Title

High-Dose Neonatal Vitamin A Supplementation Transiently Decreases Thymic Function in Early Infancy

Journal

Journal of Nutrition, 150(1)

ISSN

0022-3166

Authors

Ahmad, Shaikh M
Raqib, Rubhana
Huda, M Nazmul
et al.

Publication Date

2020

DOI

10.1093/jn/nxz193

Peer reviewed

The Journal of Nutrition
Nutritional Immunology

High-Dose Neonatal Vitamin A Supplementation Transiently Decreases Thymic Function in Early Infancy

Shaikh M Ahmad,[1] Rubhana Raqib,[1] M Nazmul Huda,[1,2,3] Md J Alam,[1] Md Monirujjaman,[1] Taslima Akhter,[1] Yukiko Wagatsuma,[4] Firdausi Qadri,[1] Melissa S Zerofsky,[2,3] and Charles B Stephensen[2,3]

[1]Immunobiology, Nutrition, and Toxicology Laboratory, Infectious Diseases Division, icddr,b, Mohakhali, Dhaka, Bangladesh; [2]USDA Western Human Nutrition Research Center at University of California, Davis, CA, USA; [3]Nutrition Department, University of California, Davis, CA, USA; and [4]Department of Clinical Trials and Clinical Epidemiology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan

ABSTRACT

Background: Vitamin A deficiency (VAD) impairs T-cell–mediated immunity. In regions where VAD is prevalent, vitamin A supplementation (VAS) reduces child mortality, perhaps by improving immune function.

Objective: Our objective was to determine if neonatal VAS would improve thymic function in Bangladeshi infants, and to determine if such effects differed by sex or nutritional status (i.e., birth weight above/below the median).

Methods: Three hundred and six infants were randomly assigned to 50,000 IU vitamin A (VA) or placebo (PL) within 48 h of birth. Primary outcomes were measured at multiple ages and included *1*) thymic index (TI) at 1, 6, 10, and 15 wk; *2*) T-cell receptor excision circles (TREC), an index of thymic output of naïve T cells; and *3*) total/naïve T cells in peripheral blood at 6 wk, 15 wk, and 2 y. A mixed linear model for repeated measures was used to assess group differences at each age and identify interactions with sex and birth weight.

Results: VAS did not significantly ($P = 0.21$) affect TI overall (i.e., at all ages) but decreased TI by 7.8% ($P = 0.029$) at 6 wk: adjusted TI means for the PL and VA groups at 1, 6, 10, and 15 wk were 4.09 compared with 3.80 cm^2, 7.78 compared with 7.18 cm^2, 8.11 compared with 7.84 cm^2, and 7.91 compared with 7.97 cm^2, respectively. VAS did not significantly ($P = 0.25$) affect TREC overall but decreased TREC by 19% ($P = 0.029$) at 15 wk: adjusted TREC means for the PL and VA groups at 6 wk, 15 wk, and 2 y were 13.6 compared with 16.1 copies/pg DNA, 19.4 compared with 15.7 copies/pg DNA, and 11.8 compared with 10.0 copies/pg DNA, respectively. VAS did not significantly affect overall total ($P = 0.10$) or naïve ($P = 0.092$) T cells: adjusted naïve T-cell means for the PL and VA groups at 6 wk, 15 wk, and 2 y were 3259 compared with 3109 cells/μL, 3771 compared with 3487 cells/μL, and 1976 compared with 1898 cells/μL, respectively.

Conclusion: In contrast to our hypothesis, VAS decreased thymic function early in infancy but health effects are presumably negligible owing to the transience and small magnitude of this effect. This trial was registered at clinicaltrials.gov as NCT01583972 and NCT02027610. *J Nutr* 2020;150:176–183.

Keywords: vitamin A, vitamin A deficiency, thymus, T-cell receptor excision circle, T-lymphocyte, neonate, infant, Bangladesh

Introduction

Vitamin A (VA) was termed "the anti-infective vitamin" early in the 20th century (1, 2) but it was not until late in the century that community intervention trials demonstrated that VA supplementation (VAS) from 6 mo to 5 y of age to those at risk of VA deficiency (VAD) reduced mortality from common infectious diseases (3), presumably by correcting the impairment of immune function caused by VAD (4–6). Results from intervention trials <6 mo of age have been mixed and some evidence of increased risk of mortality in girls has been seen (7, 8). To help resolve the question of whether VAS is beneficial <6 mo of age, 3 community intervention trials were conducted to determine if supplementation with 50,000 IU VA within 48 h of birth would decrease infant mortality (9–11). Two of the studies were in Africa and 1 in India and no overall benefit was seen, although the result in India was consistent with a modest reduction in mortality (11). The present study was designed to determine how this same intervention (i.e., 50,000 IU within 48 h of birth using the same supplement source) would affect thymic function (12). Very recently, a meta-analysis of randomized controlled trials of neonatal VAS was published which also found no benefit overall, but also reported reduced mortality through 6 mo of age among trials from south Asia, including a site in Bangladesh (13), with a prevalence of

Manuscript received March 7, 2019. Initial review completed April 15, 2019. Revision accepted July 24, 2019.
First published online August 28, 2019; doi: https://doi.org/10.1093/jn/nxz193.

maternal VAD ≥10% (defined as serum retinol <0.7 μmol/L) (14), consistent with the idea that mortality benefits will be seen in neonates born to women in a population where VAD is prevalent.

Thymic function and T-cell–mediated immunity are impaired by VAD (15, 16). T cells develop in the thymus and immunologically naïve T cells are released into the peripheral blood (17). Thymus size and the output of naïve T-cells diminish as a result of malnutrition (18–21). VAD in rodent models decreases the size and cellularity of the thymus, as well as the number of T cells found in peripheral tissues (22, 23). In the present study, we evaluated 3 primary outcomes related to thymic function: *1*) thymus size; *2*) T-cell receptor excision circle (TREC) concentrations in peripheral blood mononuclear cells (PBMCs); and *3*) peripheral blood total and naïve T-cell concentrations. TRECs are small, nonreplicating circles of DNA produced by editing of the T-cell receptor gene during T-cell development in the thymus. They are diluted by passage to daughter cells during T-cell division in the periphery, making TREC concentrations a useful index of thymic output of naïve T cells (24). We hypothesized that VAS would increase thymus size, TREC concentrations, and naïve T-cell concentrations. We have previously reported that an elevated cortisol response to a pain stimulus (vaccination) is associated with differences in thymus size and T-cell concentrations in these study participants (25).

