frequency of minor allele A tended to be lower in CP cases compared to controls (Table 12). After combining the original and replication populations and taking into account the possible confounding effect of clusters, the association between rs2735835 and CP was significant, with the minor allele A being underrepresented in CP cases compared to controls (Table 12). In a logistic regression model that controlled for GA, IVH grade II–IV and BPD grade 2–3 , the SNP association remained significant (OR 0.59, 95% CI 0.37–0.95, P = 0.028). IVH grade II–IV additively predicted CP (OR 3.81, 95% CI 1.88–7.74, P = 0.0002).

Table 12. Analysis of SNP rs2735835 in replication and combined populations.

Population	Number cases/controls	Minor allele frequency cases/controls	P	OR (95% CI)
Replication population	23/248	0.39/0.48	0.24[1]	0.69 (0.36–1.30)
Combined original and replication population	48/443	0.31/0.46	0.005[1]	0.52 (0.33–0.83)

[1]Case-control association analysis was controlled for the effect of clusters, that is, different populations (Finland and Canada). According to the Breslow–Day test, there was no heterogeneity in the ORs between the clusters (P >0.1). The P values and ORs are presented as a result of the Cochran–Mantel–Haenszel test. SNP, single nucleotide polymorphism.

5.3.1 *CCL18 polymorphisms and cord blood levels of* CCL18

We next investigated the correlation between genotype frequencies of the five *CCL18* SNPs and cord blood levels of CCL18. Due to the low number of individuals with homozygous minor allele genotypes, homozygous/heterozygous genotype groups for the minor alleles were combined and compared to those of individuals with the homozygous major allele genotype. Infants with the SNP rs2015086 TT genotype (n = 70) had lower CCL18 levels compared to carriers of the minor C allele (n = 29): mean 5869 FU vs. mean 7055 FU, respectively (mean difference −1186 FU; 95% CI −2272 to −100, P = 0.033). However, this result was not significant at the multiple testing–corrected significance threshold. The other SNPs showed no significant association with CCL18 concentration in cord blood (data not shown).

5.4 Neurocognitive, behavioral, and school outcomes in very preterm children at 9 years of age (IV)

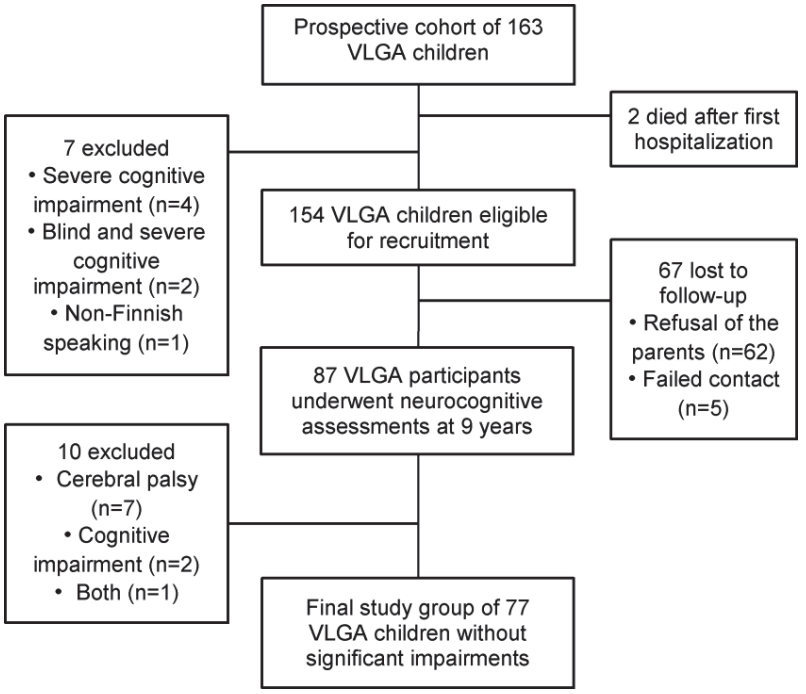

Fig. 7. Study group of children born at very low gestational age (VLGA, <32 weeks).

