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Fetal Growth Influences Lymphocyte Subset Counts at Birth: The Generation R Study

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Key Words
Birth weight · Cohort study · Cord blood · Fetal growth · Gestational age · Lymphocyte subsets

Abstract
Background: Preterm born and low-birth-weight infants are at risk for severe infections in infancy. It has been suggested that these infants have an immature immune system. ***Objective:*** To assess the associations of gestational age, birth weight and fetal growth with absolute lymphocyte subset counts at birth. ***Methods:*** This study was conducted in 571 infants participating in the Generation R Study, a population-based prospective cohort study from fetal life onwards. Gestational age and birth weight were obtained from midwives and hospital registries. Fetal growth was defined as increase in weight between late pregnancy and birth. Lymphocytes and T lymphocyte subset counts in cord blood were determined by 6-color flow cytometry. Multivariate linear regression models with adjustment for gender, maternal education, smoking, alcohol use, fever and mode of delivery were applied. ***Results:*** Per week increase of gestational age, T, B and NK lymphocyte counts increased with 3, 5 and 6%, respectively ($p < 0.05$). Helper, cytotoxic and naive T lymphocyte counts increased with 3, 4 and 5%, respectively ($p < 0.05$), but memory T lymphocyte counts did not. Increased birth weight and fetal growth were significantly associated with higher B lymphocyte counts, independent of gestational age, but not with the other lymphocyte subset counts. ***Conclusions:*** Lymphocyte subset counts increase with prolonged gestation, suggesting an ongoing development of the immune system. Birth weight and fetal growth seem to influence only B lymphocyte counts.

Introduction

Preterm born or low-birth-weight infants are at risk for severe infections in infancy [1, 2]. It has been suggested that these infants have an immature immune system. Decreased percentages or absolute counts of lymphocytes are described in infected preterm infants [3].

Previous studies, mostly small sample sized, presented lower percentage and absolute numbers of T lymphocytes and T lymphocyte subsets at an earlier gestational age, yet results on NK and B lymphocyte counts are inconclusive [4–11]. A small number of studies on birth weight and the

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development of the immune system have been performed and showed lower absolute lymphocyte subset counts in low-birth-weight infants [9, 12, 13]. However, most of these studies did not take gestational age into account. Birth weight is only a proxy for fetal growth since several fetal growth and development patterns may lead to the same birth weight. Fetal growth restriction may lead to normal birth weight if the fetus is actually supposed to grow on the upper percentiles based on the genetic growth potential. Fetal growth restriction with impaired fetal thymus and liver growth has been suggested to influence the development of the immune system [14–16]. Therefore, longitudinally measured fetal growth may be stronger related to lymphocyte counts than birth weight per se.

We examined in the Generation R Focus Study, a population-based prospective cohort study from fetal life onwards, the associations of gestational age, birth weight and longitudinally measured fetal growth with absolute numbers of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naive and memory) at birth.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, The Netherlands. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development and health and has been described previously in detail [17, 18]. Additional, more detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study [17, 18]. This subgroup is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity. Of all approached pregnant women and their partners, 79% participated in the Generation R Focus Study. Their children were born between February 2003 and August 2005. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Gestational Age, Birth Weight and Fetal Growth

Information about date of birth and birth weight was obtained from standardized midwife and hospital registries. Gestational age at birth was based on pregnancy dating by ultrasound in early pregnancy [19]. Estimated fetal weight was determined in late pregnancy (>25 weeks) using the formula of Hadlock with the parameters abdominal circumference, head circumference and femur length measured by ultrasound [20]. Fetal weight measurements were converted into gestational age-adjusted standard deviation scores (SDS) [21]. Fetal growth was defined as fetal weight gain (SDS) between late pregnancy and birth. For the latter analysis, gestational age-adjusted SDS were also constructed for birth weight measurements.

