CLINICAL RESEARCH ARTICLE

Plasma cholesterol levels and brain development in preterm newborns

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BACKGROUND: To assess whether postnatal plasma cholesterol levels are associated with microstructural and macrostructural regional brain development in preterm newborns.
METHODS: Sixty preterm newborns (born 24–32 weeks gestational age) were assessed using MRI studies soon after birth and again at term-equivalent age. Blood samples were obtained within 7 days of each MRI scan to analyze for plasma cholesterol and lathosterol (a marker of endogenous cholesterol synthesis) levels. Outcomes were assessed at 3 years using the Bayley Scales of Infant Development, Third Edition.
RESULTS: Early plasma lathosterol levels were associated with increased axial and radial diffusivities and increased volume of the subcortical white matter. Early plasma cholesterol levels were associated with increased volume of the cerebellum. Early plasma lathosterol levels were associated with a 2-point decrease in motor scores at 3 years.
CONCLUSIONS: Higher early endogenous cholesterol synthesis is associated with worse microstructural measures and larger volumes in the subcortical white matter that may signify regional edema and worse motor outcomes. Higher early cholesterol is associated with improved cerebellar volumes. Further work is needed to better understand how the balance of cholesterol supply and endogenous synthesis impacts preterm brain development, especially if these may be modifiable factors to improve outcomes.

INTRODUCTION

Children born preterm are at risk for brain injury, impaired brain development, and adverse neurological and developmental outcomes. As we improve our skills at improving survival of preterm newborns, significant strides have also been made to better understand the mechanisms for injury and impaired development, and such mechanisms have been targeted in the neonatal intensive care unit with dramatic successes. For example, optimization of ventilation settings have decreased the incidence of cystic periventricular leukomalacia.[1] With increasing understanding of the range of factors that may impact preterm brain development, the role of perinatal nutrition is becoming more important. While some studies have focused on energy intake or proportions of macronutrients,[2,3] others have focused on specific nutritional factors, such as polyunsaturated fatty acids.[4]

Cholesterol, a necessary component of the plasma cell membrane, plays an important role not only in cell membrane integrity but also in cell signaling, especially in cerebellar development.[5] In the mature human body, 25% of total body cholesterol content exists in the brain, particularly within myelin. While studies in adults have looked at the relationship between cholesterol levels and brain hemorrhage outcomes[6,7] and preterm newborns have been shown to be at risk for marked elevations in cholesterol levels due to stimulation of endogenous biosynthesis,[8] there have been no studies addressing the relationship between cholesterol and neurodevelopmental outcomes after preterm

birth. This prospective cohort study of preterm newborns uses serial blood samples and brain magnetic resonance imaging (MRI) studies to address the macrostructural (regional brain volumes) and microstructural (diffusion tensor imaging metrics) associations between postnatal cholesterol levels and brain injury and development after preterm birth. Lathosterol, a precursor in the pathway of cholesterol synthesis and a biochemical marker of endogenous cholesterol synthesis, is also assessed to better understand the relationships found. The hypothesis of this exploratory study is that higher cholesterol levels in the early postnatal period are associated with decreased risk of brain injury and improved development of the white matter and cerebellum in preterm newborns.

METHODS

Study subjects

Preterm infants born at ≤32 weeks gestational age and admitted to the neonatal intensive care units at the University of California San Francisco Benioff Children's Hospital and the British Columbia Children's and Women's Hospital between March 2010 and November 2011 were approached for this prospective cohort study, with 60 subjects enrolled. Consent was obtained following a protocol approved by the research ethics boards at both institutions. Infants were excluded if there was a congenital malformation or syndrome, congenital infection, or they were too

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Plasma cholesterol levels and brain development in preterm newborns
D Kamino et al.

