

T-lymphocyte subsets, thymic size and breastfeeding in infancy

Jeppesen DL, Hasselbalch H, Lisse IM, Ersbøll AK, Engelmann MDM. T-lymphocyte subsets, thymic size and breastfeeding in infancy. *Pediatr Allergy Immunol* 2004; 15: 127–132. © 2004 Blackwell Munksgaard

We followed the changes in concentration of T-lymphocyte subsets (CD4+ and CD8+ cells) in peripheral blood and thymus size during infancy. Previous studies have found increased thymus size in breastfed infants. The present study analyzed the association between breastfeeding and the number of CD4+ and CD8+ cells. Two different populations of infants between birth and 1 year of age were examined. Study Group I: infants with a variable duration of breastfeeding. Study Group II: long-term breastfed infants. In both groups a correlation was found between CD8+ cells and the thymic index at 10 months of age. In Group I, infants still breastfed at the 8-month examination had a higher CD8% than formula-fed infants ($p = 0.05$), and infants breastfed at the 4-month examination had a higher CD4% at 10 months of age ($p = 0.03$). Group II showed an increase in the absolute number of CD4+ and CD8+ cells from 8 to 10 months of age; and a positive correlation between the number of breastfeedings per day at 8 months of age, and an increase in CD4+ cells from 8 to 10 months of age ($p < 0.01$). In conclusion, a correlation was found between thymus size and CD8+ cells. Breastfeeding might have both a current and long-term immune-modulating effect on the developing cellular immune system.

Dorthe L. Jeppesen¹, Helle Hasselbalch², Ida M. Lisse³, Annette K. Ersbøll⁴ and Mads D. M. Engelmann⁵

Departments of ¹Pediatrics and ²Radiology, Hvidovre Hospital, Copenhagen University, ³Danish Epidemiology Science Center, Bandim Health Project, Guinea-Bissau, ⁴Department of Animal Science and Animal Health and ⁵Research Department of Human Nutrition, The Royal Veterinary and Agricultural University, Denmark

Key words: T-lymphocyte subsets; CD4+; CD8+; thymus; breastfeeding

Dorthe L. Jeppesen, Department of Pediatrics, Hvidovre Hospital, Copenhagen University, Kettegaards Alle 30, DK-2650 Hvidovre, Denmark
Tel.: +45 36323344
Fax: +45 36323770
E-mail: d.jeppesen@dadlnet.dk

Accepted 13 December 2002

The level of T-lymphocyte subsets in peripheral blood provides information about the development and function of the cellular immune defense in infancy; several reports have been published about blood lymphocyte subpopulations in infants (1–4).

The thymus provides the environment for T-lymphocyte maturation, but the significance of the size or alterations in the size of thymus in infancy in relation to the maturing immune defence is not known. Few studies have focused on the relationship between the T-lymphocyte subsets and thymic size in infancy, and the results are not clarified. A study of 31 HIV-infected children revealed a positive correlation between thymic volume (MR-scan) and the number of CD4+ cells and percentage of CD45RA+ CD4+ cells (na helper cells) (5). A Bolivian sonographic study of infants recovering from severe malnutrition demonstrated a correlation between a decrease in the number of immature T-lymphocytes from the thymic cortex and an

increase in thymic size. The same study found no correlation between mature T-lymphocytes and thymic size (6, 7).

In previous sonographic studies we have shown that the thymus grows until the age of 8 months, and then decreases to stabilize in size around the age of 12 months (8–10). We observed as in our previous study that thymic size is very much dependent on the breastfeeding status of the infant, the thymus of breastfed infants being twice the size of formula-fed infants at 4 months of age.

Very few studies report associations between breastfeeding and T-lymphocyte counts. One study found a lower percentage of CD3+ and CD4+ cells in 6-month-old breastfed infants compared with formula-fed infants of the same age (11). A recent study in Australia found a lower absolute count and percentage of CD4+ cells (CD4%) and a higher percentage of CD8+ cells (CD8%) in 6-month-old breastfed infants compared with formula-fed infants of the same

age. The absolute count of CD8+ cells was the same for each of the two populations of infants (12).

