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Relationship of thymic area with clinical-epidemiological variables and values of T-lymphocyte subpopulations in peripheral blood of children with recurrent infections

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Abstract

Background Recurrent infections in childhood are the main cause of remission to the immunology service. T lymphocytes generated in the thymus are essential for fighting infection, making the thymus area an important predictor of the immune system's competence. This study aimed to identify the possible relationship of the thymic area with clinical-epidemiological variables and values of subpopulations of T lymphocytes in the peripheral blood of children with recurrent infections.

Methods We conducted applied research using a transversal analytical design at the National Medical Genetics Center (Havana, Cuba), from January to August 2022. The study covered 73 children of which we analyzed clinical-epidemiological variables and the size of the thymus through ultrasound. Furthermore, we determined the relative and absolute values of the subpopulations of T cells using flow cytometry.

Results Of the children studied, 65.8% had thymic hypoplasia. The children who breastfed for less than 6 months showed four times the risk of developing moderate-severe thymus hypoplasia (*OR* = 3.90, 95% *CI*: 1.21–12.61). A direct relationship was found between the area of the thymus and the child's size ($r = 0.238$, $p = 0.043$) and weight ($r = 0.233$, $p = 0.047$). The relative values of CD3+ T lymphocytes decreased in the cases of mild hypoplasia ($p = 0.018$) and moderate-severe hypoplasia ($p = 0.049$). The thymus area was associated with the absolute cell count of CD8+ effector memory T cells ($rs = -0.263$, $p = 0.024$) and of the central memory T cells ($r = -0.283$, $p = 0.015$).

Conclusions Breastfeeding for less than 6 months, as well as the weight and size of the child, are related to their thymus area. The subpopulation values of T lymphocytes detected suggest that patients with thymic hypoplasia develop a contraction of CD3+ T cells, which can make them more vulnerable to infectious processes. This finding

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was combined with an expansion of the memory compartments of the subpopulations of CD8+ T cells, suggesting a greater susceptibility to intracellular viral and bacterial infections in these cases.

Keywords T lymphocytes, Thymus, Flow cytometry, Lymphocyte subgroups, Recurrent infection

Background

Recurrent infections in children may be the most noticeable expression of a possible cellular immunodeficiency [1]. The generation of T lymphocytes occurs in the thymus gland, after the arrival of lymphoid precursors that migrate from the bone marrow and the blood, towards the thymus where they proliferate, differentiate, and go through selection processes leading to the development of mature T cells [2]. After completing the central tolerance induction process, mature T lymphocytes migrate from the thymus, through the bloodstream and the lymphatic vessels, to secondary lymphoid organs. Certain studies have reported that the number of T lymphocytes in the peripheral blood offers relevant information on the development and function of the immune system during childhood [3–5]

The thymic area at birth is determined by a combination of prenatal and perinatal genetic and environmental factors [6]. A practical approach to evaluate the variability of the size of the thymus is through ultrasound. Studies estimating the area of the thymus have concluded that measuring the gland is reproducible and simple [7–9].

The area of the thymus can be an important predictor of the competence of the immune system [7, 10–14] in Cuban children between 6 months and 6 years of age with recurrent infections. However, the exact significance of its normal or altered size during childhood, in relation to clinical-epidemiological variables and the values of subpopulations of T lymphocytes in the peripheral blood, is unknown.

The identification of clinical-epidemiological variables related to the size of the thymus gland would make it possible to design prevention and promotion strategies that could contribute to the development of a more competent immune system. Moreover, the knowledge of the possible relationship of the thymic area with the values of the subpopulations of T cells in the peripheral blood would enable the implementation of mathematical models for their estimation, based on sonographic results. This application would be of great value in the design of an algorithm that would complement the assessment of clinical evolution during the treatment.

This study is aimed towards identifying the possible relationship of the normal or altered size of the thymus area with clinical-epidemiological variables and the values of the subpopulations of T lymphocytes in the peripheral blood of Cuban children ranging from 6 months to 6 years of age, who present recurrent infections.

Methods

Participants

A cross-sectional analytical design was used in an applied study covering the period from January to August of 2022. This study was carried out according to the Declaration of Helsinki principles [15]. It included 73 Cuban children attending the immunogenetics service of the National Medical Genetics Center (CNGM according to its Spanish acronym) in Havana because of their recurrent infections. The sample was divided into three groups according to the classification of thymus development, as a function of its total area [9, 16]. Hence, group 1 (G1), normal (thymic area of 1000–1500 mm^2); group 2 (G2), slight hypoplasia (thymic area of 800–999 mm^2); and group 3 (G3), moderate-severe hypoplasia (thymic area less than or equal to 799 mm^2). The latter was formed by subgroup 3a (G3a), moderate hypoplasia (thymic area of 500–799 mm^2) and subgroup 3b (G3b), and severe hypoplasia (thymic area equal to or lower than 499 mm^2). In the study groups, the sample was distributed as follows G1 = 25, G2 = 19, and G3 = 29 (G3a = 24 and G3b = 5). The patients within each group were selected according to the order of their arrival to the service.

Inclusion criteria were as follows: (1) age ranging from 6 months to 6 years of age and (2) and patients that presented one of the following recurrent infection criteria: (a) two or more severe infections in 1 year (with persistent evidence of inflammation, lack of response to oral antibiotics, and/or the need of intravenous antibiotics or hospitalization); (b) severe infection with an unusual pathogen or infections produced by bacteria, which normally do not produce disorders in children of the same age; (c) six or more infections of the respiratory tract (1 of which could be pneumonia, including severe pneumonia) during 1 year in children of 1–3 years of age; (d) five or more infections of the respiratory tract (1 of which could be pneumonia, including severe pneumonia) during 1 year in children of 3–6 years of age; (e) two mild events of pneumonia confirmed by clinical criteria and/or X-rays in 1 year; (f) completing more than four cycles of antibiotics in 1 year; and (g) and prophylactic therapy with antibiotics to prevent infections.