Methods

Study design

The design of this study and the description of the study population have been published (12) and the allocation of subjects to treatment and placebo is shown in the CONSORT (Consolidated Standards of Reporting Trials) diagram (**Supplemental Figure 1**). In brief, the study was conducted at the Maternal and Child Health Teaching Institute (MCHTI) in Dhaka, Bangladesh, among women receiving prenatal care who planned to bring their infants to MCHTI for postnatal care and routine immunizations. Laboratory work was carried out at icddr,b, located 5 miles from the MCHTI. Weight and length were measured as described (12) and converted to *z* scores using WHO Anthro software (26). Ethical approval was received from icddr,b and the WHO.

Random assignment and masking

Mothers were contacted during pregnancy to facilitate recruitment of infants soon after birth. After informed consent from a parent or guardian, singleton infants eligible for newborn immunizations were randomly assigned in a 1:1 ratio to receive a single 50,000 IU (52.5 μmol) dose of VA in oil or an identical placebo (PL) within 48 h of birth. Random assignment was balanced by sex and birth weight using 4 randomization lists (boys and girls independently, above and below expected median birth weight) as described (12). A total of 306 infants were enrolled between 15 January, 2012 and 21 March, 2013. Infants were followed through 15 wk of age with study visits occurring within 48 h of birth and at 1, 6, 10, 11, 14, and 15 wk. Mothers received VAS 6 wk postpartum, according to the standard practice in Bangladesh at the time, as has been reported (27).

Follow-up visit

After this initial study, which lasted until 15 wk of age (NCT015839720), additional funding was secured and infants were again recruited and completed visits between 14 June, 2014 and 5 May, 2015 for a follow-up visit at ∼2 y of age (NCT02027610). The initial goal was to perform this visit at ∼1 y of age, but funding was not available until infants were in their second year of life. During the follow-up visit blood was drawn, weight and height were measured, a stool sample was collected, and additional demographic and health history data were collected by the same staff and investigators.

Clinical methods

The thymic index (TI) was measured as an estimate of thymus volume at 1, 6, 10, and 15 wk by a single observer using a validated method (28), a portable ultrasound machine (Toshiba SSA 320A Justavision-200, Toshiba Medical Systems, Japan) with a PVF-745V 5.0- to 7.0-MHz probe (Toshiba Medical Systems, UK). Heparinized venous blood was collected at 6 wk, 15 wk, and 2 y. Visits were rescheduled for infants with fever, diarrhea, or respiratory infection in the preceding 2 d.

Laboratory methods

Blood was transported to icddr,b the same day. Concentrations of naïve ($CD45RA^+CD45RO^-$) and memory ($CD45RA^-CD45RO^+$) total T cells ($CD3^+$) and T helper cells ($CD3^+CD4^+$) were determined in whole blood using Multitest 4-color reagents, TruCount® tubes (Becton-Dickinson), and a FACSCalibur flow cytometer (Becton-Dickinson). PBMCs were isolated by density gradient centrifugation and a portion was frozen for later DNA extraction for signal-joint TREC analysis, which was performed using qPCR, as described (29).

Statistical analysis

Data analysis was performed using SAS 9.4 (SAS Institute Inc.). Data were examined for normality (using a Shapiro–Wilk statistic >0.96), equal variance, and to identify outliers. TI values, T-cell concentrations, and TREC concentrations were all normalized by square root transformation. The Shapiro–Wilk statistic for TREC data was 0.95, but when 3 high outliers (all in the PL group at 2 y) were removed the value was 0.98. The outliers were retained in the final analysis because the same group differences were identified with or without these outliers. Aside from primary endpoints, 2-group comparisons were made using Student's *t* test for normally distributed variables or Wilcoxon's rank-sum test for other continuous variables. Categorical variables were compared between groups using the chi-squared or Fisher's exact test.

Statistical analysis to identify treatment effects for the principal outcome variables was conducted using intention-to-treat principles. A mixed linear model approach for repeated measures was used to assess differences between the VA and PL groups. In addition to treatment group and age category, sex and a categorical variable for birth weight median (BWM; above or below) for each sex were included in all statistical analyses per the original study design (12). For boys, 77 infants were below the BWM (range: 2030–2750 g) and 76 were above (2760–3940 g). For girls, 77 infants were below (1780–2640 g) and 76 were above (2650–3870 g). The statistical model thus included categorical variables for treatment group, sex, BWM, and age, as well as all higher-order interactions, and included a random effect of subject specifying an unstructured covariance matrix across age categories. Key maternal and infant characteristics at baseline were evaluated to

Supported by WHO Project 2010168947, which was funded from the Bill and Melinda Gates Foundation (to CBS), Thrasher Research Fund grant 11488 (to CBS), and USDA-Agricultural ResearchService project 2032-53000-001-00-D (to CBS). RedCap database usage was supported by the National Center for Advancing Translational Sciences, NIH, through grant UL1TR001860.
Author disclosures: SMA, RR, MNH, MJA, MM, TA, YW, FQ, MSZ, and CBS, no conflicts of interest.
Supplemental Figure 1 and Supplemental Tables 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
Present address for MSZ: Department of Pediatrics, University of California, San Francisco, CA 94143, USA.
Address correspondence to CBS (e-mail: charles.stephensen@ars.usda.gov).
Abbreviations used: BWM, birth weight median; MCHTI, Maternal and Child Health Teaching Institute; PBMC, peripheral blood mononuclear cell; PL, placebo; TI, thymic index; TREC, T-cell receptor excision circle; VA, vitamin A; VAD, vitamin A deficiency; VAS, vitamin A supplementation.

TABLE 1 Characteristics of all enrolled study infants at birth as well as members of the placebo and vitamin A groups[1]

	All infants		Placebo group		Vitamin A group		
Characteristics	Values	*n*	Values	*n*	Values	*n*	*P* value[2]
Male sex	50.0	306	49.0	153	51.0	153	0.73
Birth weight, g	2741 ± 378	306	2752 ± 388	153	2730 ± 370	153	0.60
Birth weight <2500 g	26.8	306	24.8	153	28.8	153	0.44
Length, cm	46.6 ± 2.2	306	46.6 ± 2.2	153	46.7 ± 2.3	153	0.91
Gestational age, wk	39.1 ± 1.6	303	39.1 ± 1.6	151	39.1 ± 1.7	152	0.90
Gestational age <37 wk	7.6	303	7.9	151	7.2	152	0.82
Weight-for-length, *z* score	−0.15 ± 1.14	258[3]	−0.07 ± 1.10	129	−0.24 ± 1.17	129	0.24
Length-for-age, *z* score	−1.64 ± 1.15	306	−1.65 ± 1.14	153	−1.63 ± 1.17	153	0.94
Weight-for-age, *z* score	−1.20 ± 0.89	306	−1.17 ± 0.90	153	−1.22 ± 0.87	153	0.60
Born in hospital	96.1	306	96.7	153	95.4	153	0.71
Cesarean delivery	59.8	306	65.4	153	54.2	153	0.048