With the possibility of examining thymic size and T-lymphocyte subsets at different ages in infancy, the present study intended to associate the quantitative data of T-lymphocyte subsets to the size of thymus and the status of breastfeeding in infants less than 1 year of age.

Study populations and methods

Study Group I

The size of the thymus was assessed during the first week of life in 87 healthy term infants from the maternity ward at the Copenhagen University Hospital in Hvidovre. Blood smears were done at the same time for determination of CD4+ and CD8+ T-lymphocytes, and CD4% was obtained in all cases whereas CD8% was faulty in four cases. Information on mode of delivery, gestational age, weight, and length was collected at the time of delivery. At later follow-ups, information was collected on weight, length, and previous infectious diseases. By mail, all infants were invited to participate in a study aimed at associating thymic size with T-lymphocyte subsets at age 4, 8, 10 and 12 months. Out of 87 infants, only 33 children participated in all examinations, in spite of repeated letters of invitations. The main drop-out occurred at the 4-month examination, attended by 41 children. Thirty-five, 33 and 35 children participated in the 8-, 10- and 12-month examinations, respectively. The study took place from Autumn 1993 to Spring 1994.

At the 4-month examination, infants were divided into three groups according to breastfeeding status:

1. Exclusively breastfed (n = 17).
2. Partially breastfed (n = 12).
3. Exclusively formula fed (n = 12).

'Exclusively breastfed' allowed supplements of water, but no energy-containing fluids. An exception was made for one infant who received 250 ml of formula per week. The exception was made to ensure correspondence of the dataset with previously published data (10). Infants were classified as 'partially breastfed' as long as they were breastfed at least once a day.

At the 8-, 10- and 12-month examinations, infants were divided into two groups according to current breastfeeding status based on breastfeeding at least once a day or no breastfeeding at all.

1. Breastfeeding once or more times per day (n = 10; 8 and 5, respectively).
2. No breastfeeding (n = 25; 25 and 30, respectively).

However, data from the 8-, 10- and 12-month examinations were also analyzed according to previous breastfeeding status at the 4-month examination.

Information on breastfeeding status and pattern was collected by verbal questions. Demographic data are given in Table 1.

Study Group II

In a nutritional study performed in the Autumn of 1994 at the Research Department of Human Nutrition, The Royal Veterinary and Agricultural University, 36 children (11 girls and 25 boys) were enrolled and had the size of their thymus measured and a blood sample collected at 8 and 10 months of age. The children were not selected by sex, but by breastfeeding status. All children enrolled in the study were exclusively or partially breastfed at 8 months of age. All children participated in both examinations, 11 (31%) infants completed breastfeeding between the two examinations. Information on weight,

Table 1. Study Group I: the age, weight, length, number of infants, sex, and breastfeeding status at the different examinations

Time of examination	Newborn	4 months	8 months	10 months	12 months
Number (female, male)*	87 (35, 52)	41 (14, 27)	35 (12, 23)	33 (12, 21)	35 (12, 23)
Median age† (range)	40 (37–42)	125 (109–144)	249 (231–270)	300 (286–332)	369 (356–396)
Weight (g) median (range)	3500 (2350–4850)	7099 (5090–9476)	9218 (7100–11389)	10,100 (7500–18000)	10,753 (8259–12359)
Length (cm) median (range)	52.0 (45.0–57.0)	65 (59–69)	72 (66–78)	75 (66–80)	77 (68–82)
Breastfed‡ number (%)		17 (42%)	13 (40%)	12 (39%)	13 (40%)
Partially breastfed‡ number (%)		12 (29%)	10 (30%) [10]§	9 (29%) [8]§	10 (30%) [5]§
Exclusively formula fed‡ number (%)		12 (29%)	10 (30%)	10 (32%)	10 (30%)

*Data on breastfeeding status at the 4-month examination was missing for two of the infants seen at the 8-, 10- and 12-month examinations.

†Age: Newborns: GA in weeks; infants 4–12 months of age: age in days.

‡Breastfeeding status at the 4-month examination.

§[]The number of still partially breastfed infants at the current examination.

