ORIGINAL ARTICLE

IL-2 and the thymus/weight index are inversely correlated with gestational age: a sign of Th1/Th2 imbalance in preterm infants and a possible connection with atopic dermatitis

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Abstract

Background. Understanding the reason for the decrease in atopic dermatitis risk in preterm patients may be crucial for the development of prophylaxis and therapeutic measures. The hypotheses include a change in T-helper1/T-helper2/T-helper17 balance, thymus development, and intestinal colonization. This study was designed to compare these parameters between preterm and term patients.

Methods. The investigated population comprised 28 preterm and 19 term-born patients. On the 7th day of life, T-helper1/T-helper2/T-helper17 cytokine concentrations were assessed, thymus ultrasonographic examination was performed, and the stool was examined for the presence of pathogenic bacteria. The last two procedures were repeated at 37 weeks of post-menstrual age in the preterm group.

Results. There were no significant differences in the concentrations of interferon-γ, tumour necrosis factor-α (TNFα), interleukin (IL)-2, IL-6, or IL-10 after mitogen stimulation between the preterm and term groups. A negative correlation was found between IL-2 and the week of gestation at birth (r_s= -0.466, p=0.038) and thymus/weight ratio and week of gestation at birth (r_s= -0.592, p=0.006). IL-6 was negatively correlated with birth weight in preterm group (r_s -0.694, p=0.008), whereas IL-10 positively with birth weight in term group (r_s 0.775, p=0.041). Correlations of other investigated cytokines were statistically insignificant. The levels of IL-2 and interferon-γ after phytohemagglutinin stimulation were greater in the subgroup with pathogenic bacteria in the stool at birth (381.38 (148.7-727.4) vs. 13.23 (7.98-197.8) pg/ml;

p=0.049; 17.49 (6.53-30.54) vs. 3.37 (1.03-9.82) pg/ml; p=0.037), whereas no significant differences were found between the levels of IL-6, IL-10 or TNFα.

Conclusions. The observed associations may indicate an altered pattern of immunological development in preterm and term children.

Key words

preterm; IL-2; atopic dermatitis; Th2; thymus.

Introduction

Preterm neonates are a group of patients with a great risk of life-long morbidities and complications (1, 2). Interestingly, there is a group of conditions for which prematurity is a protective factor—atopic diseases. Allergic rhinitis, food allergies, and especially atopic dermatitis (AD) were recently proven in the literature to more frequently affect term-born babies (3, 4). Understanding the decrease in AD risk in preterm patients may be crucial for understanding the pathophysiology of all ADs and, at the same time, serve as a valuable asset in the development of prophylaxis and therapeutic measures.

One of the proposed hypotheses, explaining the decrease in AD frequency in preterm patients says, that this phenomenon may be a result of a shorter time of exposure to Th2 cytokines during pregnancy (3, 5). What is more, a greater thymus size in preterm children may play a role, due to changes in the balance of thymic cell populations in favour of Th1 cells (6, 7). T helper 17 (Th17) cell activity may also be important, as the cytokine interleukin 17A (IL-17A) is involved in the inflammatory process in older patients with AD (8). Moreover, the microbiota of the gastrointestinal (GI) tract is different between preterm-born patients and term-born patients and may influence the permeability of the intestinal wall and reactions to allergens (9).

2. Materials and methods

2.1 Study population

This prospective, observational study was conducted in the tertiary referral neonatal intensive care unit (NICU) of a paediatric hospital. Forty-seven patients were categorized into two groups according to gestational age (GA): preterm (born at $<37^0$ weeks of gestation) and term (born at 37^0 weeks of gestation or later) (Figure 1). All children with intrauterine growth retardation (IUGR), early-onset sepsis, intrauterine infection, serious life-threatening inborn defects, a family history of immunodeficiency, or severe birth asphyxia (defined as an Apgar score < 5 points, 5 minutes after birth) were excluded from the study. Based on the literature review, the

minimum total sample size was estimated to be 24, assuming a statistical power of 80%, a significance level of 0.05, and a minimum difference in the thymic index (Ti) of 3 cm^3 or the IL-10 concentration after phytohemagglutinin (PHA) stimulation of 100 pg/ml between the groups (10-14). The minimum differences were estimated by the authors, taking into account the outcomes presented in other publications (10-14), so that the research could show the change in size of thymus or cytokine level, that are of real clinical significance, a possible sign of altered immunological development and may play a role in development of AD or other allergic diseases in the future.