[1] Infants received placebo or vitamin A (50,000 IU) capsules within 48 h of birth. Values are mean ± SD or %.
[2] Comparing treatment group by Student's *t* test or chi-square test.
[3] Weight-for-length *z* scores could not be calculated from WHO standards at length <45.0 cm ($n = 48$ infants; 24 in each treatment group).

identify differences between treatment groups that should be included in the statistical model. The prevalence of delivery type (cesarean compared with vaginal) differed between groups, thus it was included as a covariate without interaction terms. (Delivery type was examined for interactions with age but none were found.) With regard to *P* value for group effects, we originally envisioned that VAS could *1*) affect all subjects similarly, *2*) affect girls and boys differentially, and *3*) affect those with a greater or lesser risk of VAD at baseline (below or above the BWM, respectively) differentially, overall or at specific ages. We thus used $P < 0.05$ for such group comparisons and used $P < 0.10$ to identify significant interactions for post-hoc examination (where we also used $P < 0.05$ for the preplanned group comparisons). Examination of all other significant interactions involving treatment group used a Bonferroni adjustment for post-hoc comparisons ($P = 0.05$/number of comparisons) because these comparisons were not envisioned as part of the initial analysis plan. For example, a significant group × sex × BWM interaction would use $P < 0.0125$ for significance of a group effect within the 4 relevant categories (i.e., boys or girls above or below the BWM; $P = 0.05/4 = 0.0125$). For the TI and TREC models, month of birth was also included as a marker for season because previous work has shown that the TI of rural Bangladeshi infants varies by month (20). Body weight at the time of TI measurement (birth weight was used at 1 wk) was also used in a second model as an allometric adjustment. T-cell concentrations were strongly associated with both month and season of blood collection at 2 y (data not shown). As a result, season (at each age) was included in T-cell models to adjust for this variability using a previous report of seasons in Bangladesh: monsoon (July–September), winter (October–February), and hot/dry (March–June) (20).

Results

Infant and maternal characteristics

Of the 306 recruited infants, 27% had birth weights <2500 g, 7.6% were <37 wk gestational age, and 60% were delivered by elective cesarean delivery (**Table 1**). These characteristics were balanced by treatment group, except the cesarean delivery rate was higher ($P < 0.05$) in the PL than in the VA group (Table 1). All infants were exclusively or partially breastfed through 15 wk: 81% of children were exclusively breastfed at 1 wk and this rate decreased to 43% at 15 wk but did not differ by treatment group (**Supplemental Table 1**). The prevalence of moderate to severe malnutrition at 6, 10, and 15 wk varied from 4% to 7% for wasting, 11% to 17% for stunting, and 10% to 12% for underweight, but did not differ by treatment group (Supplemental Table 1). Infants were examined for bulging fontanelles as a potential adverse effect but none were observed in either the VA (0 of 153) or PL (0 of 152) groups. One infant died in the VA group and 1 in the PL group during the initial 15-wk follow-up period (12). A follow-up visit was conducted at ~2 y (median age: 27 mo) where 265 children were re-enrolled (87% of the 306 infants enrolled at birth); subject characteristics were similar to baseline (**Supplemental Table 2**). Maternal education, age, weight, height, BMI, parity, VA status, and prevalence of anemia did not vary by treatment group, and 19% of mothers overall were VA insufficient 6 wk postpartum (serum retinol < 1.05 μmol/L), although only 2.5% were deficient (<0.70 μmol/L; **Supplemental Table 3**).

VAS and thymus size

TI was measured at 1, 6, 10, and 15 wk of age. VA treatment at birth did not have an overall effect on TI at all ages. However, a significant treatment-by-age interaction was seen (**Supplemental Table 4**; **Figure 1**A) with post-hoc analysis showing a marginal effect at 1 wk ($P = 0.086$), with the TI being 7.0% lower in the VA than the PL group, and a significant effect at 6 wk ($P = 0.029$), with the TI being 7.8% lower in the VA than the PL group. TI means in the VA group did not differ significantly from means in the PL group thereafter, being just 3.3% lower ($P = 0.36$) and 0.8% higher ($P = 0.83$) in the VA than in the PL group at 10 and 15 wk, respectively. Overall TI means differed significantly from one another at each age in this analysis (adjusted data not shown). TI is often allometrically scaled (19, 20) to adjust for body size but because neonatal high-dose VA may affect growth (30–32) we did not include postintervention body weight in our main statistical model, because this might obscure an effect of treatment on TI if growth were also affected by the VA intervention. However, we performed a secondary analysis to determine if the VA effect on TI was independent of body weight (Supplemental Table 4, Figure 1B) by including body weight at the time of TI measurement in the model. The magnitude of the VA effect at 6 wk in the second model (the adjusted mean values in the VA and PL groups were 7.56 and 8.05 cm^2, respectively; difference = 0.491 cm^2) was lower by 19% than in the first model (where the values were 7.18 and 7.78 cm^2, respectively; difference = 0.604 cm^2), indicating that the effect of VA on TI was largely but not completely independent of possible effects of VA on body weight. TI also differed significantly by sex (Supplemental Table 4, Figure 1C), with TI being lower in girls than in boys at all time points.