Table 2. Study Group II: data on weight and length at the two examinations

	8-month examination	10-month examination
Number (female, male)	36 (11, 25)	36 (11, 26)
Weight (g)	8966 (6623–14700)	9784 (6942–13700)
Length (cm)	71 (65–79)	74 (67–81)
Breastfed (exclusively and partially)	36	25

length, and infectious diseases was collected at each examination. Information on breastfeeding status and pattern was collected by verbal questions. Further information is given in Table 2.

Both study group infants had joined the official Danish vaccination programme, but differences in vaccine responses were not examined. Parental smoking and atopy was not taken into account. By coincidence there was a 2:1 male predominance in both study groups, see also Tables 1 and 2.

Methods

Thymic size was assessed by sonography. One radiologist (HH) measured the thymus, using a B & K sonographic scanner type 1846 and a 7 Mhz probe type 8534 (B & K Medical, Gentofte, Denmark). At sonography, the thymus appears as a well-delineated echo-poor structure in the anterior mediastinum. A trans-sternal approach was used to measure the largest transverse diameter of the thymus. Perpendicular to that, the largest sagittal area (longitudinal scan plane) was depicted on the monitor and measured by the computer. The two measurements were multiplied and recorded as the thymic index. The results were expressed as the average of two determinations. The index is an estimate of the volume of the thymus, and post-mortem examinations have shown a high correlation between thymic index, and the weight and volume (13).

The CD4% and CD8% were determined as previously described. Briefly, blood smears were prepared directly on location, as for conventional hematological examination, and air-dried. Immunocytochemical labeling was performed with monoclonal antibodies against CD4 and CD8 receptors (DAKO, Glostrup, Copenhagen, Denmark) using the alkaline phosphatase technique (14). Two hundred lymphocytes (including both T and B lymphocytes) were counted on each slide. This method requires only 2 or 3 drops of blood and determines the percentage of the CD4+ and CD8+ T-lymphocytes (CD4% and

CD8%) among all lymphocytes. The method has been shown to be closely correlated with the results obtained by flow cytometry. Comparing the slide-based method with the FACS analyzer the total counts of CD4+ and CD8+ cells were somewhat higher using the first method, but the CD4/CD8 ratio was not different when comparing the two methods (14, 15).

In addition, Study Group II had leukocytes counted in a hemocytometer according to standard procedures, and a manual differential count was performed to determinate absolute counts for the CD4+ and CD8+ lymphocytes. Due to incomplete leukocyte counts, the absolute counts of T-lymphocyte subsets were not obtained in 5 out of 36 children at the 8-month examination in Study Group II.

Owing to a skewed distribution of thymic index and T-lymphocyte subsets, a logarithmic transformation was performed (data showed then normal distribution) before the statistical analysis. The percentages of CD4+ and CD8+ T-lymphocytes, the CD4/CD8 ratio, and the sum of CD4+ and CD8+ T-lymphocytes were correlated with the sonographic thymic index, breastfeeding groups, and clinical information including the history of infectious diseases. The relationships between the T-lymphocyte subsets and other parameters were analyzed by means of covariance analysis. The differences between the T-lymphocytes at the 8- and 10-month examinations were analyzed using a paired *t*-test. All statistical analyses were performed with SAS Statistical Software (SAS Institute Inc., Cary, NC, USA) (16). A 5% significance level was used.

Informed consent was obtained from all parents, and the study was approved by the local scientific and ethical committee.

Results

Study Group I

Table 3 presents the mean values of CD4% and CD8% in relation to age and thymic index; CD4% as well as CD8% decreased from birth to 1 year of age. In newborns, we found a higher average of CD4% than reported in the literature (2, 3), and there was a tendency for a negative but not significant correlation, between the thymic index and the sum of CD4% and CD8% ($p = 0.07$). There was no correlation between the thymic index and the CD4%, the CD8%, and the CD4/CD8 ratio. There was no correlation between the thymic index and the T-lymphocyte subsets either at the 4- or 8-month examinations. At the 10-month examination, a

Table 3. Study Group I: distribution of the CD4+ and CD8+ T-lymphocytes and the thymic index from birth to 12 months of age, given as median values (range)