Immunophenotyping of Lymphocyte Subsets

Heparinized sampling of venous cord blood was carried out by midwives and obstetricians immediately after delivery and transported to the Immunology laboratory of the Erasmus Medical Center within 24 h. Umbilical cord blood samples not received within 24 h (weekend days) were excluded since flow cytometric analyses of those samples showed no reliable results in the pilot phase of the study. Flow cytometric immunophenotyping was performed to determine absolute numbers of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naive and memory T). For this, the monoclonal antibodies CD3, CD19, CD16, CD56, CD4, CD8, CD45RA and CD45RO were conjugated with the labels fluorescein isothiocyanate (FITC; Becton Dickinson, San Jose, Calif., USA), peridin chlorophyll protein (PerCP; Becton Dickinson), peridin chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5; Becton Dickinson), allophycocyanin (APC; Becton Dickinson), phycoerythrin (PE; Becton Dickinson and Dako A/S), phycoerythrin-cyanine dye (PE-cy7; Becton Dickinson), allophycocyanin-cyanine dye (APC-cy7; Becton Dickinson), and rhodamine (RD1; Beckman Coulter, Inc.). Absolute numbers of T, B and NK lymphocytes were determined with the lysed whole blood technique [22]. Subsets of the T lymphocytes were determined by 6-color flow cytometry. Therefore, erythrocytes of 1 ml whole blood were lysed using 50 ml ammonium chloride. After centrifugation, leukocytes were washed twice and suspended in 900 μl PBS/1% BSA/0.1% NaAz. Of this cell suspension ($5–10 \cdot 10^6$/ml), 50 μl was incubated for 10 min at room temperature with combinations of the optimally titrated labeled monoclonal antibodies. After incubation the cells were washed and subsequently identified by flow cytometry. Using a BD™ LSR II flow cytometer (Becton Dickinson) that had been calibrated with rainbow beads, 10,000 lymphocytes were measured. The relative count of helper T lymphocytes, cytotoxic T lymphocytes, naive T lymphocytes and memory T lymphocytes was expressed as the percentage within the total T lymphocyte population and calculated by the average of 2–4 independent incubations for each subset. The absolute counts of the T lymphocyte subsets were subsequently calculated from the absolute T lymphocyte counts as obtained by the lysed whole blood technique.

Covariates

Information about maternal education, smoking and alcohol use was obtained by postal questionnaires in early, mid and late pregnancy. Educational level was defined as highest followed education (lower or higher education) according to the classification of Statistics Netherlands [23]. Maternal smoking and alcohol use during pregnancy were categorized into 'not during pregnancy' and 'during pregnancy'. Information about maternal fever (>38°C), indicating a possible underlying infection, was recorded in late pregnancy. Mode of delivery was registered by midwives and obstetricians.

Statistical Analysis

Differences of maternal and infant characteristics between infants with and without umbilical cord blood samples were assessed by the independent sample t test for continuous normal

Table 1. Maternal and infant characteristics of the study population (n = 571)

	Infants	
	boys (n = 301)	girls (n = 270)
Mother		
Maternal age, years	31.5 (4.1)	31.9 (4.0)
Education, %		
Lower	39.6	39.0
Higher	60.4	61.0
Smoking during pregnancy, %		
No	87.4	88.5
Yes	12.6	11.5
Alcohol use during pregnancy, %		
No	29.2	39.6
Yes	70.8	60.4
Fever, %	7.2	4.1
Mode of delivery, %		
Vaginal	72.1	73.3
Forceps or vacuum-assisted	17.3	12.8
Cesarean section	10.6	14.0
Infant		
Gestational age, weeks[a]	40.4 (34.6–43.3)	40.4 (34.1–43.0)
Birth weight, g	3,613 (494)*	3,507 (472)*
Late pregnancy estimated fetal weight, g	1,638 (271)	1,613 (263)
Lymphocyte subsets, $\times 10^9$/l[a]		
Total lymphocytes	4.40 (1.15–10.91)	4.54 (0.96–12.40)
CD3+ T lymphocytes	2.80 (0.86–7.15)	2.80 (0.69–5.79)
CD19+ B lymphocytes	0.68 (0.01–2.40)	0.72 (0.0.7–2.85)
CD16–/CD56+ NK lymphocytes	0.85 (0.03–2.53)	0.89 (0.10–2.50)
CD3+/CD4+ helper T lymphocytes	1.92 (1.03–3.38)	2.05 (1.01–3.47)
CD3+/CD8+ cytotoxic T lymphocytes	0.76 (0.33–1.45)*	0.65 (0.29–1.22)*
CD3+/CD45RA+ naive T lymphocytes	2.21 (0.93–1.98)	2.19 (0.99–4.07)
CD3+/CD45RO+ memory T lymphocytes	0.18 (0–1.12)	0.21 (0–0.95)

Values are means (SD) or percentages. [a] Median (range). Data were missing on education (n = 35), maternal fever (n = 44), mode of delivery (n = 30), estimated fetal weight (n = 10), total lymphocytes (n = 2), T lymphocytes (n = 1), B lymphocytes (n = 2), CD4+ helper T lymphocytes (n = 3), CD8+ cytotoxic T lymphocytes (n = 3), CD45RA+ naive T lymphocytes (n = 3) and CD45RO+ memory T lymphocytes (n = 5).