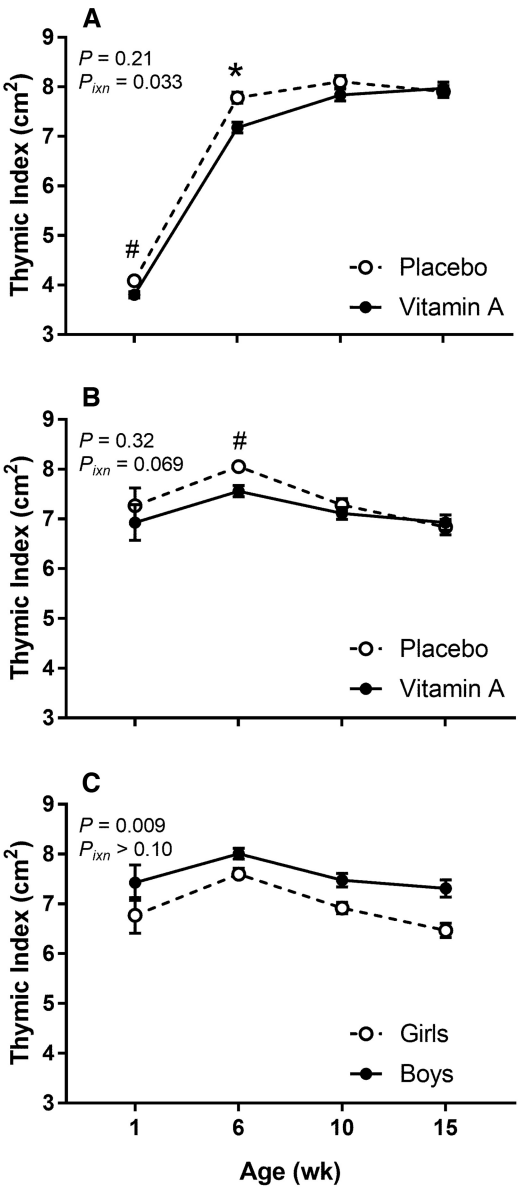

FIGURE 1 Mean ± SE thymic index between 1 and 15 wk of age by treatment group (A), treatment group adjusted for body weight at each age (B), and sex (C) in study infants receiving vitamin A (50,000 IU) or placebo capsules within 48 h of birth. *P* values indicate overall effect of treatment (A, B) or sex (C). Interaction *P* values (P_{ixn}) are shown if $P < 0.10$ and indicate interactions of age with treatment group or sex. *,#Statistical significance of group differences at each age: $^{*}P < 0.05$; $^{\#}P < 0.10$. (A, C) Values are mean ± SE, $n = 146$, 143, 142, and 143 for boys and $n = 145$, 146, 146, and 146 for girls at 1, 6, 10, and 15 wk, respectively. (B, D) Values are mean ± SE, $n = 146$, 147, 146, and 145 for the placebo group and $n = 145$, 142, 142, and 144 for the vitamin A group at 1, 6, 10, and 15 wk, respectively. Least-square means derived from the statistical analysis models (see Supplemental Table 4 and Methods) were used and the means shown here (back-transformed from square root) are thus adjusted for all covariates in the model.

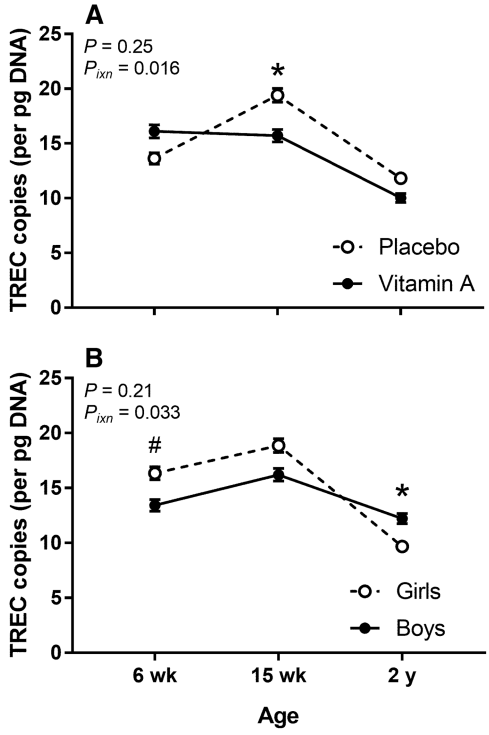

FIGURE 2 Mean ± SE TREC concentrations in peripheral blood mononuclear cells at 6 wk, 15 wk, and 2 y of age by treatment group (A) and sex (B) in study infants receiving vitamin A (50,000 IU) or placebo capsules within 48 h of birth. *P* values indicate overall effect of (A) treatment or (B) sex. Interaction *P* values (P_{ixn}) are shown if < 0.10 and indicate interactions of age with treatment group or sex. *,#Statistical significance of group differences at each age: $^{*}P < 0.05$; $^{\#}P < 0.10$. (A) Values are mean ± SE, $n = 124$, 124, and 127 for the placebo group and $n = 114$, 119, and 128 for the vitamin A group at 6 wk, 10 wk, and 2 y, respectively. (B) Values are mean ± SE, $n = 117$, 120, and 125 for boys and $n = 121$, 123, and 133 for girls at 6 wk, 15 wk, and 2 y, respectively. Least-square means derived from the statistical analysis models (see Supplemental Table 4 and Methods) were used and the means shown here (back-transformed from square root) are thus adjusted for all covariates in the model. TREC, T-cell receptor excision circle.

VAS and TREC concentrations

TREC concentrations were measured at 6 wk, 15 wk, and 2 y of age. VA treatment did not have an overall effect ($P = 0.25$) on TREC concentrations across all ages. However, a significant treatment-by-age interaction ($P = 0.016$) was seen (Supplemental Table 4; Figure 2A), with post-hoc analysis showing that VA treatment significantly ($P = 0.029$) decreased mean TREC concentrations at 15 wk but differences at other ages were not statistically significant ($P = 0.41$ and $P = 0.22$ at 15 wk and 2 y, respectively). TREC concentrations in the VA group were 18.1% higher, 19.0% lower, and 15.2% lower than in the PL group at 6 wk, 15 wk, and 2 y of age, respectively. TREC values also differed by sex (Figure 2B), with girls having marginally ($P = 0.061$) higher concentrations than boys at 6 wk, whereas the opposite was seen at 2 y ($P = 0.034$). TREC concentrations differed from one another across all ages (adjusted data not shown).

VA treatment and T-cell concentrations

VA treatment did not have an overall statistically significant effect on T cells ($P = 0.10$), naïve T cells ($P = 0.092$; Figure 3B), CD4 T cells ($P = 0.11$), or naïve CD4 T cells ($P = 0.10$; Figure 3D) (**Supplemental Table 5**), although mean concentrations in the VA group were always lower than in the PL group at each age (Figure 3B, D). For naïve T cells, the adjusted, back-transformed mean ± SE concentrations at 6, 10, and 15 wk of age in the PL group were 3259 ± 43.2,