Age	Newborn	4 months	8 months	10 months	12 months
n	87*	41	35	33	35
CD4%	64.0 (40.0–89.0)	56.0 (34.0–74.0)	52.5 (30.5–66.0)	48.5 (34.5–64.5)	48.5 (34.5–63.0)
CD8%	23.0 (10.0–58.0)	21.0 (8.0–43.0)	20.0 (11.0–38.0)	21.0 (9.5–35.0)	18.5 (6.5–37.5)
CD4/CD8 ratio	2.9 (1.0–6.7)	3.0 (1.5–6.4)	2.5 (1.3–5.2)	2.3 (1.4–6.2)	2.6 (0.9–7.7)
CD4% + CD8%	88.0 (69.0–119.0)	78.0 (55.0–109.0)	75.0 (50.0–92.5)	70.5 (52.5–88.0)	68.0 (55.0–80.5)
Thymic index	11.9 (3.8–25.0)	27.7 (12.2–83.2)	28.4 (6.3–55.2)	18.5 (9.1–48.8)	17.3 (7.4–53.0)

*The CD4% was measured in 87 infants, and the CD8% was measured in 83 infants.

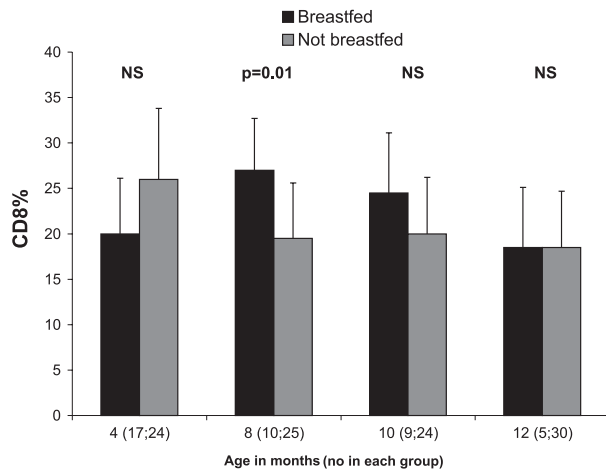


Fig. 1. CD8 percentage (median and standard deviation) and current breastfeeding status at the 4- and 12-month examinations (Study Group I).

positive correlation was observed between the thymic index and the CD8% ($p = 0.05$); this association was also reflected in the CD4/CD8 ratio ($p = 0.03$). There seemed to be no correlation between thymic size and CD4%. No correlation was found between T-lymphocytes and thymic size at 1 year of age.

Breastfeeding had no detectable influence on T-lymphocytes at 4 months of age. However, infants partially breastfed until 8 months of age had a significantly higher CD8% than infants whose breastfeeding had stopped before that age ($p = 0.01$) (Fig. 1). The higher daily breastfeeding frequency, the higher CD8% ($p = 0.03$). At 10 months of age, the CD4% was higher in infants who had been exclusively or partially breastfed until the age of 4 months ($p = 0.03$) (Fig. 2). At the 12-month examination, there was no correlation with earlier or current breastfeeding status and T-lymphocytes.

As previously reported, the thymic index at 4 months of age in the exclusively breastfed infants was twice the size as in formula-fed infants. At the 10-month examination, the breastfeeding practice at 4 months of age still influenced the size of the thymus (8, 9).

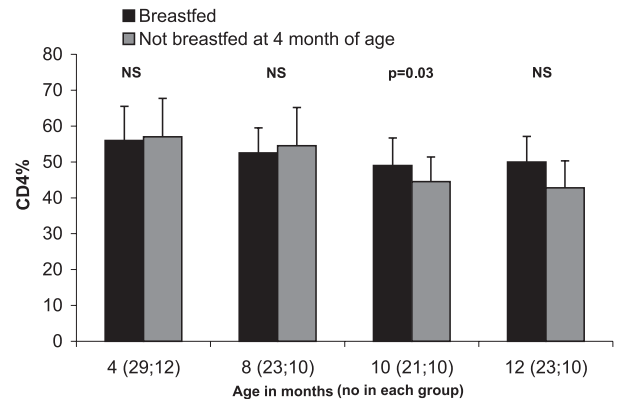


Fig. 2. CD4 percentage (median and standard deviation) at the 4- and 12-month examinations, depending on the breastfeeding status at 4 months of age (Study Group I).