2.2 Study design

All the legal guardians of the patients completed a questionnaire about their atopic history in the family and about the environmental factors that could impact the atopic risk of the baby. Data about pregnancy, birth, and NICU stay, especially concerning the type of feeding, antibiotic therapy, and infections, were collected from the medical documentation. On the 7^{th} day of life in both groups, blood was collected for the assessment of Th1, Th2, and Th17 cytokine levels. At the same time, a thymus ultrasonographic examination was performed, and the stool was examined for the presence of pathogenic bacteria. The last two procedures were repeated at 37 weeks of postmenstrual age (PMA) in the preterm group. The 7^{th} day of life was chosen because at this time, neonatological patients are usually in more stable condition, and additional blood donation for immunological tests is safer for them.

2.3 Th1/Th2/Th17 cytokine profile measurements

Blood samples for the research tests were taken simultaneously with medically necessitated diagnostic blood samples from patients in both groups on the 7^{th} day of life by a trained nurse and collected in tubes with ethylenediaminetetraacetic acid (EDTA). Blood samples were divided into 3 plates. To stimulate the production of cytokines in the cells outside of an organism, the former three samples of full blood were incubated for 24 h at 37°C as follows: 1) without mitogens (control); 2) with mitogen phytohemagglutinin; and 3) with mitogen concanavalin A (Con A). The cell-free liquid collected over the residue was stored at -80°C until analysis. A BD CBA Human Th1/Th2/Th17 Cytokine Kit (15) was used to measure the IL-2, IL-4, IL-6, IL-10, IFN-γ, TNFα, and IL-17A protein concentrations in a single sample. The CBA test involves the capture of a soluble protein by beads of known size and fluorescence conjugated with specific antibodies. The detection of different proteins was possible by using conjugated antibodies, whose fluorescence is proportional to the amount of bound protein. Flow

cytometry was used to identify particles with specific fluorescence. Finally, an FCS file for each sample was created and exported. Each sample was analysed using the FCAP Array program.

2.4 Thymus ultrasonographic measurements

On the 7th day of life, ultrasonographic measurement of the thymus was performed by a trained sonographer with a Hitachi-Aloka Arietta v70 (Hitachi, Tokyo, Japan) scanner and a linear probe of 12–5 MHz. The size of the thymus was assessed using the thymic index (16), i.e., the longest transverse dimension multiplied by the area of the sagittal cross-section of the larger lobe. Sonographic examination was performed twice by one sonographer on the same day. The procedure was repeated in the preterm group at 37 weeks PMA. To assess the thymus size-to-weight ratio, the thymus/weight index (cm^3/kg) (TWi) was calculated by dividing Ti by weight.

2.5 Stool bacterial examination

During the first week of life, stool samples were collected from the patients for bacterial examination. Samples were collected immediately after defecation from a clean diaper using a sterile container and spatula and routinely plated onto various selective and differential media (substances designed to support the growth of microorganisms through the process of cell proliferation), including selenite broth, MacConkey agar, blood agar, cetrimide agar, chromogenic agar, and Wilson Blair agar. All media were incubated at 36°C for 24 h. The colonies were further analysed with an automated system of detection or with manual identification tables. The bacterial analysis of the stool was repeated in the preterm group at 37 weeks PMA. Pathogenic stool colonization was defined as the presence of bacteria that are not a component of normal intestine microflora of a healthy neonate: alert, drug resistant pathogens (for example Gram negative extended-spectrum beta-lactamases (ESBL) (+) rod-shaped bacteria, Gram negative Klebsiella pneumoniae carbapenemase (KPC) (+) rod-shaped bacteria, Gram negative Metallo-β-lactamase (MBL) (+) rod-shaped bacteria, Gram negative OXA-48 carbapenemase (+) rod-shaped bacteria, Vancomycin-resistant Enterococcus, methicillin-resistant Staphylococcus aureus) or typical hospital-acquired ~~bacterial infection~~ bacteria (for example Klebsiella oxytoca, Enterococcus faecium, Pseudomonas aeruginosa) in the probe.

2.6 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics software, version 29.0.0.0 (241), and G*Power, version 3.1.9.6. The results are presented as descriptive statistics, such as median values with interquartile ranges (IQRs) or mean values with standard deviations (SDs) for

continuous variables and numbers with percentages for categorical variables. Statistical significance was set at a p value of <0.05. The Shapiro–Wilk test was used to confirm the normal distribution of continuous variables. The groups were compared using the Chi-square test, Fisher's exact test, independent samples t test, or Mann–Whitney U test, Wilcoxon signed-rank test and McNemara test. Correlations between the values were calculated using Spearman's rank correlation coefficient (r_s). The sonographic data were analysed for intraobserver agreement using Kendall's coefficient of concordance (Kendall's W).