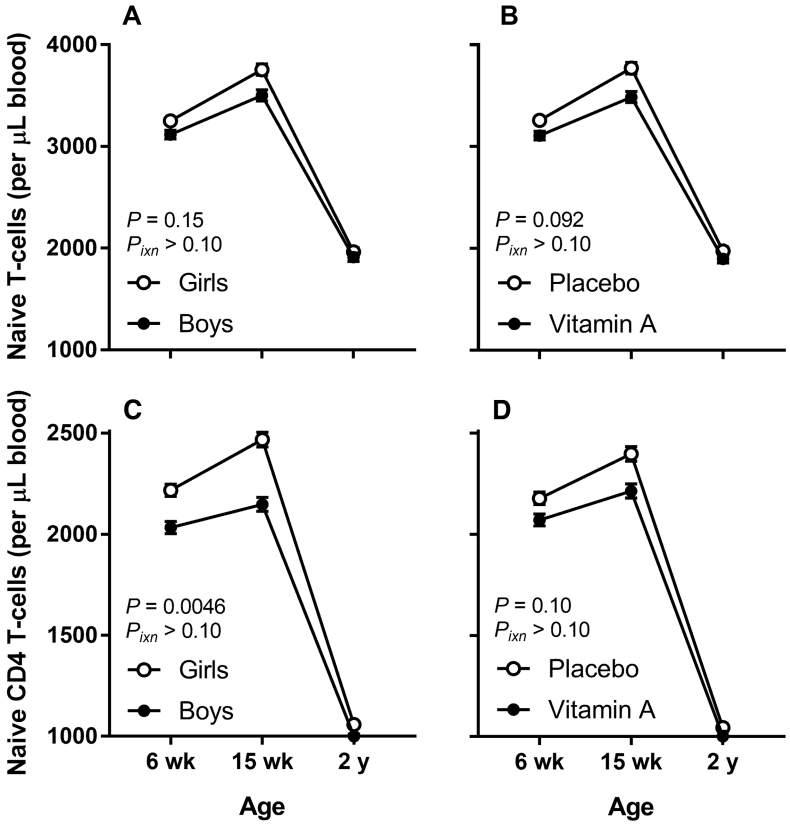

FIGURE 3 Mean ± SE peripheral blood total naïve T-cell concentrations (A, B) and naïve CD4 T-cell concentrations (C, D) by treatment group (B, D) and sex (A, C) at 6 wk, 15 wk, and 2 y of age in study infants receiving vitamin A (50,000 IU) or placebo capsules within 48 h of birth. *P* values indicate overall effect of sex (A, C) or treatment (B, D). Interaction *P* values (P_{ixn}) are shown if < 0.10 and indicate interactions of age with treatment group or sex. (A, C) Values are mean ± SE, $n = 142$, 140, and 132 for girls and $n = 134$, 143, and 126 for boys at 6 wk, 15 wk, and 2 y, respectively. (B, D) Values are mean ± SE, $n = 140$, 144, and 131 for the vitamin A group and $n = 136$, 139, and 127 for the placebo group at 6 wk, 15 wk, and 2 y of age, respectively. Least-square means derived from the statistical analysis models (see Supplemental Table 4 and Methods) were used and the means shown here (back-transformed from square root) are thus adjusted for all covariates in the model.

3771 ± 55.9, and 1976 ± 40.2 cells/μL, respectively, whereas in the VA group the corresponding means were 3109 ± 41.9, 3487 ± 54.0, and 1898 ± 39.3 cells/μL. In addition, a significant 3-way interaction (interaction *P* value < 0.10) of treatment group with sex and BWM category was seen for all 4 T-cell variables (Supplemental Table 5). However, post-hoc comparisons revealed only nonsignificant ($P > 0.0125$; using Bonferroni adjustment) differences between the PL and VA groups for girls with a birth weight below the median for CD4 T cells ($P = 0.025$) and for naïve CD4 T cells ($P = 0.020$), with T-cell concentrations being lower in the VA than in the PL groups in both cases (data not shown). Whereas total T cells and naïve T cells (Figure 3A) did not differ by sex, CD4 and naïve CD4 T-cell concentrations (Figure 3C) were higher in girls than in boys, independently of age (Supplemental Table 5).

Discussion

The thymus is the source of naïve T cells, which develop into effector and memory T cells during responses to infection and immunization (33). Such T-cell responses are important in preventing death from common childhood infections. Malnutrition, including VAD, adversely affects thymic function (6, 34) as well as thymus size (19, 20). A smaller thymus at birth (35), or later in infancy (36, 37), is associated with an increased risk of death. For thes reasons, we examined 3 measures of thymic function in the present study: thymus size using TI, concentration of recent thymic emigrant T cells in blood using TREC concentrations, and naïve T-cell concentrations in peripheral blood.

VAS at birth did not have an overall effect on TI, but decreased TI transiently at 6 wk of age. We had hypothesized that VA would increase TI because of the known positive effects of VA on thymocyte development and T-cell survival, as recently reviewed (38). However, retinoic acid, the principal active metabolite of VA, can act to increase apoptosis in thymocytes (39) and might thus decrease thymus size. High-dose VA (40) and retinoic acid treatment (41) of pregnant mice cause thymic abnormalities in the fetus and 1 case report indicates retinoic acid exposure in utero caused thymic hypoplasia and low T-cell concentrations in a human infant (42). It is thus plausible that high-dose VA postpartum could decrease thymus size in young infants and we speculate that assessment of thymic function might be a useful approach to evaluate potential postpartum toxicity. The timing of the effect in this study was consistent with a transient effect of the high dose of VA given at birth: at 1 wk there was an apparent (but not statistically significant) decrease of the TI in the VA group by 7% relative to the PL group. The effect size was similar (8%) at 6 wk when the difference was statistically significant, then the differences diminished progressively thereafter. This timing suggests a relatively rapid, although transient, effect of VA on reducing the size of the thymus which could result in a subsequent

decrease in TREC concentrations and the concentration of naïve T cells in peripheral blood. When the TI data were scaled allometrically, the magnitude (and statistical significance) of the group differences diminished somewhat (by 19% at 6 wk). However, because body size may itself be affected by a high-dose VA supplement (30–32), we still conclude that thymus size was transiently decreased by the VA treatment.

It is worth considering whether this difference in TI between the PL and VA groups might confer any increase in risk of death in the VA group, because a smaller TI has been associated with a higher risk of death (as aforementioned). An earlier study from Bangladesh showed that infants with a larger thymus at 8 wk of age had a lower risk of death (OR = 0.32 per 1-SD difference in TI; table 2A of the cited reference) from infectious disease in the first year of life (37). In other words, a child with a larger TI (by 1 SD) would have 68% lower odds of dying from an infection. In our study, the difference in TI between the PL and VA groups at 6 wk of age was ~0.13 SD. It is possible that such a difference could also indicate a differential risk of death, at least in a population with high infant mortality. On the other hand, the negative effect of VAS on TI was transient, suggesting that any increase in risk may be transient as well. In the present study, such an increase in risk would be negligible because the overall mortality rate was quite low. In summary, this finding, and the negative effects of retinoic acid and high-dose VA seen in utero (as aforementioned), both suggest that evaluation of thymic function may be useful to identify potential adverse effects of VAS in well-nourished populations.