Study Group II

An increase in the absolute number of CD4+ and CD8+ cells was observed between 8 and 10 months of age; the increase in CD8+ cells was significant ($p = 0.03$) (Table 4), and was positively correlated with the thymic index at age 8 months ($p < 0.01$). The thymic index at age 8 months was also positively correlated with the number of CD4+ cells at 10 months of age ($p = 0.03$). There was no correlation between the thymic index and the CD4/CD8 ratio.

The increase in the number of CD4+ cells from 8 to 10 months of age correlated positively with the breastfeeding frequency at the 8-month examination ($p < 0.01$) (Fig. 3). The sum of the absolute count of CD4+ and CD8+ cells at 10 months of age correlated positively with the breastfeeding frequency at 8 months of age ($p < 0.01$).

As previously reported (17), the thymic indexes at 8 and 10 months of age were not significantly different (20 and 23, respectively), but continued breastfeeding at 10 months of age had a positive influence on the size of thymus, compared with infants who were weaned between 8 and 10 months of age.

Table 4. Study Group II: T-lymphocyte counts (CD4+ and CD8+) and percentage (CD4% and CD8%), and differences between the 8- and 10-month examination, given as mean and range, and the p-values (paired *t*-test)

Difference between Number ($\times 10^9/l$) or percentage (%) of cells difference	No.	8-month examination	No.	10-month examination	8- and 10-month examination	p-value for
CD4+ count ($\times 10^9/l$)	31	4.1 (1.2–7.5)	36	4.7 (1.8–10.3)	0.3 (–2.6–5.3)	0.15
CD4%	36	53 (26–69)	36	51 (35–72)	–1 (–27–24)	0.78
CD8+ count ($\times 10^9/l$)	31	1.4 (0.5–4.1)	36	1.7 (0.6–5.4)	0.4 (–1.8–4.3)	0.03*
CD8%	36	19 (9–29)	36	22 (10–33)	1.5 (–14–21)	0.04*
CD4/CD8 ratio	36	2.8 (0.9–7.1)	36	2.5 (1.1–7)	–0.4 (–4.2–2.5)	0.26
Sum: CD4+ and CD8+ count ($\times 10^9/l$)	31	5.7 (1.7–10.2)	36	6.5 (3.0–14.2)	0.7 (–3.5–6.7)	0.04*
Sum: CD4% and CD8%	36	69 (52–84)	36	72 (54–94)	3 (–23–29)	0.24
Thymic index	36	20 (7–50)	36	23 (7–55)		

*Significant difference at a 5% level.

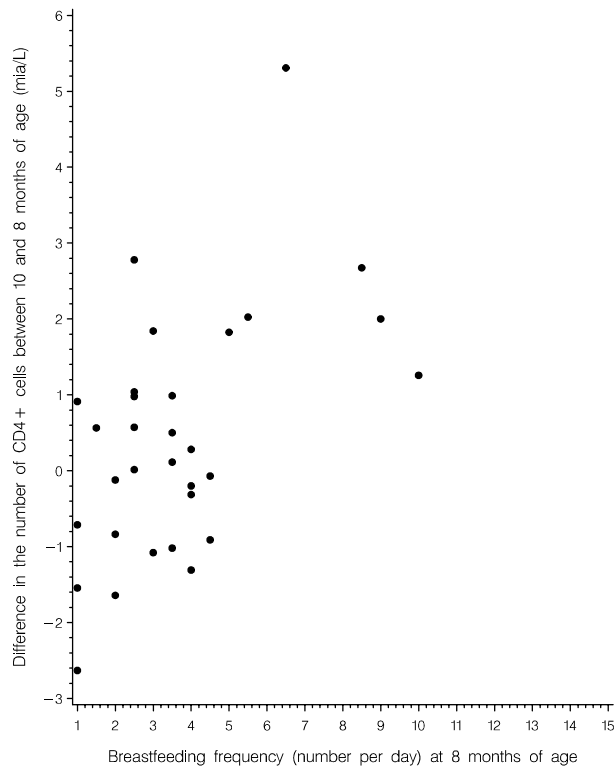


Fig. 3. The increase in the number of CD4+ cells correlated with breastfeeding frequency (number per day) at 8 months of age (Study Group II).