Differences were tested using the independent sample t test, non-parametric Mann-Whitney test or χ^2 test.

* $p < 0.01$.

distributed variables, non-parametric Mann-Whitney test for continuous non-normal distributed variables and the χ^2 test for categorical variables.

Data on all lymphocyte subsets were log-transformed to obtain normal distributed variables. Associations of continuously measured gestational age, birth weight and fetal growth in late pregnancy with absolute numbers of lymphocyte and T lymphocyte subsets were analyzed using linear regression models. Additionally, these models were adjusted for the potential confounders including gender, maternal education, smoking, alcohol use, fever and mode of delivery. Measures of associations are presented as log-transformed regression coefficients, interpreted as percentages after multiplying by 100 [24], or as back-transformed regression coefficients, with their 95% confidence interval (CI).

The statistical analyses were performed using the Statistical Package of Social Sciences Version 11.0 for Windows (SPSS, Inc., Chicago, Ill., USA).

Results

Cohort

In total, 1,232 women were enrolled in the Generation R Focus Study. Mothers with weekend deliveries (n = 202), twin pregnancies (n = 24) and pregnancies leading to perinatal death (n = 2) were excluded from the present

Table 2. Associations of gestational age, birth weight and fetal growth with absolute numbers of lymphocyte subsets at birth

	Lymphocyte subsets			T lymphocyte subsets			
	T	B	NK	helper	cytotoxic	naive	Memory
Gestational age, weeks	3 (1, 6)**	5 (2, 9)**	6 (1, 10)*	3 (1, 5)**	4 (1, 7)**	5 (2, 8)**	–2 (–9, 4)
Birth weight, kg	5 (–2, 12)	13 (2, 25)*	–5 (–19, 9)	4 (–4, 11)	8 (–2, 17)	2 (–7, 11)	14 (–8, 35)
Fetal growth (SDS)	1 (–20, 4)	6 (1, 11)*	–3 (–9, 3)	1 (–2, 4)	2 (–3, 6)	1 (–3, 5)	3 (–6, 13)

Values are log-transformed regression coefficients (95% CI) and reflect the increase of lymphocyte subsets (%) per week increase in gestational age, per kilogram increase in birth weight and per SDS increase in fetal growth between late pregnancy and birth.
* $p < 0.05$; ** $p = 0.01$.

analysis. Of the remaining 1,004 singleton live births, cord blood was collected in 889 (88.5%) infants. Immunophenotyping of lymphocytes in cord blood was not possible in 318 infants, mainly due to non-heparinized cord blood samples. Of the remaining 571 infants, 10.0% (n = 57) were siblings since mothers were allowed to participate with second or more pregnancies in the defined study period of the Generation R Focus Study. These infants were included in the present study since there were no differences in results after excluding them from the analyses.

Analyses of missing cord blood samples (n = 433) showed that infants without cord blood samples had a shorter median gestational age (40.1 and 40.4 weeks, $p < 0.01$), a lower mean birth weight (3,454 and 3,562 g, $p < 0.01$) and more often were born by cesarean delivery (20.3 and 12.2%, $p < 0.05$) than infants with cord blood samples.

Subject Characteristics

Characteristics of the mothers and their infants are presented in table 1. Of the study group, 52.7% (n = 301) were boys. The age of mothers at enrolment ranged from 18.5 to 42.9 years (mean 31.7). In general, 60.6% of the mothers had a higher education, 12.1% smoked and 65.8% consumed alcohol throughout pregnancy. Of the mothers, 72.6% had a vaginal, 15.2% a vacuum or forceps-assisted and 12.2% a cesarean section delivery. Median gestational age of the infants was 40.4 weeks (range 34.1–43.4) and was similar in boys and girls. Mean birth weight of the infants was 3,563 g (SD 487 g) and was lower in girls than boys. The medians of the lymphocyte subset counts for boys and girls are demonstrated in table 1.