VAS at birth did not have an overall effect on TREC concentrations, but TREC concentrations were significantly lower in the VA than in the PL group (by a magnitude of 19%) at 1 time point, 15 wk of age, suggesting a lower concentration of recent thymic emigrant T cells in blood. The timing of this observation is consistent with the smaller TI seen at 6 wk of age, which could result in a lower output of naïve T-cells that might not be evident in blood until 15 wk. We do not have another TREC measurement until 2 y of age, when there was no difference between the groups, but we speculate that TREC concentrations recovered relatively quickly because thymus size was equivalent between the VA and PL groups by 15 wk of age.

The TREC data indicate that there was a transient decrease in the thymic output of naïve T cells into peripheral blood that was detectable at 15 wk of age. We thus might expect to see lower naïve T-cell concentrations in the peripheral blood of the VA compared with the PL group, if the decreased thymic output were of sufficient magnitude. Although naïve T-cell concentrations were somewhat lower in the VA than in the PL group at each age, this overall difference of 5.5% was not statistically significant ($P = 0.092$). This finding suggests that the magnitude of the effect on thymic function was not sufficient to affect the body's reservoir of naïve T cells, at least to the extent where this would be reflected by peripheral blood concentrations of these cells. In addition, it is worth noting that both the mean TREC concentrations and mean T-cell concentrations of these infants are well within normal reference ranges for healthy infants (43), suggesting that any differences seen between the PL and VA groups are unlikely to be associated with an increase in risk.

Sex differences were seen in thymic function and peripheral blood CD4 T-cell concentrations. Girls had smaller thymuses than boys in early infancy, even when adjusted for body weight. However, TREC concentrations tended to be higher in girls than in boys at 6 wk of age, although the difference was not statistically significant ($P = 0.061$). At 2 y, however, TREC concentrations were lower in girls than in boys. However, naïve T-cell concentrations (which includes both CD4 and CD8 T cells) did not differ by sex at any age, although both total and naïve CD4 T-cell concentrations were higher in girls than in boys at all ages, suggesting that thymic output (or peripheral survival or distribution) of these cells differed by sex. The occurrence of these differences in early infancy is interesting because girls and boys transiently have increased concentrations of estrogen or testosterone, respectively, at this age (44, 45). Estrogen has recently been found to decrease thymic deletion of T cells (46), which could account for the higher concentrations of CD4 T cells seen in girls in the present study. Whether such a transient effect would have a long-lasting effect on adaptive immunity is not clear. It is also worth noting that thymic function is sensitive to other environmental factors, such as stress, in a sex-specific manner. For example, in these same infants we have reported that an elevated stress response, as indicated by a higher cortisol response to a pain stimulus (vaccination), is associated with a smaller TI in boys, and with lower naïve CD4 T-cell concentrations in boys and girls (25).

Although VAS did not improve thymic function as we had hypothesized, it is worth noting that VAS did have beneficial effects on development of the intestinal microbiome in this cohort (47). Bifidobacteria are important commensals in infancy and we found that boys in this cohort had a lower relative abundance of bifidobacteria than girls. However, boys in the VA group had significantly higher bifidobacteria abundance than did boys in the PL group, suggesting that VAS beneficially affected development of the intestinal microbiome, perhaps via effects on the intestinal immune system. Higher bifidobacteria abundance may have survival benefits for children in a population with a high risk of death from infectious diseases. Specifically, we recently reported (48) from this cohort that a higher abundance of bifidobacteria in early infancy, when key childhood vaccines are given, correlates positively with higher responses to these vaccines when measured at 2–3 y of age. This finding suggests that bifidobacteria enhance immunologic memory which would improve resistance to potentially life-threatening infections.

Our study was initiated in conjunction with, but before completion of, 3 recent trials that evaluated the effect of neonatal VAS on infant mortality (9–11) and was designed to evaluate effects of the same intervention (50,000 IU within 48 h of birth) on measures of immune function known to be sensitive to VA which we felt could plausibly affect infant survival in the context of a high risk of death from infectious diseases. Results of these and other neonatal VAS trials have recently been evaluated in a meta-analysis that used participant-level data to evaluate contextual differences between the 11 published trials (14). Although no overall decrease in 6-mo mortality was seen from these 11 trials, the authors of this meta-analysis identified several factors associated with the effect of VAS on mortality at 6 mo of age. Reviewing these factors will help to define the applicability of our results to other settings. One factor associated with a difference in effect of VAS on mortality was geographic region: trials in south Asia, including rural Bangladesh, showed that VAS decreased mortality, whereas no benefit (and a trend toward increased risk) was seen in Africa. Although the present study was conducted in Bangladesh, it was in an urban population in Dhaka where mothers and infants were receiving good medical care pre- and postpartum; such care is much more limited in rural areas of the country (27), including the sites where the neonatal VAS mortality studies have been conducted in Bangladesh (13). Another factor

was maternal VAD; the 3 trials (all in Asia) with ≥10% of mothers at baseline with VAD (serum retinol <0.7 μmol/L) showed lower mortality with VAS, whereas the others did not. Although we did not assess maternal VA status at baseline in the present study, we do have plasma retinol from mothers at 6 wk postpartum and only 2.5% were deficient, whereas 19% had insufficient status. Maternal education was a factor as well, with a benefit of VAS seen in populations where >32% of mothers reported no schooling. In our population maternal education was quite different, with the median number of years of education being 9. Finally, VAS decreased mortality in settings with higher (≥30/1000) as compared with lower (<30/1000) infant mortality by 6 mo in the control groups, or in settings where ≥75% of infant deaths occurred by 6 mo. In our study we do not have directly comparable data but because only 2 deaths occurred by 15 wk of age (a rate of 6/1000), the rate in 6 mo would certainly be <30/1000. However, the overall infant mortality in urban settings in Bangladesh is 34/1000 compared with 40/1000 in rural areas (27), so neonatal VAS might be beneficial in some urban settings in Bangladesh (based on this single criterion). In summary, the present study population shows major contextual differences from the studies where decreased mortality was seen with neonatal VAS. In particular, our study population was in an urban area where mothers had higher levels of education, better nutritional status, and better access to medical care than in rural areas of Bangladesh where the benefits of VAS have been seen.

Given these context differences, particularly in VA status, it is reasonable to ask whether different results, perhaps the improvement in thymic function that we hypothesized would occur in infants at greater risk of VAD, would have been found in a population of infants with a higher prevalence of maternal VAD. This is plausible, although we don't know the answer to that question. Newborn infants are born with low VA stores even in areas with a very low risk of maternal VAD (49, 50) and we felt at the outset that neonatal VAS might have immunological benefits even in infants with a relatively low risk of VAD, although that was not the case in the present study. The transient decreases in TI and TRECs were not accompanied by decreases in T-cell concentrations (although there was a trend in that direction, including in girls with birth weights below the median) and we thus speculate that no permanent change in thymic function resulted from this intervention. Finally, urban settings such as Dhaka have been and may continue to be sites of maternal and child VAS programs (27) and evaluating the impact of VAS on infant health, including aspects of immune function, provides useful information for evaluating the potential impact on the recipients of such interventions.