Exclusion criteria for patients included the following: (1) nutritional value less than percentile 3 and higher than percentile 97; (2) under steroid treatment for up to 45 days before the start of the study; (3) treatment with immunomodulators at the time of the study; (4) personal history of genetic chromosomal diseases and/or congenital malformation, of thymic neoplasia, leukemia, lymphoma, and or chronic hepatic diseases; (5) history of partial or total surgical treatment of the thymus; and (6) and those received mediastinal radiotherapy.

Thymus echography

We assessed the thymic area of the selected patients after receiving the informed consent of the parents and/or legal tutors. The area was measured using mediastinal echography, by the same researcher and echography method in all cases, at the "Dr. Ángel Arturo Aballí Arellano" Hospital. For the thymus echography, we used a real-time mobile SAL30A device with a flat surface linear pediatric transducer of 5 mHz. The parasternal line was the cutting plane used, and the area of the longitudinal echography section of both thymus lobules was determined between the upper edge of the second rib and the lower edge of the fourth rib [9, 13]

The thymus area was calculated using the following formula: $Total\ area\ of\ the\ thymus(mm^2) = (lengthRL \times widthRL) + (lengthLL \times widthLL)$, where RL refers to the right lobule and LL is the left lobule. We determined severe hypoplasia when the thymus area was < 500 mm^2, moderate hypoplasia when the range was of 500–799 mm^2, and slight hypoplasia at the range of 800–999 mm^2. The normal area was recorded as having values in the range of 1000–1500 mm^2, since values above 1500 mm^2 were classified as thymic hyperplasia.

Immunophenotyping using flow cytometry

Cellular immunophenotyping was made by flow cytometry (*eight-color Gallios flow cytometry, Beckman Coulter*, France) with peripheral blood obtained through venous puncture, using the anti-coagulant K2-EDTA and the lysis solution VersaLyse (*Beckman Coulter*, France). According to the manufacturer's recommendations (*Beckman Coulter*, France), we used a protocol of red blood cell lysis without washing [17]. To rapidly and accurately identify and count the subpopulations of T cells, we designed a polychromatic tube with six lymphocyte antigens. The following monoclonal antibodies conjugated with fluorochromosomes of *MACS Miltenyi Biotec* (*Germany*) were added: 0.5 µL of anti-CD45 PE-Vio770 (Clone 5B1), 2.5 µL of anti-CD3 PE (Clone BW264/56), 0.5 µL of anti-CD4 APC-Vio770 (Clone M-T466), 0.5 µL of anti-CD8 PerCP-Vio700 (Clone BW135/80), 1.0 µL of anti-CD45RA APC (Clone T6D11), and 1.0 µL of anti-CD27 FITC (Clone M-T271). To each volume of the conjugate, we added 100 µL of blood; they were mixed for 3 s and incubated in a dark chamber for 15 min at room temperature. Later, we included 1 mL of the lysis buffer VersaLyse™ (Beckman Coulter, Francia) and incubated this for 10 min under the same conditions as the previous step. Finally, the next step was to immediately acquire the sample through the cytometer. Quality control was carried out accordingly (see Supplementary material). For data acquisition, we used the Kaluza Acquisition v1.0 software through which we obtained a minimum of 50,000 total events. For the analysis and results output, we used Kaluza Analysis v1.5a. The absolute cell counts of the lymphocyte populations were made through a dual platform. A manual selection and sequential window strategy were designed with biparametric graphs (see Supplementary material).

Identification of subpopulations of T cells

The immunophenotypes of T cells were characterized by taking into account the antigenic marking on the lymphocyte selection window in the biparametric graph of CD45 vs SS (*side scatter*). We quantified the subpopulations of T lymphocytes $CD3^+$ ($CD45^+$, $CD3^+$), T $CD4^+$ ($CD45^+$, $CD3^+$, $CD4^+$) and T $CD8^+$ ($CD45^+$, $CD3^+$, $CD8^+$). Additionally, we quantified the extended immune phenotypes where we included the following: (a) *naïve* T cells, T $CD4^+$ *naïve* ($CD45^+$, $CD3^+$, $CD4^+$, $CD45RA^+$, $CD27^+$) and T $CD8^+$ *naïve* ($CD45^+$, $CD3^+$, $CD8^+$, $CD45RA^+$, $CD27^+$), (b) central memory T cells (T_{CM}), T_{CM} $CD4^+$ ($CD45^+$, $CD3^+$, $CD4^+$, $CD45RA^-$, $CD27^+$) and y T_{CM} $CD8^+$ ($CD45^+$, $CD3^+$, $CD8^+$, $CD45RA^-$, $CD27^+$), and (c) effector memory T cells (T_{EM}), T_{EM} $CD4^+$ ($CD45^+$, $CD3^+$, $CD4^+$, $CD45RA^-$, $CD27^-$) and T_{EM} $CD8^+$ ($CD45^+$, $CD3^+$, $CD8^+$, $CD45RA^-$, $CD27^-$), as well as terminally differentiated effector memory T cells known as T_{EMRA} cells (*T-effector memory re-expresses CD45RA*), T_{EMRA} $CD4^+$ ($CD45^+$, $CD3^+$, $CD4^+$, $CD45RA^+$, $CD27^-$), and T_{EMRA} $CD8^+$ ($CD45^+$, $CD3^+$, $CD8^+$, $CD45RA^+$, $CD27^-$).

Statistical analysis

The summary measures used here were percentage and proportion for qualitative variables. The comparison of the distribution of the qualitative variables was made through the Pearson χ^2 test. The odds ratio (OR) was used as a measure of association and their 95% confidence intervals (CI). The quantitative variables were analyzed through central trend measures of position and dispersion. We evaluated the normal distribution of the continuous quantitative variables using the Shapiro Wilks test from group samples with $n < 50$ and through the Kolmogorov-Smirnov test for samples with $n > 50$.