300

clinically unstable to transport to the MRI scanner within the study timeframe. Clinical history was collected prospectively from patient charts. Feeding protocols in these infants are as previously described, including parenteral nutrition and intravenous fat emulsion as needed, followed by enteral feeds of expressed breast milk or formula, sometimes supplemented for protein.[4]

MRI studies
MRI studies were performed soon after birth as soon as clinically stable and at term-equivalent age on 1.5-T MRI scanners (General Electric Sigma, GE Medical Systems, Milwaukee, WI or Siemens Avanto, Siemens Medical Solutions, Malvern, PA). Sequences obtained include volumetric three-dimensional T1-weighted, T2-weighted, and diffusion tensor imaging (DTI) sequences as previously described.[4] All imaging analysis was performed blinded to patient clinical history. Scans were visually graded for brain injury, including intraventricular hemorrhage (IVH),[9] white matter injury (WMI),[10] and cerebellar hemorrhage,[11] by a single neuroradiologist at each study site (A.J.B., Kenneth J. Poskitt). The highest injury score from both scans was selected as the injury severity score for each infant.

Prespecified regions of interest were measured on DTI sequences for apparent diffusion coefficient (ADC), fractional anisotropy (FA), axial diffusivity (AD, first eigenvector), and radial diffusivity (RD, average of second and third eigenvector). Regions of interest included subcortical white matter, posterior limb of the internal capsule, optic radiations, and deep gray matter (including basal ganglia and thalamus) (Fig. 1). Data were inadequate for assessment of cerebellar and brainstem regions of interest. Measurements of left and right hemispheres were averaged for each scan.

Brain tissue volumes were automatically segmented into tissue classes (cortical gray matter, white matter, deep gray matter, cerebellum, and brainstem) as previously described, with Dice Similar Coefficients of 0.883 across tissue classes.[12,13]

Blood samples
Two blood samples were drawn from each subject, obtained as close in time as possible within a week of each MRI scan, timed with clinically indicated bloodwork. Samples were separated into plasma and red blood cell fractions and stored at −70 °C until analysis. Cholesterol and lathosterol (a marker of de novo cholesterol synthesis[14]) levels in plasma were analyzed by gas chromatography–mass spectrometry.
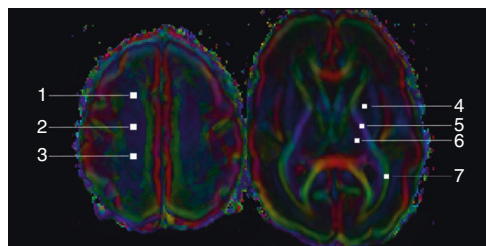
Fig. 1 Diffusion tensor imaging and regions of interest. The anisotropy color maps of an infant born at 26–6/7 weeks gestational age and scanned at 29–3/7 weeks postmenstrual age are presented in two axial planes to demonstrate the regions of interest. Regions of interest in the (1) anterior, (2) central, and (3) posterior cortical white matter were averaged for analysis of cortical white matter. Regions of interest in the (4) basal ganglia and (6) thalamus were averaged for analysis of deep gray matter. Other regions of interest included the (5) posterior limb of the internal capsul and the (7) optic radiations. Colors display the predominant diffusion directions, with red representing right–left, green representing anterior–posterior, and blue presenting superior–inferior directions

Neurodevelopmental outcomes
Infants were reassessed between 30 and 36 months corrected age by a developmental psychologist or physiotherapist blinded to the child's perinatal course. Standardized assessment was performed using the Bayley Scales of Infant Development, Third Edition, and composite cognitive, language, and motor scores were assigned based on the child's corrected age.

Statistical analysis
Statistical analysis was performed using R version 3.3.[15] All analyses were adjusted for study site to account for variations between sites. Ordinal logistic regression analysis was used to study the association between cholesterol or lathosterol levels and measures of brain injury (including IVH, WMI, and cerebellar hemorrhage). These analyses adjusted for known confounders for brain injury including gestational age at birth, chorioamnionitis (suspected or confirmed), antenatal glucocorticoid exposure, 5-min Apgar score, patent ductus arteriosus (diagnosed by echocardiogram), hypotension requiring medical intervention, intubation, and neonatal sepsis (presumed or confirmed, including necrotizing enterocolitis).