Gestational Age, Birth Weight, Fetal Growth and Lymphocyte Subsets

Table 2 presents the unadjusted associations of gestational age, birth weight and fetal growth with absolute numbers of lymphocyte subsets at birth. Per week longer gestation, infants had a 3% increase of T lymphocyte counts (p = 0.002), 5% increase of B lymphocyte counts (p = 0.003) and 6% increase of NK lymphocyte counts (p = 0.005). Of the T lymphocyte subsets, per week longer gestation an increase of 3, 4 and 5% was observed for helper, cytotoxic and naive T lymphocyte counts, respectively (all $p < 0.01$). Gestational age was not associated with memory T lymphocyte counts (p = 0.49). Per kilogram increase in birth weight, adjusted for gestational age, infants had 13% higher B lymphocyte counts (p = 0.01). An increase of 6% of B lymphocyte counts was found per SDS increase in fetal growth between late pregnancy and birth (p = 0.02). Total T and NK lymphocyte counts and T lymphocyte subset counts were not significantly associated with birth weight or fetal growth.

After adjusting for gender, maternal education, smoking, alcohol use, fever and mode of delivery the effect estimates of gestational age, birth weight and fetal growth on absolute T, B, NK and T lymphocyte subset counts did not materially change. The adjusted associations are graphically presented in figures 1–3.

Discussion

This prospective cohort study showed that with increasing gestational age at birth absolute numbers of T, helper, cytotoxic and naive T lymphocytes, B and NK lymphocytes increase. Increased birth weight and fetal growth in late pregnancy were associated with higher ab-

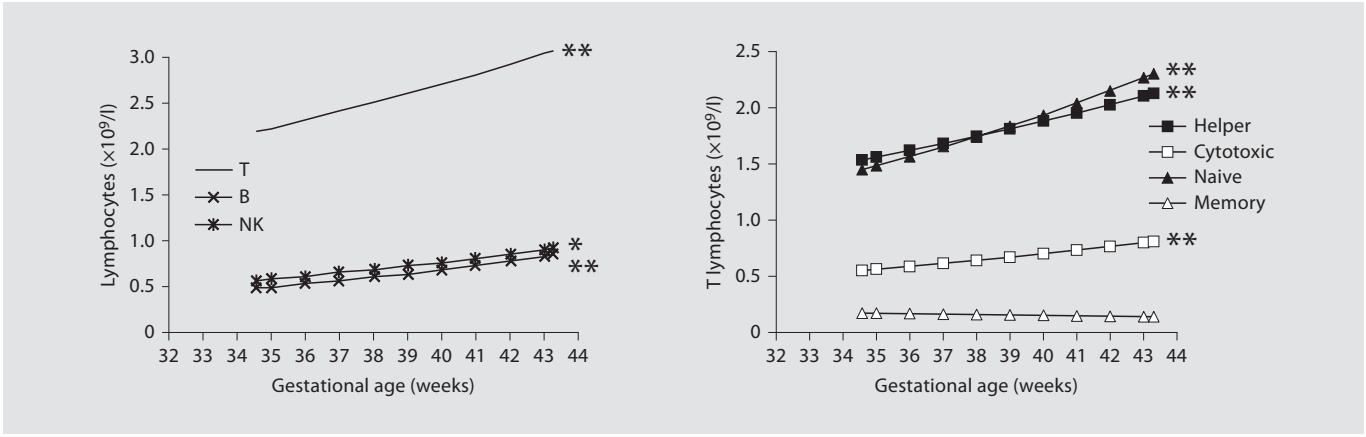

Fig. 1. Absolute numbers of lymphocyte subsets in infants of different gestational ages. The estimated back-transformed regression lines reflect the number of lymphocyte subsets per week increase of gestational age. All models were adjusted for gender, maternal education, smoking, alcohol use, fever and mode of delivery. * $p < 0.05$; ** $p = 0.01$.