The CD4% and CD8% at 8 and 10 months of age did not differ between the two study groups.

Discussion

To our knowledge, correlations between the CD4+ cells and CD8+ cells and the thymic size in healthy infants have not been investigated before. Our aim was to follow the changes in T-lymphocyte subsets and thymus size during infancy, correlate the mutual immunological markers, and to correlate T-lymphocyte subsets to breastfeeding status.

T-lymphocytes and thymic size

In Study Group I involving infants with standard duration of breastfeeding, the CD4% and CD8% both declined during the observation period, from birth to 1 year of age. The median CD4% at 64% at birth is higher than in previous reports (2, 3). This difference might be due to the chosen method of analysing, but the results from The European Collaborative Study are based on uninfected children born to human immunodeficiency virus 1-infected women (3). These children might have lower values than the children of the present study who were born to healthy women.

In contrast to the CD4% and the CD8% the thymic index increased from birth to 4 months before decreasing again after 8 months of age.

A correlation was found between the proportion of CD8+ cells and the CD4/CD8 ratio and the thymic index at 10 months of age; at any other times no significant correlation between the T-lymphocyte subsets and the thymic index was found.

In Study Group II, comprising long-term breastfed infants, there was a positive correlation between the thymic index at 8 months of age, and an increase in the numbers of CD8+ cells from 8 to 10 months. A correlation with the numbers of CD4+ cells at age 10 months and the thymic size at age 8 months was also seen.

The correlation found between the thymus and T-lymphocyte subsets was seemingly related to later infancy, when both thymus and the CD8+ T-lymphocytes were decreasing. However, in HIV-positive infants the absolute number of CD4+ cells correlated positively with the thymic size (5), and in the Bolivian study of infants suffering from malnutrition no correlation was found between mature T-lymphocytes and thymic size (6, 7). In conclusion, the findings in healthy infants seem to differ from those in infants with a depressed immune system.

T-lymphocytes and breastfeeding

In Study Group I, the CD8% was higher in infants who had been breastfed until the age of 8 months, and there was evidence of a long-term effect of breastfeeding at 4 months of age on the CD4% at age 10 months. In long-term breastfed infants in Study Group II, we observed that the absolute number of both CD4+ and CD8+ cells increased from 8 to 10 months of age. This could be due to the effect of breastfeeding and the rise in CD4+ counts being significantly and positively correlated with the numbers of breastfeedings per day at age 8 months. Overall, there might be a direct increasing effect of breastfeeding on the CD8+ cells in early infancy. The observed alterations of the CD8+ cells are supported by a recent Australian study of infants at 6 months of age that demonstrated a higher CD8% in breastfed than formula-fed infants (12). However, that study found a lower number of CD4+ cells and a lower CD4/CD8 ratio in breastfed than formula-fed infants. This finding partially conflicts with our finding of an increasing effect of long-term breastfeeding on the CD4+ cells, and a long-lasting effect of breastfeeding at 4 months of age, leading to a higher CD4% even after the infants were weaned at age 10 months.

The study by Carver et al. also reported a lower CD3+ percentage (CD3%) and CD4% in breastfed 6-month-old infants than in formula-fed infants (11). Our findings in both study groups show delayed effects of breastfeeding on the CD4+ cells.

Several studies have analyzed the relationship between breastfeeding and the infant's immune system, some suggesting a possible transfer of cellular immunity from the mother to the child via human milk; however, the content of immune modulating factors in human milk is well known (18–20).

The significance of the observed influence of breastfeeding on T-lymphocytes is not entirely clear, but the findings of the present study support the speculations of the possibility of both a current and a long-term immune-modulating effect of human milk on the developing cellular immune system.

Acknowledgments

The study was supported by grant nos 11/052-95 and 11/063-96 Sygekassernes Helsefond.