Table 13. Clinical characteristics of the children in study IV.

Factor	VLGA (n = 77)	Term (n = 27)
Gestational age (wks+days)[1]	29 (24+1 – 31+6)	39+3 (37+2 – 41+4)
Birth weight (grams)[1]	1202 (370–2295)	3336 (2655–4040)
Male[2]	40 (52)	12 (44)
Multiple pregnancy[2]	20 (26)	4 (15)
Gestational age <28 weeks[2]	22 (29)	
Fetal growth restriction[2]	18 (23)	
Histologic chorioamnionitis[2]	27 (35)	
Administration of antenatal corticosteroids[2]	66 (86)	
Apgar score at 5 minutes <7[2,3]	28 (38)	
Surfactant therapy[2]	47 (61)	
Respiratory distress syndrome[2]	49 (64)	
Intraventricular hemorrhage (IVH), grade II-IV[2,4]	5 (7)	
Sepsis, at age > 72 hours[2]	23 (30)	
Bronchopulmonary dysplasia, grade 2–3[2]	17 (23)	
Postmenstrual age at discharge (weeks)[1]	39.4 (35.0–50.1)	

[1]Data are given as mean (minimum-maximum), [2]Data are given as frequency; n (%), [3]Data is missing for four cases, [4]Distribution of the IVH cases: grade II; n = 2, grade III; n = 3, grade IV; n = 0.
VLGA, Very low gestation age.

The 87 children who participated represented 56.5% of the eligible VLGA population. The participants and non-participants did not differ in GA, BW, gender distribution, FGR, IVH, CP or other factors tested (data not shown). Ten of the VLGA participants were further excluded from the analyses because of CP or cognitive impairment, leaving 77 VLGA children for the primary study (Figure 7). Clinical data of the study population are shown in Table 13. Of the term-born comparison population who were approached, 27 (30%) participated. The comparison group did not differ significantly from the VLGA participants in terms of gender distribution or maternal education (data not shown). The birth weights of all term-born control children were appropriate for gestation.

5.4.1 *Association of prematurity and fetal growth restriction with neurocognitive outcomes*

VLGA children scored worse in visuospatial–sensorimotor processing and attention–executive functions compared to term-born children (Table 14). The results remained significant after correction for multiple comparisons (Table 14) and after adjustment for gender and maternal education (regression coefficient [β] −1.5; 95% CI −2.3 to −0.6; P = 0.001 for visuospatial–sensorimotor processing and β −1.2; 95% CI −1.9 to −0.5; P = 0.001 for attention–executive functions). Low maternal education, VLGA and male gender explained 27% of the variance in the visuospatial–sensorimotor processing scores and VLGA and male gender explained 19% of the variance in the attention–executive functions scores.

Table 14. Mean scores of psychological test domains in VLGA children and in term-born children.

Domain	VLGA[1] (n = 77)	Term[1] (n = 27)	Mean difference (95% CI)	P[2]	P[3]	Cohen's d[4]
Visuospatial–sensorimotor processing	9.1 (1.9)	10.6 (2.5)	−1.5 (−2.4 to −0.6)	0.002	0.005	0.68
Attention–executive functions	8.8 (1.7)	10.1 (1.4)	−1.3 (−2.0 to −0.6)	<0.001	<0.001	0.83
Language	8.9 (2.5)	9.7 (2.5)	−0.7 (−1.8 to 0.4)	0.19	0.31	0.32
Memory–learning	8.8 (2.3)	8.7 (3.1)	0.1 (−1.3 to 1.4)	0.94	0.94	0.04
Social perception	9.5 (2.5)	10.2 (2.5)	−0.6 (−1.7 to 0.5)	0.25	0.31	0.28

[1]Data are given as mean (standard deviation), [2]Student's t-test, [3]Corrected for multiple comparisons (Benjamini–Hochberg). [4]Effect sizes calculated in terms of Cohen's d.
The psychological subtest scores have a normed mean of 10 and a standard deviation of 3 (range 1–19). These values can be used when evaluating mean scores of the psychological test domains.
VLGA, Very low gestational age.