2.7 Ethical approval

Written, informed, formal consent was obtained from all the legal guardians of the patients. The study was performed following the tenets of the Helsinki Declaration and was approved by the Ethics Committee of the local university.

3. Results

3.1 Study population

The investigated population comprised 28 preterm and 19 term-born patients. The characteristics of the cohorts are shown in Table I. The most common reason for hospitalization in both groups was respiratory failure: 89% in the preterm and 58% in the term group.

3.2 Th1/Th2/Th17 cytokines

Blood samples for Th1/Th2/Th17 cytokine profile measurements were taken on the 7th day of life in both groups. The number of patients with cytokine results differed from that of the whole group due to technical problems with sample obtainment or volume. The production of cytokines was analysed in three ways for every patient: in a sample without mitogens (control), incubated with mitogen phytohemagglutinin and incubated with the mitogen concanavalin A. The levels of all cytokines in the control samples were below the detection limits (our own, unpublished data). Therefore, it was not possible to properly interpret and draw conclusions from these data and they were omitted from the analysis. After stimulation with PHA and Con A, the concentrations of IFNγ, TNFα, IL-2, IL-6, and IL-10 were much greater than the detection levels. However, we still did not observe significant production of IL-17A or IL-4. There were no statistically significant differences in the concentrations of IFNγ, TNFα, IL-2, IL-6, or IL-10 after PHA or Con A stimulation between the preterm and term groups (Figure 2).

In the whole study population, significant correlations were found between IL-2 level and the week of gestation at birth (r_s -0.466, p=0.038). This result is shown on Figure 3 – the lower the gestational age, the higher the concentration of interleukin 2. R_s -0.466 points to the relatively strong effect size of the correlation. Furthermore, we showed that IL-6 was negatively correlated with birth weight in preterm group (r_s -0.694, p=0.008), whereas IL-10 positively with birth weight in term group (r_s 0.775, p=0.041). On the basis of r_s values we can state that both these associations present a strong effect size. Taking into account low values of p and not a very large research group, all these correlations have a great chance to be clinically meaningful, what is discussed below.

3.3 Thymic index and thymus/weight index

A high level of intraobserver agreement in ultrasonographic measurements was confirmed using Kendall's W (W=0,95). The study showed no significant difference in the thymic indices between the groups in the first week of life and at 37 weeks of postmenstrual age. However, when we compared the thymic indices in the preterm group in the first week of life and at 37 weeks postmenstrual age, the study revealed significant thymus growth with age (Table II, Figure 3). The TWi was significantly greater in the preterm group at birth (Figure 3). Week of gestation was significantly correlated with TWi at birth (Figure 3) in the whole study population (p=0.006), with a relatively strong effect size r_s=(-0.548). In the term group, Ti at birth was negatively and also strongly correlated with Hbd (r_s=-0.78, p=0.039).

3.4 Bacteriological stool examination

In the first week of life, pathogenic bacterial colonization of the gastrointestinal tract was present in 25% of preterm patients and 44.4% of term patients. No statistically significant difference was found between the groups. Nevertheless, when we compared both groups at 37 weeks postmenstrual age, we found that pathogenic colonization was more common in preterm infants (Table III). After dividing the population according to the presence of pathogenic bacteria in the stool at birth and at 37 PMA, we compared the cytokine levels in both groups. We found that the levels of IL-2 and IFNγ after PHA stimulation were greater in the group with pathogenic stool colonization at birth. There were no statistically significant differences between the levels of IL-6, IL-10 or TNFα. The size of the research group was too small to determine the influence of a specific microbe on cytokine levels. There were no significant differences in thymus size between the groups with pathogenic and non-pathogenic intestinal colonization.

4. Discussion

In our study, we observed a significant, relatively strong, inverse correlation between the IL-2 concentration and gestational age at birth. IL-2 is a cytokine with multiple functions in the human immune system, and its activity is closely connected with T-cell activity. IL-2 promotes Th1 differentiation via various metabolic and genetic pathways (17). Importantly, IL-2 also seems to downregulate, through the STAT5 pathway, the differentiation and function of Th17 cells as well as the expression of IL-17 (17,18). Both of the abovementioned factors are associated with skin inflammation in atopic dermatitis (8). Thus, by promoting Th1 bias and reducing the activity of Th17 cells, IL-2 could reduce the risk of AD development in preterm infants, but this hypothesis needs further research. Moreover, it has been proven in the recent literature that a crucial function of IL-2 is controlling the immune response by maintaining the regulatory T cells and protecting the organism against autoimmunity (19). From the practical, clinical point of view, it has been lately reported that a new medication, T cell-selective IL-2 receptor agonist – rezpegaldesleukin, is currently tested in clinical trials (20). On the other hand, it was shown that IL-2 signalling is also crucial for the development of Th2-type atopic reactions because it promotes the expression and function of IL-4. IL-2 enhances the accessibility of the IL-4 locus and promotes the transcription of the *IL4RA* gene (21). However, we hypothesize that this effect could be reduced in the smallest preterm infants by a higher concentration of IL-6, which is associated with frequent infections in this group. In our study in the preterm group, birth weight was negatively correlated with IL-6, and Bachus et al. showed that IL-6 promotes SOCS3-dependent suppression of IL-2 signalling and, in this way, reduces Th2 cell polarization (22).