Acknowledgments

We thank Jan Peerson for providing statistical support and Xiaowen Jang for providing laboratory support. The authors' responsibilities were as follows—SMA, RR, and FQ: were co-investigators of the study and contributed to the development of the study protocol; SMA: directed research activities at the clinical site and in the laboratory in Bangladesh, oversaw the overall study operations including providing feedback to the ethical committee, data and safety monitoring board, and funders, and mentored MNH to perform laboratory analysis; MNH: oversaw participant enrolment and follow-up, data collection, and performed laboratory analysis; MJA, MM, and TA: participated in the data collection, laboratory analysis, and editing of the manuscript; YW: consulted on the study design and oversaw the training of the ultrasound specialist for assessment of the thymic index; MSZ: performed statistical analysis; CBS: was principal investigator, helped develop the study protocol, and performed and oversaw the statistical analysis and manuscript writing; and all authors: read and approved the final manuscript.

References

1. Mellanby E, Green HN. Vitamin A as an anti-infective agent: its use in the treatment of puerperal septicaemia. Br Med J 1929;1:984–6.
2. Semba RD. Vitamin A as "anti-infective" therapy, 1920–1940. J Nutr 1999;129:783–91.
3. Imdad A, Mayo-Wilson E, Herzer K, Bhutta ZA. Vitamin A supplementation for preventing morbidity and mortality in children from six months to five years of age. Cochrane Database Syst Rev 2017;3:CD008524.
4. Brown CC, Noelle RJ. Seeing through the dark: new insights into the immune regulatory functions of vitamin A. Eur J Immunol 2015;45:1287–95.
5. Larange A, Cheroutre H. Retinoic acid and retinoic acid receptors as pleiotropic modulators of the immune system. Annu Rev Immunol 2016;34:369–94.
6. Stephensen CB. Vitamin A, infection, and immune function. Annu Rev Nutr 2001;21:167–92.
7. Benn CS, Diness BR, Roth A, Nante E, Fisker AB, Lisse IM, Yazdanbakhsh M, Whittle H, Rodrigues A, Aaby P. Effect of 50,000 IU vitamin A given with BCG vaccine on mortality in infants in Guinea-Bissau: randomised placebo controlled trial. BMJ 2008;336:1416–20.
8. Haider BA, Sharma R, Bhutta ZA. Neonatal vitamin A supplementation for the prevention of mortality and morbidity in term neonates in low and middle income countries. Cochrane Database Syst Rev 2017;2:CD006980.
9. Edmond KM, Newton S, Shannon C, O'Leary M, Hurt L, Thomas G, Amenga-Etego S, Tawiah-Agyemang C, Gram L, Hurt CN, et al. Effect of early neonatal vitamin A supplementation on mortality during infancy in Ghana (Neovita): a randomised, double-blind, placebo-controlled trial. Lancet 2014;385:1315–23.
10. Masanja H, Smith ER, Muhihi A, Briegleb C, Mshamu S, Ruben J, Noor RA, Khudyakov P, Yoshida S, Martines J, et al. Effect of neonatal vitamin A supplementation on mortality in infants in Tanzania (Neovita): a randomised, double-blind, placebo-controlled trial. Lancet 2014;385:1324–32.
11. Mazumder S, Taneja S, Bhatia K, Yoshida S, Kaur J, Dube B, Toteja GS, Bahl R, Fontaine O, Martines J, et al. Efficacy of early neonatal supplementation with vitamin A to reduce mortality in infancy in Haryana, India (Neovita): a randomised, double-blind, placebo-controlled trial. Lancet 2015;385:1333–42.
12. Ahmad SM, Raqib R, Qadri F, Stephensen CB. The effect of newborn vitamin A supplementation on infant immune functions: trial design, interventions, and baseline data. Contemp Clin Trials 2014;39:269–79.
13. Klemm RD, Labrique AB, Christian P, Rashid M, Shamim AA, Katz J, Sommer A, West KP, Jr. Newborn vitamin A supplementation reduced infant mortality in rural Bangladesh. Pediatrics 2008;122:e242–50.
14. West KP, Wu LS, Ali H, Klemm RDW, Edmond KM, Hurt L, Kirkwood B, Newton S, Shannon C, Taneja S, et al. Early neonatal vitamin A supplementation and infant mortality: an individual participant data meta-analysis of randomised controlled trials. Arch Dis Child 2019;104:217–26.
15. Ahmad SM, Haskell MJ, Raqib R, Stephensen CB. Vitamin A status is associated with T-cell responses in Bangladeshi men. Br J Nutr 2009;102:797–802.
16. Stephensen CB, Rasooly R, Jiang XW, Ceddia MA, Weaver CT, Chandraratna RAS, Bucy RP. Vitamin A enhances in vitro Th2 development via retinoid X receptor pathway. J Immunol 2002;168:4495–503.
17. Kurd N, Robey EA. T-cell selection in the thymus: a spatial and temporal perspective. Immunol Rev 2016;271:114–26.
18. Chevalier P, Sevilla R, Sejas E, Zalles L, Belmonte G, Parent G. Immune recovery of malnourished children takes longer than nutritional recovery: implications for treatment and discharge. J Trop Pediatr 1998;44:304–7.