The following subtest scores of visuospatial–sensorimotor processing and attention–executive functions were significantly lower in VLGA children compared to term children after adjusting for multiple testing: coding, block design, inhibition/naming and inhibition/switching. Although VLGA children scored worse in visuospatial–sensorimotor processing and attention–executive

functions, their mean scores in these domains and in the specific subtests were in the low average range.

Those with FGR, n = 18/77 (23%), had a poorer outcome in language and memory–learning scores compared to VLGA children without FGR (Table 15). These results remained significant after correction for multiple comparisons (Table 15) and after adjustment for GA, gender and maternal education (β −1.7; 95% CI −3.0 to −0.5; P = 0.006 for language and β −1.6; 95% CI −2.8 to −0.4; P = 0.008 for memory–learning). Low maternal education and FGR explained 17% of the variance of the language scores in the VLGA cohort. Furthermore, FGR alone explained 9% of the variance of the memory–learning scores. The degree of prematurity or the other clinical factors tested had no detectable effect on neurocognitive outcome in VLGA children (data not shown).

Table 15. Mean scores of psychological test domains in VLGA children with FGR and in VLGA children without FGR.

Domain	FGR[1] (n = 18)	No FGR[1] (n = 59)	Mean difference (95% CI)	P[2]	P[3]	Cohen's d[4]
Language	7.7 (2.2)	9.3 (2.4)	−1.6 (−2.8 to −0.3)	0.017	0.04	0.69
Memory–learning	7.6 (2.8)	9.2 (2.0)	−1.6 (−2.8 to −0.4)	0.009	0.04	0.66
Visuospatial–sensorimotor processing	8.9 (2.0)	9.2 (1.9)	−0.3 (−1.3 to 0.8)	0.62	0.67	0.15
Attention–executive functions	8.5 (1.8)	8.9 (1.6)	−0.4 (−1.3 to 0.5)	0.34	0.57	0.23
Social perception	9.2 (2.1)	9.6 (2.6)	−0.4 (−1.8 to 1.0)	0.67	0.67	0.17

[1]Data are given as mean (standard deviation), [2]Student's t-test, [3]Corrected for multiple comparisons (Benjamini–Hochberg). [4]Effect sizes calculated in terms of Cohen's d.

The psychological subtest scores have a normed mean of 10 and a standard deviation of 3 (range 1–19). These values can be used when evaluating mean scores of the psychological test domains.

FGR, Fetal growth restriction; VLGA, Very low gestational age.

5.4.2 *School achievement*

Teachers graded overall school achievement more often as average to poor (3–5) in VLGA children compared to term children (33% vs. 8%; P = 0.01), especially in mathematics (39% vs. 0%; P <0.001). These results remained significant after correction for multiple comparisons and after adjustment for gender and maternal

education (data not shown). Reading and writing skills did not differ between VLGA and term children (data not shown). Ten (13%) VLGA children had repeated a preschool year and six (8%) VLGA children had repeated a school year. None of the comparison group had repeated a preschool or school year. All VLGA and term children went to mainstream schools, although 3 (4%) VLGA children needed a special education class.

5.4.3 *Behavior and social interaction skills*

The results of the questionnaire that comprised items of the FTF were compared between VLGA and term children. After adjusting for multiple testing, VLGA children had significantly more attention difficulties and hypoactive symptoms, deficits in relation in space and internalizing problems than the comparison group (Table 16).

Table 16. Results of the questionnaire on children's behavior and social interaction skills.