Early plasma levels were compared to neuroimaging measures on both scans. Linear mixed effects analysis was used to study the association between early cholesterol or lathosterol levels and DTI regions of interest, to account for repeated scans in each subject. Linear mixed effects analysis was used to study the association between early cholesterol or lathosterol levels and brain volumes, which were cube-root transformed for normality. Term-equivalent age plasma levels were compared to neuroimaging measures on the term-equivalent age scan only, as it would not be relevant to study the relationship between MRI scans done earlier than the plasma levels. For these analyses, simple linear regression models for DTI and non-linear regression models for brain volumes were used. All analyses were adjusted for postmenstrual age (PMA) at MRI, sex, IVH, WMI, days intubated (log-transformed), sepsis, and patent ductus arteriosus.

Plasma cholesterol and lathosterol levels were also compared to Bayley cognitive, language, and motor scores at 30–36 months using linear regression analyses.

Associations were considered significant at $P \leq 0.050$.

RESULTS
A total of 60 preterm newborns were enrolled from the two study centers (37 males), demographics described in Supplemental Table S1, as previously reported.[4] Plasma cholesterol and lathosterol levels, as well as brain MRIs were successfully completed early in 59/60 (98%) subjects (mean 31.44 ± 2.15 weeks PMA), while near-term MRI and blood sampling were completed in 43/60 (72%) of subjects (mean 37.68 ± 3.01 weeks PMA). Mean early plasma cholesterol levels were 2.50 ± 0.76 mmol/L (range 1.3–6.1 mmol/L) and lathosterol levels 5.85 ± 2.96 µmol/L (range 1.5–15.5µmol/L). Mean near-term plasma cholesterol levels were 2.34 ± 0.78 mmol/L (range 1.2–5.1 mmol/L) and lathosterol levels 3.71 ± 1.53 µmol/L (range 1.2–8.5 µmol/L).

Brain injury
Ordinal logistic regression analysis adjusting for confounding factors found no associations between early or near-term cholesterol and lathosterol levels and IVH, WMI, or cerebellar hemorrhage (Table 1).

Diffusion tensor imaging
Mixed effects models adjusting for confounders, including study site, demonstrated that early plasma lathosterol levels were associated with increased AD (0.02, 95% confidence interval (CI) 0.002–0.047, P = 0.035) and RD (0.012, 95% CI 0.00004–0.02, P = 0.049) in the subcortical white matter on both MRI scans but in no

Plasma cholesterol levels and brain development in preterm newborns
D Kamino et al.

301

Table 1. Association between plasma cholesterol and lathosterol levels and brain injury, including IVH, WMI, and cerebellar hemorrhage

	IVH		WMI		Cerebellar hemorrhage	
	OR[b]	P value[a]	OR[b]	P value[a]	OR[b]	P value[a]
Early cholesterol (mmol/L)	1.12	0.78	1.77	0.14	1.95	0.49
Early lathosterol (µmol/L)	1.10	0.31	0.89	0.32	1.02	0.94
Near-term cholesterol (mmol/L)	0.25	0.068	1.21	0.67	0.15	0.31
Near-term lathosterol (µmol/L)	0.73	0.42	0.95	0.84	4.09	0.29

IVH intraventricular hemorrhage, OR odds ratio, WMI white matter injury
[a]Ordinal logistic regression analyses adjusted for gestational age at birth, chorioamnionitis, antenatal glucocorticoid exposure, 5-min Apgar score, patent ductus arteriosus, hypotension, intubation, and sepsis
[b]ORs are per 1-point increase in injury score

Table 2. Association between early plasma cholesterol and lathosterol levels and diffusion tensor metrics