The variables with a normal distribution were expressed through their mean and standard deviation, while for those with a different type of distribution, we used the median and interquartile range (IQR). Univariate analyses were made using parametric methods (*t-test and ANOVA test*) and nonparametric methods (*Mann-Whitney U- and Kruskal-Wallis test*). The correlation coefficient between the values of the T-cell subpopulations identified by flow cytometry and other variables of interest was analyzed using parametric (*Pearson*) or nonparametric (*Spearman*) tests, accordingly. Multivariate analyses of logistic regressions were made to evaluate the influence of the type of delivery, weight at birth, duration of breastfeeding, size, and weight (adjusted by age and sex) on the development of the thymus according to their total area (normal, mild hypoplasia, and moderate-severe hypoplasia). Statistical significance was considered when $p < 0.05$. For data analysis, we used *IBM SPSS Statistics (version 26.0 for Windows, NY, USA)*. The graphs were obtained through *GraphPad Prism* (*version 9.0 for Windows, CA, USA)*.

Results

Demographic, clinical, and epidemiological characteristics

Table 1 shows the clinical-demographic and anthropometric characteristics of children with recurrent infections according to the area of the thymus. Hypoplasia of the thymus was found in 65.8% of the children, and half of these showed moderate thymic hypoplasia. The median (IQR, interquartile range) of the patients' age was 36 (22.5–50.5) months. The analysis related to the sex of the patients in the sample showed a male-to-female ratio of 1:1. The frequency of the cases considering white skin color was 44 (60.3%), while the number of patients who had black skin was similar to those of mixed ancestry, with 13 (17.8%) and 16 (21.9%) cases, respectively. The comparison of the distribution of birth weight did not reveal any difference between individuals with a normal thymus and any form of thymus hypoplasia. This contrasted with the distribution of the anthropometric variables of size (ANOVA: $F = 2.780$, $p = 0.048$) and weight (ANOVA: $F = 2.828$, $p = 0.045$). Differences were found for the means of the size of the children, between the groups of patients with mild and with moderate-severe hypoplasia at 2–4 years of age ($p = 0.031$), where the latter group had the smallest mean. The comparison of the distribution of weight according to the age group did not reflect the differences between the pairs of the groups of interest. The median time of pregnancy at birth of the children included in the study, which was 39.5 weeks, as well as the distribution of type of delivery, both showed similar behavior in groups of patients with normal or altered thymus areas. In 95.9% of the cases, the children were breastfed for a median period of 7 months (*IQR*: 3–12 months). Respiratory infections were the most frequent ones in all comparison groups, and affected 97.3% of the patients, followed by dermatological infections, which affected 38.4% of the cases. The rest of the recurrent infections are shown in Table 1, in the order of the highest to the lowest frequencies observed.

Association between the clinical variables and thymus area

The analysis of the relationship between the development of the thymus according to its area, and the duration of breastfeeding, is shown in Fig. 1. Of all patients breastfed for at least 6 months, 21.4% ($n = 6$) showed a normal thymus area, while 21.4% ($n = 6$) and 57.1% ($n = 16$) presented slight hypoplasia and moderate-severe hypoplasia of the thymus, respectively. In the case of children that were breastfed for 6 to 11 months, 50.0% ($n = 10$) showed a normal thymus size, 20.0% ($n = 4$) had a thymus size of between 800 and 999 mm^3, and 30.0% ($n = 6$) had a thymus size of less than 799 mm^3. In the sample, 34.2% ($n = 25$) were breastfed for 12 months or more. From this group, 36.0% ($n = 9$) showed normal thymus area, while two-thirds had thymic hypoplasia, of which 36.0% ($n = 9$) had mild hypoplasia and 28.0% ($n = 7$) had moderate-severe hypoplasia. The analysis of the *odds ratio* (OR) revealed a greater tendency towards thymic hypoplasia in children that were breastfed for less than 6 months, within which the risk of developing moderate-severe thymic hypoplasia was four times greater than those with a normal thymus area (Fig. 1A). This risk is maintained when comparing the individuals presenting moderate-severe thymic hypoplasia with the group that included cases with normal and mild thymus hypoplasia (*OR* = 3.28, 95% *CI*: 1.22–8.81). In patients that were breastfed for 6–11 months, there was a greater trend of presenting a normal size thymus (Fig. 1B); while in those under breastfeeding for 12 months or more, there was no relationship between breast milk consumption and whether the child developed or not thymic hypoplasia (Fig. 1C).

The analyses of the linear correlation between the thymus area and the anthropometric variables revealed an association, since there was a direct relationship with size ($r = 0.238$, $p = 0.043$) and weight ($r = 0.233$, $p = 0.047$). These results contrast with those found in studying the correlation between the area of the thymus and variables such as birth weight ($rs = 0.211$, $p = 0.073$) and gestational age at delivery ($rs = 0.007$, $p = 0.950$). The association studies (*odds* ratio) between the development of the thymus according to its area, and the type of delivery, did not reveal any relationship in both cases (Supplementary Table S1).