Domain Subdomain	VLGA[1] (n = 76)	Term[1] (n = 26)	P[2]	P[3]	Cohen's d[4]
Attention–executive functions	0.38 (0.32)	0.19 (0.24)	0.005	0.03	0.67
Attention	0.52 (0.40)	0.29 (0.3)	0.01	0.03	0.65
Hyperactive/impulsive	0.29 (0.37)	0.17 (0.27)	0.14	0.18	0.37
Hypoactive	0.30 (0.33)	0.14 (0.25)	0.02	0.04	0.55
Planning	0.35 (0.37)	0.29 (0.40)	0.54	0.54	0.16
Perception					
Relation in space	0.12 (0.19)	0.05 (0.10)	0.01	0.03	0.46
Social skills	0.17 (0.23)	0.08 (0.15)	0.08	0.11	0.46
Emotional–behavioral problems	0.21 (0.22)	0.11 (0.17)	0.05	0.08	0.51
Internalizing	0.21 (0.21)	0.10 (0.18)	0.02	0.04	0.56
Externalizing	0.20 (0.27)	0.12 (0.21)	0.18	0.20	0.33

[1]Data are given as mean (standard deviation), [2]Student's t-test, [3]Corrected for multiple comparisons (Benjamini–Hochberg). [4]Effect sizes calculated in terms of Cohen's d.
VLGA, Very low gestation age.

6 Discussion

6.1 Chemokine CCL18 and its potential role in the pathogenesis of brain injury (I, III)

The present study revealed that VLGA infants developing IVH had lower levels of cord serum CCL18 than VLGA infants without IVH or with a small GM hemorrhage (I). CCL18 has not previously been associated with the risk of IVH. Another novel finding was the association between *CCL18* SNP rs2735835 and predisposition to CP among VLGA children (III).

CCL18 belongs to the CC-chemokine family and is known to be constitutively present at high levels in adult blood. Further, CCL18 levels have been found to increase in inflammatory conditions, such as in atopic dermatitis, pneumonitis, arthritis, vasculitis, and, interestingly, in CA (Schutyser *et al.* 2005). In brain biopsies from adult patients with traumatic brain injuries, elevated CCL18 expression was evident in several cell types, including microglia (Chang *et al.* 2010). The gene encoding CCL18 is only present in primates. During evolution a fusion and exon inactivation of two duplicated *CCL3*-like genes may have generated *CCL18* and adapted the new gene to critical functions (Schutyser *et al.* 2005).

CCL18 functions as an antagonistic ligand for CCR3 (Nibbs *et al.* 2000), a G-protein-coupled transmembrane receptor previously found in microvascular endothelial cells of adult brain (Berger *et al.* 1999). Both neurons and glial cells of term-born, but not preterm infants have been shown to express CCR3 (Van Der Meer *et al.* 2001). Remarkably, we found CCR3 to be detectable in the periventricular area and in the neurons of the hippocampus of preterm infants already at 23 weeks of gestation (I). Many other CC-chemokines, such as CCL5, CCL7, CCL8, CCL11, CCL13, CCL24, and CCL26, are also recognized by CCR3. These chemokines activate CCR3 mediated intracellular signaling (Elsner *et al.* 2004). Considering the antagonistic function of CCL18, it is possible that a high concentration of CCL18 may block the action of agonistic ligands on CCR3, thereby inhibiting the leukocyte degranulation and proinflammatory activation (Fujisawa *et al.* 2000) that are known to influence the endothelial permeability and, thus, the risk of bleeding. However, this putative direct action of CCL18 on CCR3 remains to be demonstrated.

CCL18 is suggested to have other anti-inflammatory properties: it has been shown to attract regulatory T cells (Chenivesse *et al.* 2012) and to reduce activation of other chemokine receptors CCR1, CCR2, CCR4, and CCR5, which are also related to inflammation, by displacing the glycosaminoglycan-bound chemokine (Krohn *et al.* 2013). Recently, CCL18 was found to be a ligand for another receptor, CCR8 (Islam *et al.* 2013). In an earlier study, CCR8 expression was associated with phagocytic macrophages and activated microglia in the human brain (Trebst *et al.* 2003). Human microglia participate in both innate and adaptive immune responses and are responsive to alternative macrophage stimuli (Melief *et al.* 2012). CCL18 has been shown to induce an alternative macrophage phenotype that associates with tissue repair, expression of IL-10 and termination of the inflammatory response (Schraufstatter *et al.* 2012). Activation of microglia serves as an interface for pathogenetic mechanisms that are thought to underlie brain injury in VLGA infants, including infection/inflammation and ischemia as well as excitotoxicity and free radical attack (Khwaja & Volpe 2008). A recent study suggested that activated microglia play an important role in mediating the influence of IVH on brain development in premature infants (Supramaniam *et al.* 2013).