		Cholesterol (mmol/L)			Lathosterol (µmol/L)		
		Coefficient	95% CI	P value	Coefficient	95% CI	P value
Cortical white matter	ADC	0.027	−0.050 to 0.103	0.49	0.014	−0.0008 to 0.005	0.06
	FA	0.011	−0.011 to 0.033	0.35	0.0004	−0.004 to 0.006	0.88
	AD	0.090	−0.019 to 0.198	0.11	0.024	0.002 to 0.047	0.035*
	RD	0.018	−0.045 to 0.082	0.57	0.012	0.00004 to 0.024	0.049*
PLIC	ADC	−0.027	−0.081 to 0.028	0.33	0.003	−0.009 to 0.015	0.66
	FA	0.008	−0.026 to 0.043	0.64	−0.005	−0.012 to 0.003	0.20
	AD	−0.022	−0.094 to 0.049	0.54	−0.0003	−0.016 to 0.016	0.97
	RD	−0.030	−0.088 to 0.029	0.32	0.004	−0.009 to 0.018	0.50
OR	ADC	0.006	−0.078 to 0.090	0.88	0.005	−0.014 to 0.024	0.60
	FA	−0.014	−0.069 to 0.042	0.63	−0.004	−0.016 to 0.008	0.48
	AD	−0.003	−0.136 to 0.131	0.97	0.002	−0.028 to 0.031	0.92
	RD	0.020	−0.077 to 0.116	0.69	0.007	−0.014 to 0.028	0.51
Deep gray matter	ADC	−0.007	−0.060 to 0.046	0.80	0.007	−0.004 to 0.019	0.23
	FA	0.010	−0.015 to 0.034	0.43	−0.003	−0.008 to 0.003	0.33
	AD	0.001	−0.081 to 0.061	0.78	0.001	−0.014 to 0.016	0.87
	RD	NA	NA	NA	NA	NA	NA

Analyses adjusted for postmenstrual age at MRI, sex, IVH, WMI, days intubated, sepsis, and patent ductus arteriosus
AD axial diffusivity, ADC apparent diffusion coefficient, FA fractional anisotropy, IVH intraventricular hemorrhage, OR optic radiations, PLIC posterior limb of internal capsule, RD radial diffusivity, WMI white matter injury, NA not available.
*$P \leq 0.050$

other brain regions measured. Early plasma cholesterol and lathosterol associations with DTI measures are summarized in Table 2. Linear regression analyses showed no associations between near-term cholesterol or lathosterol levels and DTI measures in the regions assessed ($P > 0.1$, data not shown).

Brain volumes
Mixed effects models adjusting for confounders demonstrated that, per 1 mmol/L increase in early plasma cholesterol levels, there is an associated 1.11 cm^3 increase in cerebellar volumes on both MRI scans (95% CI 0.37–1.86 cm^3, $P = 0.009$). Meanwhile, per 1 mmol/L increase in early plasma lathosterol levels, there is an associated 1.98 cm^3 increase in subcortical white matter volume on both MRI scans (95% CI 0.53–3.46 cm^3, $P = 0.018$). As well, per 1 mmol/L increase in near-term plasma cholesterol levels, there is an associated 8.00 cm^3 decrease in near-term cortical gray matter volumes (95% CI 0.52–15.48 cm^3, $P = 0.048$). Associations between plasma levels and brain volumes are summarized in Table 3.

Neurodevelopmental outcome
Mean scores on neurodevelopmental testing were 104 ± 14 on cognitive testing (range 80–145), 101. ± 18 on language testing (range 65–138), and 98 ± 14 on motor testing (range 64–124). Using linear regression analysis, each 1-µmol/L increase in early plasma lathosterol levels was associated with a 2.0-point decrease in Bayley motor scores (95% CI 0.3–3.6, $P = 0.018$), but other plasma levels were not associated with outcomes (Table 4).

DISCUSSION
Although cholesterol plays an important role in cell signaling through neurodevelopment and is a key component of myelin, there has been little research into its impact on preterm brain injury, growth, and neurodevelopment. This current study demonstrates associations between cholesterol and lathosterol levels and brain development in the subcortical white matter, cortical gray matter, and cerebellum, as well as associations with motor outcomes at 3 years corrected age.

Plasma cholesterol levels and brain development in preterm newborns
D Kamino et al.