A multivariate analysis of logistic regression was conducted using the clinical-epidemiological and anthropometric

Table 1 Clinical-demographic and anthropometric characteristics of children with recurrent infections in relation to thymus development according to its area

Characteristics	Development of the thymus according to area†ζ			
	Normal thymus $n = 25$	Slight hypoplasia of the thymus $n = 19$	Moderate hypoplasia of the thymus $n = 24$	Severe hypoplasia of the thymus $n = 5$
Demographic aspects				
Age				
In months — median (IQR)	34.0 (29.0–58.5)	39.0 (19.0–46.0)	28.5 (22.3–39.8)	35.0 (13.0–71.0)
Groups — no. (%)				
<12	0 (0.0)	1 (5.3)	2 (8.3)	1 (20.0)
12–23	4 (16.0)	5 (26.3)	7 (29.2)	1 (20.0)
24–35	7 (28.0)	2 (10.5)	5 (20.8)	1 (20.0)
36–47	3 (12.0)	8 (42.1)	7 (29.2)	0 (0.0)
48–59	5 (20.0)	1 (5.3)	2 (8.3)	0 (0.0)
≥ 60	6 (24.0)	2 (10.5)	1 (4.2)	2 (40.0)
Sex — no. (%)				
Female	11 (44.0)	10 (52.6)	12 (50.0)	4 (80.0)
Male	14 (56.0)	9 (47.4)	12 (50.0)	1 (20.0)
Skin color — no. (%)				
White	11 (44.0)	12 (63.2)	17 (70.8)	4 (80.0)
Mixed	7 (28.0)	2 (10.5)	6 (25.0)	1 (20.0)
Black	7 (28.0)	5 (26.3)	1 (4.2)	0 (0.0)
Anthropometric aspects				
Weight at birth (g) — median (IQR)	3454 (3202–3700)	3120 (2812–3400)	3220 (2688–3621)	3234 (2941–3631)
Size (cm) — mean (DS)	99.6 (±14.3)	94.5 (±14.8)	88.5 (±10.8)	89.8 (±21)
Weight (kg) — mean (DS)	17.4 (±5.5)	15.1 (±4.8)	13.8 (±3.3)	13.3 (±4.6)
Perinatal and postnatal aspects				
Gestational age at delivery in weeks — median (IQR)	39.5 (38.4–40.3)	40.1 (39.1–40.4)	39.3 (38.2–40.2)	40.1 (38.3–41.5)
Type of delivery — no. (%)				
Eutocic	11 (44.0)	6 (31.6)	11 (45.8)	2 (40.0)
Dystocia requiring cesarean section	14 (56.0)	13 (68.4)	11 (45.8)	3 (60.0)
Dystocia requiring instrumentation	0 (0.0)	0 (0.0)	2 (8.3)	0 (0.0)
History of BF — mo. (%)	24 (96.0)	18 (94.7)	23 (95.8)	5 (100.0)
Duration of BF in months — median (IQR)	8.00 (5.50–13.0)	10.0 (3.0–12.0)	5.00 (3.0–8.75)	6.00 (4.5–13.5)
Localization of infections — no. (%)φ				
Respiratory	24 (96.0)	19 (100.0)	23 (95.8)	5 (100.0)
Dermatologic	8 (32.0)	7 (36.8)	12 (50.0)	1 (20.0)
Digestive	5 (20.0)	4 (21.1)	5 (20.8)	3 (60.0)
Systemic	4 (16.0)	3 (15.8)	3 (12.5)	1 (20.0)
Genitourinary	1 (4.0)	1 (5.3)	2 (8.3)	0 (0.0)
Myo-osteoarticular infection	0 (0.0)	1 (5.3)	0 (0.0)	0 (0.0)

Abbreviations: *IQR* inter-quartile range, *g* gram, *cm* centimeter, *kg* kilogram, *BF* breastfeeding

†The comparison of the quantitative variables that do not follow a normal distribution did not reveal differences between groups on applying the Kruskal-Wallis test with a significance level of $p < 0.05$. The comparison of the weight and size variables did reveal differences

ζThe comparison of the distribution of the qualitative variables did not reveal differences between the groups on applying Pearson's χ^2 test with a level of significance of $p < 0.05$

φThere was no history of neurologic infections or cardiovascular infections in the participants