The periventricular area in VLGA infants is prone to hypoxia–reperfusion associated injuries (du Plessis 2008). The expression of CCL18 is inhibited by hypoxia in immature dendritic cells (Ricciardi *et al.* 2008). Moreover, we detected CCR3 immunopositivity in the brain capillary endothelium of VLGA infants. High CCR3 expression may be a characteristic of capillary endothelium during active angiogenesis (Hillyer *et al.* 2003, Salcedo *et al.* 2001). Fragility of the GM vasculature is also a proposed risk factor of IVH. Astrocyte endfeet likely provide structural integrity to GM blood vessels. Glial fibrillary acidic protein and other support structures of GM vessels, such as collagen, may be deficient in IVH (Ballabh 2010). CCL18 has been shown to increase the synthesis of collagen in vitro (Atamas *et al.* 2003). Theoretically, CCL18 deficiency could partly attenuate the support structure of the vasculature.

During the first week after very preterm birth, the CCL18 levels increased, as the risk of new IVH events decreased. We also found that the cord blood concentrations of CCL18 were higher in term infants compared to VLGA infants. We suggest that immaturity related paucity in CCL18 expression delays the increase of CCL18 in the blood in premature infants. Low blood levels of CCL18 could also result from excessive consumption of CCL18 in inflammatory processes. Since the proposed protective effect of CCL18 is insufficient,

inflammatory injury to the BBB may increase the fragility of the GM vasculature, and thus, the risk of bleeding. The low cord blood concentration of CCL18 was associated with CP in a previous study (Kaukola *et al.* 2004), whereas the present study (II) did not show a similar association. This might be partly explained by the differences in treatment practices between the 1992–1993 and 1998–2002 cohorts. The increased use of antenatal corticosteroids and advanced intensive care may have modified the brain injury of premature infant into a less severe disease. While the rates of severe IVH and cPVL have declined (Baud *et al.* 1999, Kari *et al.* 1994), diffuse, subtle alterations in WM are common among preterm infants. Simultaneously, the spectrum of CP has changed into a less disabling condition among preterm born children (van Haastert *et al.* 2011).

In conclusion, our findings support the hypothesis that CCL18 might play a role in the complex pathways leading to brain damage. A common polymorphism in the *CCL18* gene, that was associated with predisposition to CP in the present study, might influence, together with other polymorphisms within the *CCL18* gene, the expression of CCL18 in specific brain cells. Since CCL18 is known to participate in repair and immunoregulation in microglia (Melief *et al.* 2012) by inducing an alternative macrophage phenotype (Schraufstatter *et al.* 2012), we propose that CCL18 could protect against brain injury and CP by inhibiting locally the signaling of inflammatory receptors in the periventricular cells.

6.2 Biomarkers contributing to the risk of CP (II)

Previous studies of cytokine profiles in both term and preterm children with CP support the current finding (II) that clusters of blood proteins are associated with or predispose infants to CP (Carlo *et al.* 2011b, Kaukola *et al.* 2004, Nelson *et al.* 1998). The present study focuses on CP among infants born before 32 weeks of gestation. This population accounts for 20–25% of all CP cases, although they represent only 0.9–1.5% of all live births (Himmelmann *et al.* 2005).

Using factor analysis, the cord blood cytokines were arranged into clusters that significantly explained the overall variation among the cytokines. The two most significant clusters (i.e. factor 1 and factor 2 shown in Table 9) were independently associated with the risk of CP. Most cytokines within the clusters were higher in CP than in gestational controls. These two clusters included chemokines, growth factors, TNF-family cytokines, and hematopoietin family cytokines that have a range of functional characteristics. Some of them may be involved in neural damage affecting OLs, neurons, or microglia. Others may be

associated with the repair of such injuries, while some cytokines may have either several or no known roles in damage/repair.