302

Table 3. Association between early and near-term plasma cholesterol and lathosterol levels and regional brain volumes

Brain region (cm^3)	Mean volume at 40 weeks (cm^3)	Plasma sample	Cholesterol (mmol/L)			Lathosterol (µmol/L)		
			Coefficient[c]	95% CI	P value	Coefficient[d]	95% CI	P value
Cortical gray matter	145.42 ± 5.71	Early[a]	2.01	−2.80 to 6.84	0.46	0.34	−0.71 to 1.41	0.57
		Term[b]	−8.00	−15.48 to −0.52	0.048*	−0.39	−5.99 to 5.20	0.89
Cortical white matter	161.62 ± 7.25	Early[a]	6.54	−0.15 to 13.41	0.09	1.98	0.53 to 3.46	0.018*
		Term[b]	2.08	−8.12 to 12.28	0.69	0.68	−6.66 to 8.03	0.86
Deep gray matter	21.41 ± 0.73	Early[a]	0.76	−0.07 to 1.59	0.10	0.10	−0.08 to 0.29	0.32
		Term[b]	−0.50	−1.62 to 0.61	0.39	0.12	−0.65 to 0.89	0.77
Cerebellum	21.45 ± 0.89	Early[a]	1.11	0.37 to 1.86	0.009*	0.13	−0.05 to 0.30	0.19
		Term[b]	−0.45	−1.70 to 0.80	0.49	−0.52	−1.36 to 0.31	0.23
Brainstem	6.10 ± 0.21	Early[a]	0.23	−0.03 to 0.49	0.12	0.05	−0.01 to 0.11	0.12
		Term[b]	−0.17	−0.51 to 0.16	0.32	−0.13	−0.36 to 0.10	0.27

All analyses adjusted for postmenstrual age at MRI, sex, IVH, WMI, days intubated, sepsis, and patent ductus arteriosus. Mean volumes at 40 weeks are calculated for the absence of IVH, WMI, intubation, sepsis, or patent ductus arteriosus
IVH intraventricular hemorrhage, *WMI* white matter injury
[a]Early plasma levels are compared with both MRI scans using mixed effects models
[b]Near-term plasma levels are compared with near-term MRI only using linear regression models
[c]Units of coefficients are cm^3 per mmol/L cholesterol
[d]Units of coefficients are cm^3 per µmol/L lathosterol
*$P \leq 0.050$

Considering that 25% of the total body cholesterol is found in the brain, concentrated in the myelin, it would be expected that variations in cholesterol supply would have consequences on the developing subcortical white matter. Indeed, preterm newborns have been found to be at risk for marked elevations in plasma cholesterol levels due to upregulation of endogenous cholesterol synthesis.[8] Here we show that higher early plasma lathosterol levels are associated with higher AD and RD, without significant change in ADC or FA, and higher regional volumes in the subcortical white matter. Interestingly, other regions of interest in the white matter, including the posterior limb of the internal capsule and the optic radiations, do not seem to be involved. It has been demonstrated that, with increasing gestational age, FA increases while ADC, AD, and RD decrease.[16] The constellation of increasing AD and RD and increasing volume could suggest regional tissue swelling and impaired or delayed development of the cortical white matter. This theory is further supported by the corresponding worsened Bayley motor scores at 3 years with higher early lathosterol. Since lathosterol is a marker of endogenous cholesterol synthesis, this may suggest that this may represent an increased endogenous production either in response to inadequate cholesterol supplies or an endogenous drive for higher cholesterol levels. Since higher early plasma cholesterol levels were not found to be associated with worse DTI metrics, this suggests that inadequate cholesterol supply may be more likely. This fits with what has previously been described in preterm newborns.[8] With standardized feeding protocols postnatally, cholesterol supply becomes more standard, which may explain the lack of associations found with later lathosterol levels.

The most statistically significant finding in this study was an association between early plasma cholesterol levels and increased cerebellar volumes. Unfortunately, the DTI measures could not be accurately assessed in the posterior fossa using the sequences obtained for this study,[17] thus only volumetric data are available for the cerebellum and brainstem. Cholesterol is known to play a critical role in upregulating Sonic hedgehog signaling,[5,18–21] which in turn is a key pathway supporting the development of the cerebellum.[22] Cholesterol covalently modifies hedgehog proteins and is a necessary posttranslational modification to initiate the function of Sonic hedgehog protein.[5] Inhibition of cholesterol synthesis has been shown to result in abnormalities in cerebellar development.[23–25] Thus there is mechanistic evidence linking cholesterol and cerebellar development to support our observed association.