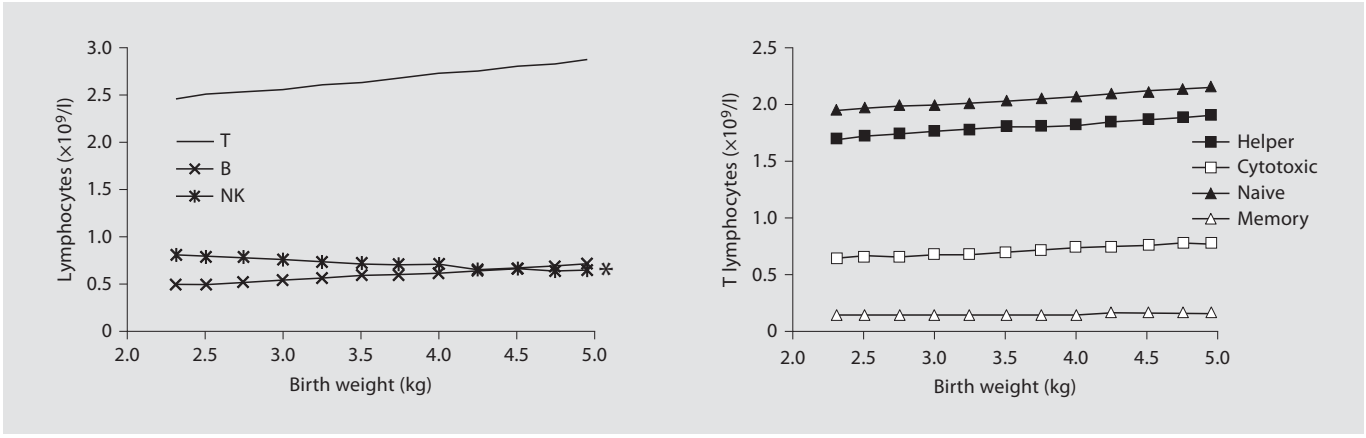

Fig. 2. Absolute numbers of lymphocyte subsets in infants with different birth weights. The estimated back-transformed regression lines reflect the number of lymphocyte subsets per kilogram increase in birth weight. All models were adjusted for gender, maternal education, smoking, alcohol use, fever and mode of delivery. * $p < 0.05$.

solute numbers of B lymphocytes, but not with T, NK and T lymphocyte subset counts.

To our knowledge, no large population-based prospective cohort studies have assessed the effects of gestational age, birth weight and fetal growth in late pregnancy on different lymphocyte subsets at birth. Previous studies of smaller sample sizes (50–120 subjects) showed that preterm birth infants had lower mean T lymphocyte counts and lower mean helper, cytotoxic and naive T lymphocyte counts compared to infants born at term [5–7, 25, 26]. Furthermore, it was shown that T lymphocyte subsets in fetal blood, obtained with cordocentesis during pregnancy, were lower in preterm than in term born infants [25, 27, 28]. Our results, showing an influence of gestational age at birth on T lymphocyte subset counts, are in line with these previous studies and suggest an ongoing development of the immune system during pregnancy. For the association of gestational age with B and NK lymphocyte counts, previous studies showed inconsistent results [5–7, 9, 10, 25, 26]. One large study of cord

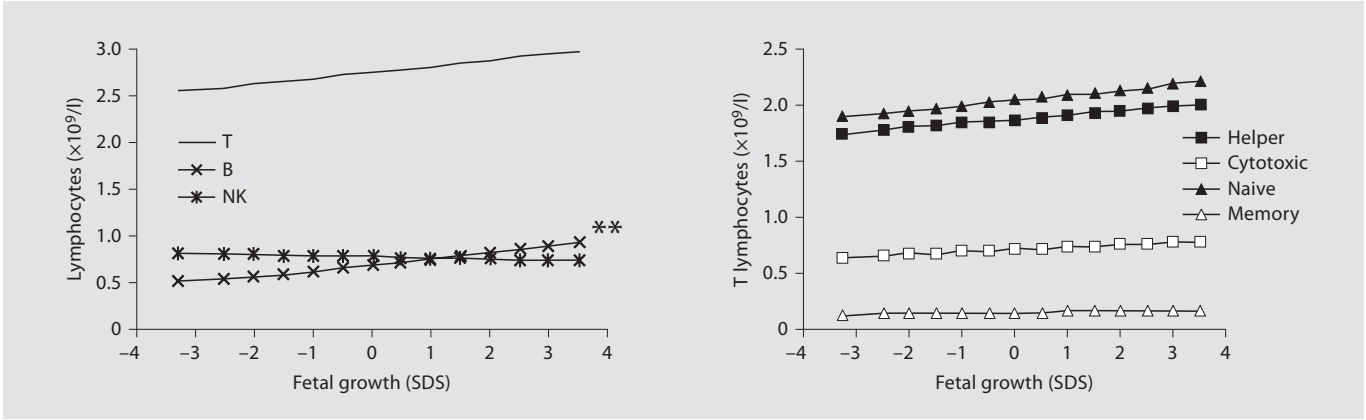

Fig. 3. Absolute numbers of lymphocyte subsets in infants with different fetal growths. The estimated back-transformed regression lines reflect the number of lymphocyte subsets per standard deviation increase in fetal growth. All models were adjusted for gender, maternal education, smoking, alcohol use, fever and mode of delivery. ** p = 0.01.