Cytokines that are activated during an injurious process, such as inflammation or hypoxia/ischemia, may influence the integrity of the BBB (McAdams & Juul 2012). Furthermore, astrocytes may lose their supportive function in the brain vasculature in response to hypoxia, and thus contribute to BBB breakdown (Mani *et al.* 2005). In the present study, birth asphyxia was not more common among infants who developed CP, nor was there a direct association between CP and CA. However, most of the VLGA children with CP were born after spontaneous preterm labor, which is commonly associated with antenatal infection and inflammatory cytokines (Challis *et al.* 2009). We therefore propose that several of the immunoproteins identified in this study may have a role in mediating early detrimental pathways that lead to the development of CP. The exact mechanisms that enable the immunoproteins to cross the BBB in human infants remain unknown.

In the present study, two inflammatory chemokines, CCL27 and CX3CL1, that are known to be upregulated in response to mitochondrial damage (Haines *et al.* 2010), and seven members of the TNF family, TRAIL-R1, CD30, CD27, FASL, HVEM, RANK, and TRAIL-R4, that are known to participate in apoptosis (Choi & Benveniste 2004, Hase *et al.* 2002, Matysiak *et al.* 2002, Pasero *et al.* 2009), were included in the cytokine clusters predicting CP. Immature OLs are the cells that are most prone to the detrimental effects of inflammation, hypoxia/ischemia, and oxidant damage. Cytokines participating in these processes, e.g. TNF and interferon family members have been shown to cause damage to OLs (Buntinx *et al.* 2004, Matysiak *et al.* 2002). Cytokines may further contribute to the production of toxic substances, such as nitric oxide and free oxygen radicals (McAdams & Juul 2012). Ischemia and hypoxia may lead to mitochondrial pathways of apoptosis.

We did not detect any association between cord blood proinflammatory cytokines and CP. This is consistent with a recent study (Varner *et al.* 2014). These somewhat unexpected findings may be due to early referral and active treatment of pregnant mothers with suspected antenatal infection. In addition, individual cytokines and their soluble receptors are expressed within a certain time window emphasizing the time point of the sample drawing. Further, several factors can affect serum cytokine levels including GA and perinatal morbidities.

We showed that the risk profile of inflammatory cytokines was different at birth than during the first week after birth. IL-12p70 (day 1) and IL-8 (day 7)

were higher in children with CP compared to children without CP. Consistently, high IL-8 concentration measured in neonatal blood has been associated with the development of CP in a study comprising ELBW children (Carlo *et al.* 2011b). In our study, IL-12p70 remained a significant predictor of CP after adjusting for clinical and biochemical risk factors of CP. IL-12 is a proinflammatory cytokine that induces another proinflammatory cytokine, IFN-γ, in peripheral lymphocytes. Circulating IFN-γ levels have been found to be associated with WMI in very preterm children (Hansen-Pupp *et al.* 2005). In VLGA infants, postnatal inflammation also correlates with reduced cortical growth (Kaukola *et al.* 2009).

To summarize, we identified a group of cord blood cytokines that was associated with the development of CP in children born very preterm. The inflammatory cytokine profile changed after birth, and the elevated concentration of IL-12 was associated with an increased risk of CP, both singly and additively with the protein cluster defined at birth in cord blood.

6.3 Fetal growth restriction and prematurity were associated with neurocognitive development at school-age (IV)

The present longitudinal follow-up study demonstrated that a subgroup of VLGA children, those with FGR, had specific difficulties in language as well as in memory and learning. However, they did not differ from VLGA children with appropriate fetal growth in visuospatial and sensorimotor skills, attention and executive functioning or social perception.