References

- WESTERMANN J, PABST R. Lymphocyte subsets in the blood: a diagnostic window on the lymphoid system? *Immunol Today* 1990; 11: 406–10.
- COMANS-BITTER WM, DE GROOT R, VAN DEN BEEMD R, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997; 130: 388–93.
- THE EUROPEAN COLLABORATIVE STUDY. Age-related standards for T lymphocyte subsets based on uninfected children born to human immunodeficiency virus 1-infected women. *Pediatr Infect Dis J* 1992; 11: 1018–26.
- LISSE IM, AABY P, WHITTLE H, JENSEN H, ENGELMANN M, CHRISTENSEN LB. T-lymphocyte subsets in West African children: Impact of age, sex, and season. *J Pediatr* 1997; 130: 77–85.
- HASSELBALCH H, ERSBØLL AK, JEPPESEN DL, NIELSEN MB. Thymus size in infants from birth until 24 months of age evaluated by ultrasound. *Acta Radiol* 1999; 40: 41–4.
- HASSELBALCH H, JEPPESEN DL, ERSBØLL AK, ENGELMANN MDM, NIELSEN MB. Thymus size evaluated by sonography: A longitudinal study on infants during the first year of life. *Acta Radiol* 1997; 38: 222–7.
- HASSELBALCH H, JEPPESEN DL, ENGELMANN MDM, MICHAELSEN KF, NIELSEN MB. Decreased thymus size in formula-fed infants compared to breastfed infants. *Acta Pediatr* 1997; 85: 1029–32.
- VIGANO A, VELLA S, PRINCIP N, et al. Thymus volume correlates with the progression of vertical HIV infection. *AIDS* 1999; 13: F29–F34.
- CHEVALIER P, SEVILLA R, ZALLES L, SEJAS E, BELMONTE G. Study of thymus and thymocytes in Bolivian preschool children during recovery from severe protein energy malnutrition. *J Nutr Immunol* 1994; 3: 27–39.
- CHEVALIER P. Thymic ultrasonography in children, a non-invasive assessment of nutritional immune deficiency. *Nutrition Res* 1997; 17: 1271–6.
- CARVER JD, PIMENTEL B, WIENER DA, LOWELL NE, BARNES LA. Infant feeding effects on flow cytometric analysis of blood. *J Clin Laboratory Anal* 1991; 5: 54–6.
- HAWKES JS, NEUMANN MA, GIBSON RA. The effect of breast feeding on lymphocyte subpopulations in healthy term infants at 6 months of age. *Pediatr Res* 1999; 45: 648–51.
- HASSELBALCH H, NIELSEN MB, JEPPESEN DL, PEDERSEN JF, KARKOV J. Sonographic measurement of the thymus in infants. *Eur Radiol* 1996; 6: 700–3.
- LISSE IM, WHITTLE H, AABY P, et al. Labelling of T-cell subsets under field conditions in tropical countries. *J Immunol Methods* 1990; 129: 49–53.
- LISSE IM, BOTTIGER B, CHRISTENSEN LB, et al. Evaluation of T cell subsets by an immunocytochemical method compared to flow cytometry in four countries. *Scand J Immunol* 1997; 45: 637–44.
- THE SAS STATISTICAL SOFTWARE. SAS/STAT User's Guide, Version 6, 4th edn, Volumes 1 and 2. Cary, NC: SAS Institute Inc., 1989.
- HASSELBALCH H, ENGELMANN MDM, ERSBØLL AK, JEPPESEN DL, MICHAELSEN KF. Breast-feeding influences thymic size in late infancy. *Eur J Pediatr* 1999; 158: 964–7.
- HANSON LÅ. Breastfeeding provides passive and likely longlasting active immunity. *Ann Allergy Asthma Immunol* 1998; 81: 523–37.
- PABST HF, SPADY DW, PILARSKI LM, et al. Differential modulation of the immune response by breast or formula feeding of infants. *Acta Pediatr* 1997; 86: 1291–7.
- WOLD AE, ADLERBERTH I. Does breastfeeding affect the infant's immune responsiveness? *Acta Pediatr* 1998; 87: 19–22.