We have previously reported from this cohort that larger cortical and deep gray matter, cerebellar, and brainstem volumes were associated with improved language scores at 30–36 months corrected age. Also, larger cerebellar and brainstem volumes were associated with improved motor scores.[12] Although we did not find that cholesterol levels were associated with improved language or motor scores, an association between cholesterol levels and cerebellar volumes suggests that these larger volumes may also be contributing to improved outcomes.

The association of higher endogenous synthesis with worse subcortical white matter development and that of higher overall cholesterol levels with better cerebellar development seems superficially contradictory. However, one could postulate that higher endogenous synthesis can be a response to lower supply, creating a need to synthesize more endogenously to meet demand. This endogenous synthesis may be adequate to normalize plasma levels, while remaining inadequate to maintain subcortical white matter development. Thus, in such a way, higher cholesterol levels and lower need to synthesize endogenously are both associated with improved subcortical white matter and cerebellar development.

Another aspect of the relationship between cholesterol and preterm brain development is the issue of the blood–brain barrier. The fetus has been shown to receive cholesterol supply via the placenta.[26] However, there is evidence in sheep that cholesterol carried in low-density lipoproteins are not incorporated into the brain.[27] In the adult brain, it is known that the blood–brain barrier prevents transport of cholesterol,[28] but the exact timing of the closure of the blood–brain barrier to cholesterol is not known in humans. The associations found in this study suggest that there may be either some level of transfer of cholesterol into the brain or at least signaling effects that alter brain development. Indeed, it is unknown what factors result in variability in cholesterol and lathosterol levels in preterm newborns, and these factors may also be important in driving these observed associations.

Finally, we also found in this study that higher term cholesterol levels were associated with decreased cortical gray matter volumes at term age. The biochemical basis of this association is

Plasma cholesterol levels and brain development in preterm newborns
D Kamino et al.

303

Table 4. Association between plasma cholesterol and lathosterol levels and Bayley Scales of Infant Development, Third Edition scores at 30–36 months corrected age

	Motor score		Cognitive score		Language score	
	Coefficient[a]	P value	Coefficient[a]	P value	Coefficient[a]	P value
Early cholesterol (mmol/L)	2.06	0.52	−1.71	0.65	0.63	0.89
Early lathosterol (μmol/L)	−1.98	0.02*	−0.40	0.63	−1.29	0.20
Near-term cholesterol (mmol/L)	5.70	0.06	2.36	0.86	5.40	0.14
Near-term lathosterol (μmol/L)	−1.41	0.35	1.34	0.33	−2.86	0.11

[a]Units of coefficients are points per mmol/L cholesterol or μmol/L lathosterol
*$P \leq 0.050$

unclear, as there is no mechanistic link previously demonstrated between cholesterol and this brain region. It is reassuring, however, that term cholesterol levels were not associated with neurodevelopmental outcomes. However, cognitive impacts may be more evident on longer developmental follow-up.

This study is limited by its exploratory nature and small sample size. However, consistent findings associating early lathosterol levels and macrostructural and microstructural measures of brain development and cholesterol's key role in myelin formation suggest that a true relationship exists between early lathosterol levels and subcortical white matter development. Also, strong mechanistic ties between cholesterol and cerebellar development support the plausibility of these reported associations.

It has been well established that cholesterol is a key component of myelin and that cholesterol is necessary for the Sonic hedgehog pathway, which is key to cerebellar development. However, there has been very little research into the implications of cholesterol exposure antenatally and postnatally on brain injury and development in the preterm newborn. This study brings forth new questions regarding the relationship between endogenous cholesterol synthesis and impaired development of the subcortical white matter, as well as cholesterol levels and cerebellar growth. Indeed more work is needed to understand the relationship between plasma and brain cholesterol levels. This study sheds light on an important and poorly understood topic, suggesting that more research is needed to better understand the complex role of cholesterol supply and endogenous synthesis on preterm neurodevelopment.

AUTHOR CONTRIBUTIONS
All authors made substantial contributions to the conception and design of the study, acquisition of data, or analysis and interpretation of data, drafted the article or revised it critically for important intellectual content, and approved the final version of the article to be published.

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Plasma cholesterol levels and brain development in preterm newborns
D Kamino et al.

304

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