We found no significant differences in the concentrations of Th1 and Th2 cytokines between the groups. Similarly, Frezza et al. (23) did not observe a significant difference in IFNγ levels after PHA stimulation between preterm and term children. However, the basal levels of IFNγ were greater in preterm infants, and after stimulation, the IFNγ concentration increased only in the term group. Furthermore, in a study by Matoba et al. (24), preterm and term groups did not differ in terms of IL-6, IFNγ, or TNFα levels in cord blood, but the concentrations of IL-2, IL-4, and IL-10 were greater in preterm infants. Importantly, these above-mentioned two studies were carried out on cord blood, not on peripheral blood of the newborn, what may be one of the reasons for discrepancy in results. What is more, in the research by Matoba et. al. (24), the

preterm group included also term-born children, but with birth weight <2500g. In contrast to our findings, Lusyati et al. (25) reported lower levels of IL-2, IL-6, IL-10, IFNγ and TNFα in preterm patients than in term patients. However, the exclusion criteria of this study were less strict than ours and the preterm group comprised only children with gestational age 30 weeks or more. The level of cytokines was also assessed without mitogen stimulation. These factors could have had impact on getting different results.

In our study, in the term group, birth weight was positively associated with the IL-10 level. This finding is in agreement with studies that showed an increased risk of AD in children with greater birth weights or who were born post-term (5, 26, 27), as IL-10 is widely correlated with atopy and Th2 responses in the literature (21).

The thymus is an important organ responsible for T-cell maturation and for the development of the immune system (28). The thymus and its size are also widely described as indicators of foetal and neonatal growth and general well-being (29), as high levels of the stress hormone cortisol and infection induce its involution (30, 31). Ngom et al. reported that differences in thymic size were also reflected in changes in thymocyte output as assessed by measuring signal-joint T-cell receptor–rearrangement excision circles (32). This may point to a direct connection between thymus size and function. Godfrey et al. and Barbarot et al. suggested that changes in thymus growth in the foetal and neonatal periods may be connected to the risk of atopy, especially atopic dermatitis, which affects the Th1/Th2 balance in the organism (6, 7). In line with that finding, Thyssen et al. demonstrated a lower risk of AD in children after thymectomy (33). From comparisons of TWi in the present study, we observed that preterm patients have a relatively larger thymus at birth than at term birth. Moreover, the week of gestation was negatively correlated with the TWi at birth. There was no difference in the sheer thymic index between the groups. A possible explanation for the observed tendency is that the thymus grows constantly and proportionately to weight and gestational age in foetal life and immediately after birth in both preterm and term babies. This was already shown in the literature (11, 12) and our study (Table II). However, it seems that the thymus in preterm infants is relatively larger, and hence, there was no difference in its size immediately after birth between the groups. This may be due to an altered immunological milieu during pregnancy that favours prematurity, but this hypothesis needs further investigation. In contrast to our study, Eriksen et al. reported lower Ti and TWi in low birth weight (LBW) children than in normal-birth-weight children (34). However, the study was conducted in a developing country, had much less restrictive exclusion

criteria and included only 16% of premature infants in the LBW group, which indicates that IUGR strongly influenced the results.