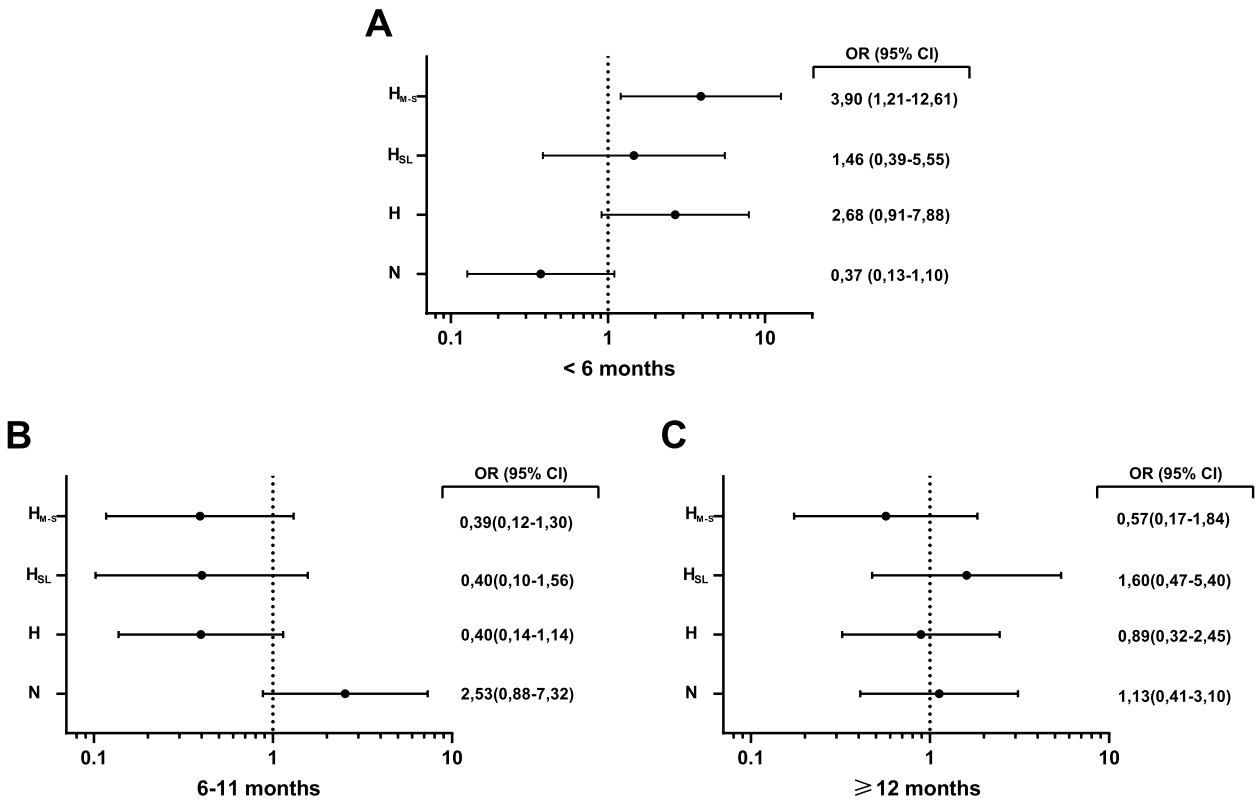

Fig. 1 Odds ratio (OR) and 95% confidence intervals (95% CI) of the development of the thymus according to its area in relation to the duration of breastfeeding in children with recurrent infections. The duration of breastfeeding of less than 6 months in **A**, 7 to 11 months in **B**, and 12 or more months in **C**. The values of OR and 95% CI are shown in each graph. Abbreviations: N normal, H hypoplasia, HSL slight thymus hypoplasia, HM-S moderate-severe thymus hypoplasia

variables of interest for the model (dystocia, birth weight of less than 2500 g, breastfed for less than 6 months, size and weight adjusted according to age and sex). We identified that breast milk consumption for less than 6 months is a risk factor for the development of moderate-severe thymus hypoplasia (adjusted *OR* = 5.06, 95% *CI*: 1.19–21.60) when compared to individuals with a normal size thymus (Supplementary Table S2). There was no evidence of developing mild hypoplasia on considering the variables of interest in the logistic regression model analyzed.

Association between values of T-lymphocyte subpopulations and thymus area

Figure 2 shows the distributions of the absolute and relative values of the cellular immunophenotypes using flow cytometry, according to the area of the thymus in children with recurrent infections. The comparison of the distribution between the groups of patients with normal thymus area and with hypoplasia showed differences in the relative values of lymphocytes T CD45+ CD3+ ($p = 0.015$) with their decrease in the cases of thymus hypoplasia. These differences were also observed in the relative values of lymphocytes T_{CM} CD8+ ($p = 0.035$) with their increase in the cases in which the area of the thymus was lower.

The distributions of the absolute and relative values of the cellular immune phenotypes by flow cytometry, in relation to the development of the thymus according to area, are shown in Fig. 3. There were differences in the relative values of the lymphocytes T CD45+ CD3+ between the cases of normal thymus and mild hypoplasia ($p = 0.018$) and those that were moderate-severe ($p = 0.049$). A decrease in these values was observed in cases showing one of the forms of hypoplasia. Furthermore, differences were observed in the memory compartment of the T CD8+ cells, showing an increase of absolute values of the T CD8+ central memory cells and effector memory cells in the cases with moderate-severe hypoplasia.

The analysis of linear correlation between the thymus area and values of the subpopulations of T cells in children with recurrent infections revealed a weak relationship. This finding was identified by showing an increase in the absolute cell count of T_{EM} CD8+ ($rs = -0.263$, $p = 0.024$) and T_{CM} CD8+ ($r = -0.283$, $p = 0.015$) as the area of the thymus decreases. A similar correlation was observed on