The most common cause of FGR is placental insufficiency. It is accompanied by lack of nutrients, harmful alterations in hemodynamics (e.g. hypoxic events) and changes in placental and fetal endocrine function, all of which can disrupt fetal brain development (Tolsa *et al.* 2004). Considering the cognitive outcomes of the present and previous studies, prematurity and FGR potentially cause distinct disturbances in brain development. In fact, Padilla *et al.* demonstrated that FGR was associated with altered brain structure and with neurodevelopmental impairments among preterm children (Padilla *et al.* 2011). Further, Morsing *et al.* found no correlation between the degree of prematurity and developmental outcome in children born very preterm, whereas cognitive abilities were particularly poor in FGR boys with absent or reversed end-diastolic umbilical artery blood flow (Morsing *et al.* 2011).

Several studies have reported that cognitive impairments increase with decreasing GA in preterm-born children (Anderson 2014, Bhutta *et al.* 2002,

Serenius *et al.* 2013). However, we detect no influence of the degree of prematurity on neurocognitive outcome in VLGA children. Our results are in agreement with those of recent studies (Morsing *et al.* 2011, Reidy *et al.* 2013). The compromised outcomes in preterm-born children may not be inevitably due to prematurity, but rather to factors that operate during the ante- or neonatal period or beyond the newborn period (Boardman *et al.* 2007, Charkaluk *et al.* 2010, Flacking *et al.* 2012, Smith *et al.* 2011). In accordance with the previous studies (Wong & Edwards 2013), we confirmed the association of maternal education with cognitive outcome.

Many recent epidemiological studies include only children born extremely preterm, among whom cognitive and neurologic impairments are common. The rates of major neurodevelopmental impairments in ELGA and VLGA cohorts are shown in table 2. The present study started from a different premise. Our aim was to evaluate specific neurocognitive abilities in VLGA children without apparent impairments.

We showed that the VLGA children free of CP or cognitive impairment had deficits in visuospatial–sensorimotor skills and in attention–executive functions compared to term-born children. However, they did not differ from term children in language, memory, learning, or social perception tasks. In addition, teachers reported no significant differences in language skills between the VLGA and term children. These findings are in agreement with other studies showing that verbal skills are less affected than performance functions in preterm-born children (Lind *et al.* 2011, Mikkola *et al.* 2005). The profile of neurocognitive impairments in the present study resembles that found in the EPICure-study; the extremely preterm children without CP and attending mainstream schools had a high prevalence of visuospatial, perceptuomotor and attention–executive problems (Marlow *et al.* 2007). In turn, very preterm children without CP or severe sensory impairment had mild delays in several areas of development at 2 years of age in the Epipage cohort (Charkaluk *et al.* 2010).

Furthermore, the VLGA children in our study had poorer school performance, especially in mathematics, compared to term children. This result is in accordance with the findings of previous studies (Anderson 2014, Johnson *et al.* 2011). Impairments in visuospatial and sensorimotor skills, attention and executive functioning as well as in working memory and processing speed may lie behind poor academic achievement (Johnson *et al.* 2011, Mulder *et al.* 2010). According to the FTF questionnaires completed by the parents, VLGA children had significantly more attention difficulties and hypoactive symptoms, deficits in

relation in space and internalizing problems compared to the term children. These dysexecutive characteristics have been observed in preterm-born children in previous studies (Aarnoudse-Moens *et al.* 2009, Johnson & Marlow 2011).

Neurogenesis and neuronal migration to form the neo-cortex of the fetus continue until the end of the second trimester. Synaptogenesis, vascularization, brain folding and myelination continue well beyond term age. These processes are still in progress after very preterm birth and thus prone to aberrations in cortical connectivity, cell death and myelination disorders (Ment *et al.* 2009). Consequently, late-developing fibers are most vulnerable to disorganization or maldevelopment (Skranes *et al.* 2007). Advanced MRI studies have revealed neuroanatomical correlates of developmental impairments related to prematurity (Ment *et al.* 2009). In our cohort of school-age VLGA children, the specific pattern of neurocognitive problems may well reflect disturbed programming in brain development.