blood samples (>8,000 cord blood units), collected at birth and banked for future umbilical cord blood transplantation, showed small positive partial correlations of gestational age at birth with NK, helper T and cytotoxic T lymphocyte counts. The exact amount of change in lymphocyte subset counts was not presented [9]. Percentages or absolute counts of lymphocytes seem decreased in infected preterm infants or immunocompromised children with severe respiratory syncytial virus infections [3, 29]. However, neonatal lymphocytes may have a near-normal capacity to proliferate when needed [28]. The precise functional role and consequences for morbidity of low lymphocyte counts in preterm infants remains to be studied.

Previous studies found that infants with a lower birth weight had lower or similar lymphocyte subset counts compared to term infants [9, 12, 13, 30]. These studies did not examine birth weight independent of gestational age. One study assessed the effect of birth weight on numbers of different lymphocytes taking gestational age into account, and found that small-for-gestational-age (SGA) infants had lower T and B lymphocyte counts and lower helper and cytotoxic T lymphocyte counts than appropriate-for-gestational-age infants or preterm SGA infants [31]. Studies assessing the association of fetal growth restriction and lymphocytes found a reduction of T lymphocyte counts, helper and cytotoxic T lymphocyte counts, neutrophils and monocytes in fetal growth-restricted infants [16, 32]. An explanation for the different results with our study could be that studies of low birth weight and fetal growth were performed in small sample sizes (19–104 subjects) with another definition of fetal growth restriction (fetal abdominal circumference <5th percentile for gestation and abnormal Doppler ultrasound measurements of the uterine or umbilical arteries) and without adjustment for potential confounders.

We found an association of reduced birth weight and fetal growth with lower B lymphocyte counts. A biological mechanism might be that low-birth-weight and fetal-growth-restricted infants have a reduced abdominal circumference, the so-called 'brain-sparing effect' [33]. The reduced abdominal circumference can partly be explained by a reduced fetal liver size that may be caused by fetal malnutrition. Since B lymphocytes in fetal life are primarily produced by the fetal liver, a reduced fetal liver size might consequently lead to a decrease in B lymphocyte production. In line with this, we observed a 2% decrease of immature B lymphocytes (CD5+) per SDS decrease of fetal growth ($p < 0.01$). Similarly, an 8% decrease of immature B lymphocytes per kilogram decrease of birth weight was found ($p < 0.01$). However, the exact underlying pathophysiological mechanism of birth weight and fetal growth with different maturational stadia of B lymphocytes needs to be studied in more detail. Our study found no effect of birth weight or fetal growth on T lymphocyte counts. During pregnancy, T lymphocytes are mainly produced by the fetal thymus. Lower birth weight is associated with a reduced thymus size at birth; however, size of the fetal thymus relevant to its T lymphocyte production and function needs to be further explored [34, 35].

Some methodological issues should be considered. In our study, excluding participants with missing data would lead to selection biased results if the associations of gestational age, birth weight and fetal growth with lymphocyte subsets differ between participants with and without data. Of all single live births, information about gestational age and birth weight were not missing. Information about fetal growth was only missing in 1.5% of the infants. Participating infants without cord blood samples more often had a lower gestational age and lower birth weight, but not a lower estimated fetal weight in late pregnancy, than participating infants with cord blood samples. Therefore, it is likely that due to these selective missing of cord blood samples the observed effects of gestational age and birth weight with lymphocyte subsets may be underestimated.

In summary, this population-based prospective cohort study implies that with increasing gestational age development of the various lymphocyte subsets progresses. Birth weight and fetal growth seem to only influence B lymphocyte counts. These results contribute to a better understanding of the early development of the immune system and to conditions that might affect this process. Long-term effects of gestational age, birth weight and fetal growth on lymphocyte subsets and their consequences remain to be studied.

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