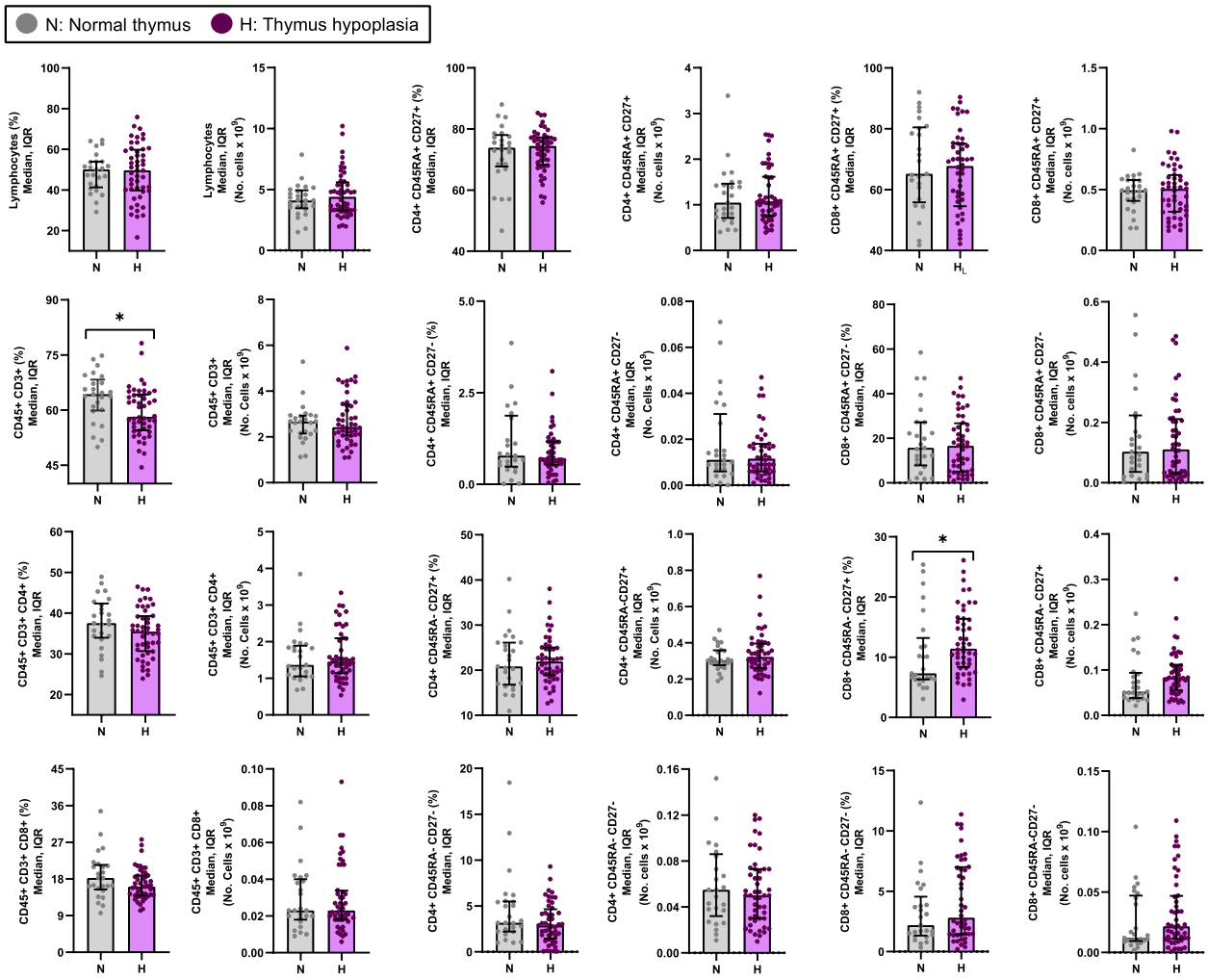

Fig. 2 Cellular immunophenotypes by flow cytometry according to the thymus area in children with recurrent infections. The multiparametric analysis (relative and absolute frequencies) is based on the values of the flow cytometry of cell subpopulations CD45+ CD3+, CD45+ CD3+ CD4+, and CD45+ CD3+ CD8+ and cell subpopulations T CD4+ and T CD8+ naïve (CD45RA+ CD27+) and memory cells (central memory cells, CM, CD45RA− CD27+; effector memory cells, EM, CD45RA− CD27−; terminally differentiated effector T cells, TEMRA, CD45RA+ CD27−). Each point represents an individual with a normal thymus area (N, gray, $n = 25$) and thymus hypoplasia (H, purple, $n = 48$). The analysis of distribution comparison between groups was carried out using the U Mann-Whitney test, with a level of significance of $p < 0.05$. The p-value, and its significance, was $^*p < 0.05$. No differences were found in the CD4/CD8 index on comparing the groups. Abbreviations: *IQR* interquartile range

analyzing the relative values of the T_{EM} CD8+ ($rs = -0.241$, $p = 0.039$) and T_{CM} CD8+ ($r = -0.281$, $p = 0.015$) cells.

The percentages and absolute values of the T-cell subpopulations are related to age, except for lymphocytes T_{CM} CD8+ and the T_{EMRA} in the compartments CD4+ and CD8+ (Supplementary Table S3). The correlation analysis revealed that the absolute count of total lymphocytes ($rs = -0.442$, $p = 0.0001$), T CD45+ CD3+ ($rs = -0.306$, $p = 0.009$) cells, T CD4+ ($rs = -0.341$, $p = 0.003$) cells, T CD4+ *naïve* ($rs = -0.369$, $p = 0.001$) cells, and T CD8+ *naïve* ($r = -0.301$, $p = 0.01$) cells decrease when the age of the children with recurrent infections increases. Moreover, the increase in age was associated with the absolute number of T_{EM} CD4+ ($rs = 0.373$, $p = 0.002$) cells in these cases.

The correlation of the sex of the patients with the percentages and absolute values of T-cell subpopulations (Supplementary Table S4) did not reveal associations with the thymus area, except for the lymphocytes T_{CM} and T_{EM} in the CD8+ compartment of male patients.

Discussion

On analyzing the demographic characteristics of the cases included in the study, it was noteworthy that in children with recurrent infections presenting a normal