To conclude, fetal growth restriction and prematurity place school-age children without significant neurologic or cognitive impairments at an increased risk of specific neurocognitive deficits. The present data are important for VLGA children and their parents as well as for educators in order to detect specific difficulties, plan appropriate educational support and finally make adequate educational and professional choices.

6.4 Significance of the study and future perspectives

We propose that CCL18 is involved in suppression and resolution of proinflammatory responses in the periventricular area. However, clarifying the function of CCL18 in the immature brain and the factors that regulate its expression remain a challenge. The first aim is to study the influence of the CP-predisposing polymorphism of *CCL18* on the expression levels of CCL18 in specific brain cells. Preterm infants, who have low expression of CCL18, may be a potential target group for prevention of brain injuries.

A group of cord blood cytokines, that was associated with the development of CP, included several growth factors and chemokines that have potential roles in brain development or injury mechanisms. These proteins may influence early pathways, resulting in neuronal cell death and WMI. Our further aim is to evaluate MRI images in schoolchildren born very preterm, using advanced image analysis tools, and to investigate, whether the perinatal cytokine clusters are associated with sophisticated MRI findings at school-age. Genome-wide

association studies may offer further ways to investigate causative genetic markers of brain injury and CP.

Despite improved perinatal treatment practices, both the burden of being born very preterm and the superimposed burden of fetal growth restriction are associated with deviations in the pattern of cognitive development among school-age children without apparent impairments. These children may have subtle neural abnormalities that restrict brain plasticity and normal development of complex skills. As for future perspectives, sophisticated image analysis techniques could be used to identify possible structural correlates of the specific neurocognitive or language deficits, occurring in this high-risk population.

7 Conclusions

1. Low cord blood concentration of chemokine CCL18 was an independent risk factor of IVH in VLGA infants. During the first week after very preterm birth, the concentration of CCL18 increased, as the risk of new IVH cases decreased. CCL18 is an inhibitory ligand of the CCR3 receptor that was detectable at the bleeding site.
2. A cluster of cord blood cytokines was associated with the risk of CP among VLGA children. This cluster included several growth factors and chemokines, and most of them were higher in children with CP than in children without CP. An inflammatory cytokine associating with CP, IL-12 emerged after birth.
3. A common *CCL18* polymorphism had an influence on CP susceptibility in populations of VLGA children. IVH additively predicted the risk of CP in these children.
4. Cytokine clusters and specific cytokines may influence the pathways leading to early insult in the immature brain and to subsequent CP. Genetic factors likely have an additional influence on these processes. We further propose that a developmentally regulated CCL18, confined to primates, is expressed locally in specific brain cells. CCL18 may be protective against injury by inhibiting the signaling of inflammatory receptors in the periventricular cells.
5. In the era of antenatal corticosteroids and surfactant therapies, otherwise normal VLGA children still had a specific combination of visuospatial, sensorimotor and executive deficits when they reached school age. We highlight the finding that fetal growth restriction, as an additional risk factor of this preterm population, independently predicted distinct dysfunctions in language, memory and learning.

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Original articles

I Kallankari H, Kaukola T, Ojaniemi M, Herva R, Perhomaa M, Vuolteenaho R, Kingsmore SF, Hallman M (2010) Chemokine CCL18 predicts intraventricular hemorrhage in very preterm infants. Ann Med 42: 416–25.

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III Kallankari H, Huusko JM, Kaukola T, Ojaniemi M, Mahlman M, Marttila R, Kingsmore SF, Haataja L, Lavoie PM, Synnes A, Hallman M (2014) Cerebral palsy and polymorphism of the chemokine CCL18 in very preterm children. Manuscript.

IV Kallankari H, Kaukola T, Olsén P, Ojaniemi M, Hallman M (2014) Very preterm birth and foetal growth restriction are associated with specific cognitive deficits in children attending mainstream school. Acta Paediatr. doi: 10.1111/apa.12811.

Original publications are not included in the electronic version of the dissertation.

1265. Manninen, Anna-Leena (2014) Clinical applications of radiophotoluminescence (RPL) dosimetry in evaluation of patient radiation exposure in radiology : determination of absorbed and effective dose

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