19. Collinson AC, Moore SE, Cole TJ, Prentice AM. Birth season and environmental influences on patterns of thymic growth in rural Gambian infants. Acta Paediatr 2003;92:1014–20.
20. Moore SE, Prentice AM, Wagatsuma Y, Fulford AJ, Collinson AC, Raqib R, Vahter M, Persson LA, Arifeen SE. Early-life nutritional and environmental determinants of thymic size in infants born in rural Bangladesh. Acta Paediatr 2009;98:1168–75.
21. Moore SE, Collinson AC, Tamba N'Gom P, Aspinall R, Prentice AM. Early immunological development and mortality from infectious disease in later life. Proc Nutr Soc 2006;65:311–18.
22. Stephensen CB, Borowsky AD, Lloyd KC. Disruption of *Rxra* gene in thymocytes and T lymphocytes modestly alters lymphocyte frequencies, proliferation, survival and T helper type 1/type 2 balance. Immunology 2007;121:484–98.
23. Zhou X, Wang W, Yang Y. The expression of retinoic acid receptors in thymus of young children and the effect of all-transretinoic acid on the development of T cells in thymus. J Clin Immunol 2008;28: 85–91.
24. Ribeiro RM, Perelson AS. Determining thymic output quantitatively: using models to interpret experimental T-cell receptor excision circle (TREC) data. Immunol Rev 2007;216:21–34.
25. Huda MN, Ahmad SM, Alam MJ, Khanam A, Afsar MNA, Wagatsuma Y, Raqib R, Stephensen CB, Laugero KD. Infant cortisol stress-response is associated with thymic function and vaccine response. Stress 2018;22(1):36–43.
26. WHO. WHO Anthro for personal computers: software for assessing growth and development of the world's children. [Internet]. Version 3.2.2. Geneva: WHO; 2011. Available from: http://www.who.int/childgrowth/software/en/.
27. National Institute of Population Research and Training (NIPORT), Mitra and Associates, and ICF International. Bangladesh Demographic and Health Survey 2014. Dhaka, Bangladesh, and Rockville, MD: NIPORT, Mitra and Associates, and ICF International; 2016.
28. Hasselbalch H, Nielsen MB, Jeppesen D, Pedersen JF, Karkov J. Sonographic measurement of the thymus in infants. Eur Radiol 1996;6:700–3.
29. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, Polis MA, Haase AT, Feinberg MB, Sullivan JL, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature 1998;396:690–5.
30. Fisker AB, Benn CS, Diness BR, Martins C, Rodrigues A, Aaby P, Bibby BM. The effect of 50 000 IU vitamin A with BCG vaccine at birth on growth in the first year of life. J Trop Med 2011:570170.
31. Gannon BM, Davis CR, Nair N, Grahn M, Tanumihardjo SA. Single high-dose vitamin A supplementation to neonatal piglets results in a transient dose response in extrahepatic organs and sustained increases in liver stores. J Nutr 2017;147:798–806.
32. Humphrey JH, Agoestina T, Juliana A, Septiana S, Widjaja H, Cerreto MC, Wu LS, Ichord RN, Katz J, West KP, Jr. Neonatal vitamin A supplementation: effect on development and growth at 3 y of age. Am J Clin Nutr 1998;68:109–17.
33. Murphy K, Weaver C. Janeway's immunobiology. 9th ed. New York, NY: Garland Science/Taylor & Francis Group, LLC; 2016.
34. Savino W, Dardenne M. Nutritional imbalances and infections affect the thymus: consequences on T-cell-mediated immune responses. Proc Nutr Soc 2010;69:636–43.
35. Aaby P, Marx C, Trautner S, Rudaa D, Hasselbalch H, Jensen H, Lisse I. Thymus size at birth is associated with infant mortality: a community study from Guinea-Bissau. Acta Paediatr 2002;91:698–703.
36. Garly ML, Trautner SL, Marx C, Danebod K, Nielsen J, Ravn H, Martins CL, Bale C, Aaby P, Lisse IM. Thymus size at 6 months of age and subsequent child mortality. J Pediatr 2008;153:683–8 .e3.
37. Moore SE, Fulford AJ, Wagatsuma Y, Persson LA, Arifeen SE, Prentice AM. Thymus development and infant and child mortality in rural Bangladesh. Int J Epidemiol 2014;43:216–23.
38. Bono MR, Tejon G, Flores-Santibañez F, Fernandez D, Rosemblatt M, Sauma D. Retinoic acid as a modulator of T cell immunity. Nutrients 2016;8:E349.
39. Szondy Z, Reichert U, Bernardon JM, Michel S, Toth R, Ancian P, Ajzner E, Fesus L. Induction of apoptosis by retinoids and retinoic acid receptor γ-selective compounds in mouse thymocytes through a novel apoptosis pathway. Mol Pharmacol 1997;51:972–82.
40. Mulder GB, Manley N, Grant J, Schmidt K, Zeng W, Eckhoff C, Maggio-Price L. Effects of excess vitamin A on development of cranial neural crest-derived structures: a neonatal and embryologic study. Teratology 2000;62:214–26.
41. Mulder GB, Manley N, Maggio-Price L. Retinoic acid-induced thymic abnormalities in the mouse are associated with altered pharyngeal morphology, thymocyte maturation defects, and altered expression of *Hoxa3* and *Pax1*. Teratology 1998;58:263–75.
42. Cohen M, Rubinstein A, Li JK, Nathenson G. Thymic hypoplasia associated with isotretinoin embryopathy. Am J Dis Child 1987;141:263–6.
43. Garcia-Prat M, Álvarez-Sierra D, Aguiló-Cucurull A, Salgado-Perandrés S, Briongos-Sebastian S, Franco-Jarava C, Martin-Nalda A, Colobran R, Montserrat I, Hernández-González M, et al. Extended immunophenotyping reference values in a healthy pediatric population. Cytometry B Clin Cytom 2019;96(3):223–33.
44. Kliegman RM, Stanton BMD, St Geme J, Schor NF. Nelson textbook of pediatrics: expert consult. 20th ed. Saint Louis. MO: Elsevier Health Sciences; 2015.
45. Lamminmäki A, Hines M, Kuiri-Hänninen T, Kilpeläinen L, Dunkel L, Sankilampi U. Testosterone measured in infancy predicts subsequent sex-typed behavior in boys and in girls. Horm Behav 2012;61:611–16.
46. Dragin N, Bismuth J, Cizeron-Clairac G, Biferi MG, Berthault C, Serraf A, Nottin R, Klatzmann D, Cumano A, Barkats M, et al. Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. J Clin Invest 2016;126:1525–37.
47. Huda MN, Ahmad SM, Kalanetra KM, Taft DH, Alam MJ, Khanam A, Raqib R, Underwood MA, Mills DA, Stephensen CB. Neonatal vitamin A supplementation and vitamin A status are associated with gut microbiome composition in Bangladeshi infants in early infancy and at 2 years of age. J Nutr 2019;149:1075–88.
48. Huda MN, Ahmad SM, Alam MJ, Khanam A, Kalanetra KM, Taft DH, Raqib R, Underwood MA, Mills DA, Stephensen CB. *Bifidobacterium* abundance in early infancy and vaccine response at 2 years of age. Pediatrics 2019;143:e20181489.
49. Olson JA, Gunning DB, Tilton RA. Liver concentrations of vitamin A and carotenoids, as a function of age and other parameters, of American children who died of various causes. Am J Clin Nutr 1984;39:903–10.
50. Olson JA. Liver vitamin A reserves of neonates, preschool children and adults dying of various causes in Salvador, Brazil. Arch Latinoam Nutr 1979;29:521–45.