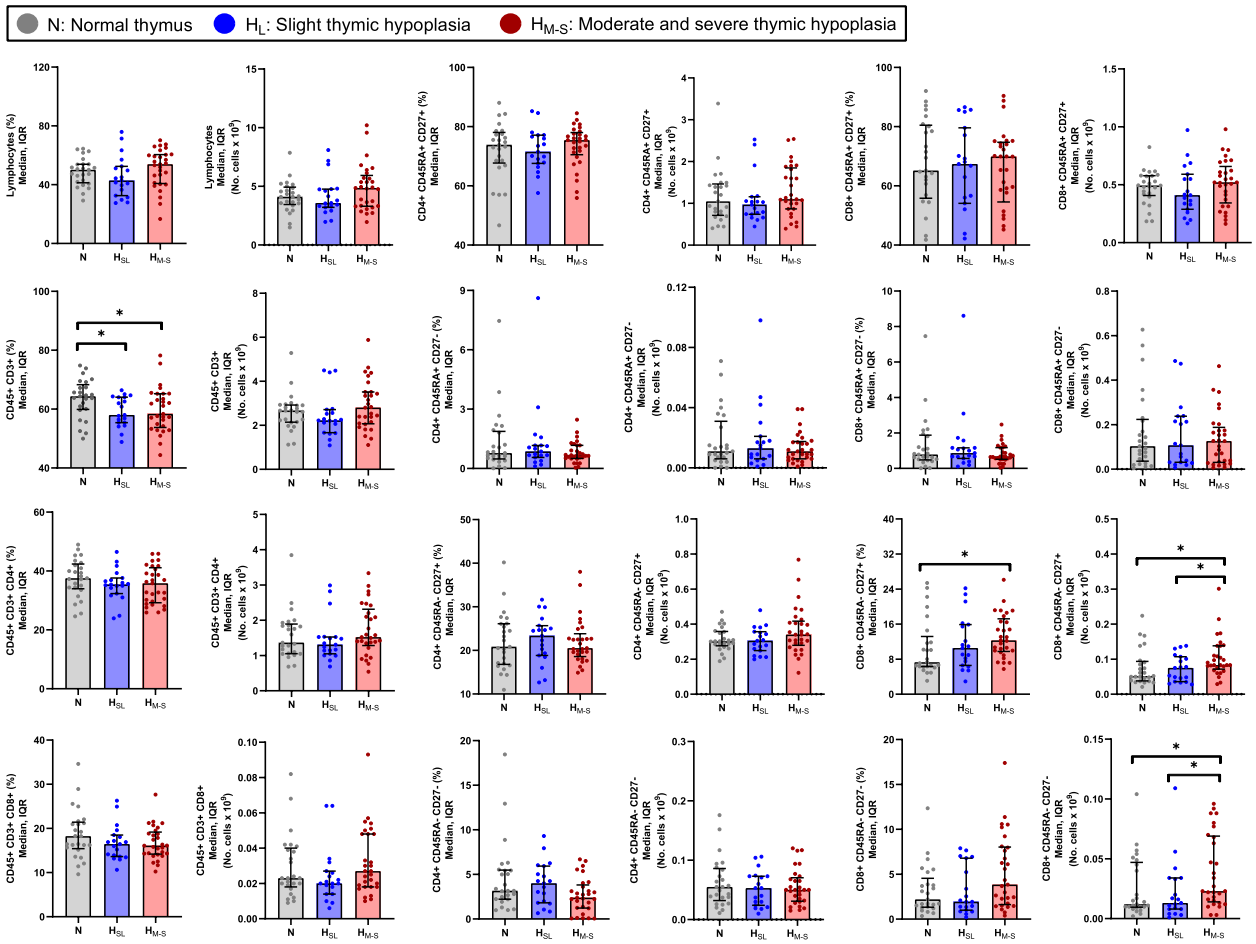

Fig. 3 Cellular immunophenotypes by flow cytometry according to thymus development depending on its area in children with recurrent infections. The multiparametric analysis (relative and absolute frequencies) is based on the values of flow cytometry of cellular subpopulations, CD45+ CD3+, CD45+ CD3+ CD4+, CD45+ CD3+ CD8+ and of subpopulations of cells T CD4+ and T CD8+ naïve (CD45RA+ CD27+) and of memory cells (central memory cells, CM, CD45RA- CD27+; memory effector cells, EM, CD45RA- CD27−; terminally differentiated T effector cells, TEMRA, CD45RA+ CD27−). Each point represents an individual with a normal thymus area (N, gray, $n = 25$), slight thymic hypoplasia (HSL, blue, $n = 19$), and moderate and severe thymus hypoplasia (HM-S, red, $n = 29$). The analysis of distribution comparison between groups was made using the U Mann-Whitney test with a level of significance of $p < 0.05$. The p-values and their significance were $^*p < 0.05$. No differences were found in the CD4/CD8 index on comparing the groups. Abbreviations: *IQR* interquartile range

thymus area, the individuals with white skin were less frequent, on comparing them with the frequencies reported in the last Cuban census, which stated that 64.1% of the population had white skin, 26.6% were of mixed descent, and 9.3% had black skin [18]. Thymus size showed variations between individuals in subjects of the same species, as well as, within individual variations, when evaluated at different points of development [19]. Studies show that dimensions have slight variations according to the ethnic diversity of the populations [9, 10, 14, 20]. This is a strong argument for new research on intrinsic factors that predispose individuals to the development of thymic hypoplasia within the Cuban pediatric population, where ancestral origin is very heterogeneous [21].

The dimensions of the thymus are determined by the combination of intrinsic and extrinsic factors. These factors include breastfeeding that offers outstanding natural nutrition to the newborn and lactating child. Its premature interruption can become a risk factor to many chronic diseases [22]. Many of the previous studies that have suggested that there is a positive association between breastfeeding and thymus size have been carried out in the temporary context of breast milk consumption [11, 12, 23–25]. These results could indicate that this association is stronger at an earlier age, at which children are still fed exclusively with breast milk. However, in our study, which also included children that had ended their breastfeeding period, results show that breastfeeding

for a period of less than 6 months is a risk factor for the development of moderate-severe thymus hypoplasia in children with recurrent infections. This finding could be due to the well-established effect of breast milk microbiota, its bioactive oligosaccharides, and the extracellular vessels that transport the mARN, miARN, and cytosolic proteins, in the establishment of neonatal microbiome and the consequent potential for the modulation of the development of the neonatal immune system [26]. The incipient intestinal microbiota poses a unique challenge for the developing immune system. A clear interrelationship is required between the immune cells of the mucosa and the microorganisms, to form healthy microbial communities and promote the development of productive mucosal immunity. Breast milk plays a relevant role in this process.

In relation to the anthropometric variables, results show that on increasing weight and size, there is an increase of the thymus area. These anthropometric measures are found to decrease in children that are immunologically compromised, which is probably linked to environmental stress factors, produced by infectious agents that enter through different routes and affect growth and metabolism [24, 27]. The same state of immunological compromise could justify a low-energy intake, and, as a consequence, it can produce a decrease in longitudinal growth and weight gain [28]. Secondary malnutrition due to a lower protein-calorie, vitamin, and mineral intake results in variations of the size of the thymus, given by hormonal unbalance that implies a decrease of leptin and an increase of glucocorticoid levels in the serum. This state can lead to hypoplasia or atrophy of the thymic gland, through the depletion of thymocytes induced by apoptosis and the decrease of cell proliferation, which affects the immature cells of the CD4+ and CD8+ type and perpetuates the compromised immune system. The microenvironment of the thymus is also affected during acute infections, with an increase of cytoplasmic inclusions that are rich in free cholesterol and cholesteryl ester in cells of the cortical and medullary epithelial network, which substitute cytoplasmic inclusions of the thymulin hormone, thus producing a reduction of the epithelial volume of the gland [29].

This study showed that the values of the subpopulation of T CD3+ lymphocytes are lower in the cases of thymus hypoplasia (mild and moderate-severe), compared those with a normal size gland. The contraction of the glandular parenchyma volume with a decrease of the number of immunological cells is produced through different cell death mechanisms. This includes apoptosis as the main pathogenesis of thymic atrophy induced by infection, although necrosis, autophagy, and pyroptosis have also been described [30, 31]. The atrophy of the thymus with a central depletion of thymocytes and peripheral depletion of CD3+ cells was reported in infections by different pathogens in histopathological and immunophenotyping studies [32].

In the analyses, we did not find any correlation of the values of the cellular subpopulations of CD4+, CD8+, and the CD4/CD8 ratio with the thymus area in children having a history of recurrent infections. These results may be explained by the contribution of *naïve* T cells, whose values were within the normal range and showed no relation with the thymus area. In line with these results, it is considered that the population of peripheral T cells remains at a relatively constant number through life, through the addition of recent thymic emigrants (RTE), and through the homeostatic control of the number of peripheral T cells [33]. Although the effects of the decrease in the size of the thymus do not support an absolute correlation between the exportation of RTE and the number of peripheral T cells, its possible influence on thymopoiesis may produce a decrease of the number of RTE. This would lead to a consequent loss of T-cell receptor (TCR) diversity and the replacement of clones of older *naïve* T lymphocytes that would probably be redundant or auto-reactive [34]. This would then generate clones that do not suitably respond to different pathogens, which would explain the occurrence of recurrent infections. Thymic emigration is the only mechanism that increases the levels of *naïve* T cells, and their release from the thymus does not depend on the set of peripheral T cells [35, 36]. It is known that the inflow of the RTE population is required to enrich the diversity of peripheral T cells in chronic infections. The new *naïve* T cells promote the specific diversity of the antigen and the homeostatic proliferation that is lost in T cells that have responded chronically [37]. Additional research on the development of thymus size and its relationship with RTE is required to demonstrate the possible hypothesis and offer additional information on its function.

The analysis of the values of T CD4+ central and effector memory populations showed a larger number and percentage in the former. The presence of a larger subpopulation of T_{CM} will lead to a reserve of T_{EM} precursors that cooperate with B lymphocytes for the generation of antibodies and the performance of its effector capacity *per se* [38]. For the case of CD8+ memory cells, we observed a similar distribution.

The increase of T CD8+ cells in the memory compartment of the cases with moderate-severe thymus hypoplasia is due to constant antigenic exposures that involve recurrent infections by intracellular pathogens. The T_{CM} CD8+ have a limited capacity of performing effector functions when they meet with the antigen, but they produce very rapid proliferative responses and generate

many effector cells when meeting with it. The population of T_{EM} CD8+ cells enables early and efficient actions (large amounts of cytokines produced in less time) in the cases of intracellular infections, compared to those generated from activated *naïve* cells [39]. It is important to stress that the expansion of the clones of T CD8+ memory cells in children with moderate-severe hypoplasia, could respond to a homeostatic regulation mechanism of peripheral cells, as a result of a decrease of the RTE and its repercussion on the number and quality of *naïve* cells.

These results offer new opportunities to investigate the influence of the nutritional status, specific infections, and other environmental agents in thymic development and to explore the role of the thymus in the integrity of the health of pediatric patients.

Conclusions

The fact of being breastfed for less than 6 months and the weight and size of the child are related to thymic area. Modifications identified in the values of subpopulations of T lymphocytes suggest that patients with thymic hypoplasia develop a contraction of T CD3+ cells that can lead to more vulnerable infectious processes. This finding was combined with the expansion of the memory compartments of subpopulations of T CD8+ cells, which suggests a greater predisposition to virus and intracellular bacterial infections in these cases.

Abbreviations

T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
T_{EMRA}	T-effector memory re-expresses CD45RA (terminally differentiated effector memory T cells)
RTE	Recent thymic emigrants
TCR	T-cell receptor

Supplementary Information

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Additional file 1: Supplementary Table S1. Frequencies and odds ratio of thymic development according to its area and in relation to the type of delivery, in children with recurrent infections. **Supplementary Table S2.** Logistic multivariate regression analysis in patients with moderate-severe thymic hypoplasia. **Supplementary Table S3.** Correlations of absolute and relative values of T cell subpopulations with age in months. **Supplementary Table S4.** Correlations of absolute and relative values of T cell subpopulations with thymic area in boys and girls with recurrent infections.

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Authors' contributions

FSL, YZR, HER, and HFH, conceptualization, methodology, and writing—original draft. KRG, LTR, and IGD, investigation. EAV, validation. FSL and BTR, data curation and supervision. YMG, CVV, and ICH, formal analysis. OPM, CMA, and BMT, review and editing. YHR and ASP, resources and investigation. FSL, project administration. All authors critically reviewed the manuscript for its theoretical content, approved the final version, and accepted their responsibility in all aspects of the study.

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Availability of data and materials

The data supporting the findings in this study are not publicly available due to Cuban laws in relation to the general regulation for data protection. However, the data are available through the corresponding author (F. S. L.), after a reasonable request and permission from the National Center of Medical Genetics of Cuba (CNGM, according to its Spanish acronym). The Medical and Scientific Research Ethics Committee of the CNGM must be contacted to access the data within the legal Cuban framework, (cngm@infomed.sld.cu). Requests must be addressed to the vice president of the committee, Dr. Hilda Roblejo Balbuena.

Declarations

Ethics approval and consent to participate

This study was carried out according to the Declaration of Helsinki principles. The Scientific Council and the Medical Ethics and Scientific Research Committee of the National Medical Genetics Center reviewed and approved the protocol of the research according to the established regulations and norms (protocol number 17/05-2022). We obtained the written informed consent from the parents and/or legal tutors of the children participating in the study. All tutors gave their informed consent in relation to the publication of the data. For the analyses, we used anonymous data.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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