Microbiological colonization of the gastrointestinal tract in preterm infants varies greatly from that in term infants and is vital for multiple immunological processes (35, 36). It was shown that preterms in most cases present a state of gut dysbacteriosis. In comparison with term born children, in their intestine there is lower bacterial diversity, with abundant Proteobacteria (for example Enterobacteriaceae, Escherichia coli, Shigella, Klebsiella), but scarce Actinobacteria, and Bacteroides. Colonization with Bifidobacterium is delayed. The reason for such a state lays in many fields, for example delivery mode, chorioamnionitis and strains causing it, way of feeding, degree of prematurity, antibiotics, length of NICU stay (35). Many authors suggest that an abnormal microbiome in preterm infants is connected with a proinflammatory state, probably due to a discrepancy in cross-talk between the gut microbiota and immune system (36, 37). They hypothesize that state of dysbiosis stimulates microorganism associated molecular patterns (MAMPs) to induce pro-inflammatory cytokines expression, and subsequent Th1and Th17 bias, proinflammatory impact of IL-12, IFN-γ, TNFα, IL-17, increased neutrophil recruitment, IgG production and in the end protection against pathogens, but also inadequate inflammation - necrotizing enterocolitis (NEC), late onset sepsis (LONS), autoimmune and allergic diseases (37). What is more, NEC in the literature is usually associated with reduced diversity and one pathogen domination, as well as with abundance of Proteobacteria (35). A few studies identified one specific pathogen possibly responsible for NEC pathogenesis – Enterobacteriaceae group (38) or Clostridium perfringens (39). LONS is also associated with dysbiosis – abundant Proteobacteria, Firmicutes and reduced Bifidobacteria were found in the gut of the premature children just before LONS diagnosis (40). The evidence for the association of microbiota with inflammation in preterms is strong, but the mechanism of specific bacterial strains or patterns of colonisation have not been fully clarified yet. In line with these findings, in our study, we found a higher concentration of proinflammatory cytokines, IL-2 and IFNγ, in children with pathogenic colonization of stool at birth. Together, these cytokines may contribute to the Th1 response, which may be the beginning of the disruption of gut colonization. Further observation in a longitudinal study with follow up is needed to combine the findings with clinical implications.

This study is the first part of a research project that also involves a follow-up in the second year of life, with the repetition of the abovementioned tests and verification of the presence and

intensity of atopic dermatitis, shown in the future publications. The main strength of the study is the comprehensive prospective analysis of neonatal factors that may impact the development of AD, combining both immunological and microbiological assessments. The other advantage is the methodology of cytokine measurement involving ex vivo cell stimulation in full blood samples, which ensures that most of the results are much above the detection level. The main limitation is that the study does not conclude about the connection of thymus size and cytokines with atopic dermatitis. However, the authors mention atopic dermatitis in the article to explain the main point and underline the future direction of undertaken research project. The other limitation is small sample size, but as mentioned above, it is sufficient to show assumed differences in the investigated parameters. With a greater number of patients, it could be demonstrated that cytokine levels differ slightly between the groups. However, these small differences likely have limited clinical implications. The next limitations are relatively high morbidity in term patients and microbiological assessment performed by stool culture instead of more precise molecular tests.

There are several main conclusions that can be stated on the basis of the presented results. There was a significant inverse correlation between the IL-2 concentration and gestational age at birth. We found no significant differences in the concentrations of Th1 or Th2 cytokines between the preterm and term groups. In term-born children, birth weight is positively associated with the IL-10 level. The TWi is greater at birth in preterm patients than in term patients. The levels of IL-2 and IFNγ are greater in children with pathogenic stool colonization at birth than in those with non-pathogenic stool colonization.

As the nature of the study was exploratory, the authors cannot yet draw conclusions about the impact of the obtained results on the further health and disease history of premature and full-term infants. However, these findings, showing the clear immunological differences between the groups, open the field for further longitudinal research. It is advisable for further studies to analyze these parameters and their connection with development of AD and other allergic diseases in a follow-up in the next years of childhood, what may lead to establishing new early-life markers of future AD. The existence of such markers would allow for earlier prevention and treatment of atopic dermatitis. Perhaps showing new pathophysiological mechanisms could also one day be a target for new AD medications.

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Ethical approval: Written, informed, formal consent was obtained from all legal guardians of the patients. The study was performed following the tenets of the Helsinki Declaration and was approved by the local Ethics Committee.

Author contribution:

Study design: AK, PK; Data collection: AK, ZK, KW, MS; Statistical analysis: AK; Data interpretation: AK, PK; Manuscript preparation: AK, KW, MS, PK; Literature search: AK, ZK, MC. All authors have read and approved the final version of the paper.

References

1. Kim SJ, Port AD, Swan R, Campbell JP, Chan RVP, Chiang MF. Retinopathy of prematurity: a review of risk factors and their clinical significance. Surv Ophthalmol. 2018;63(5):618-37. doi: 10.1016/j.survophthal.2018.04.002.
2. Williams JE, Pugh Y. The Late Preterm: A Population at Risk. Crit Care Nurs Clin North Am. 2018;30(4):431-43. doi: 10.1016/j.cnc.2018.07.001.
3. Zhu T, Zhao J, Qu Y, Zhang L, Mu D. Association of very preterm birth with decreased risk of eczema: A systematic review and meta-analysis. J Am Acad Dermatol. 2018;78(6):1142-48.e8. doi: 10.1016/j.jaad.2017.12.015.
4. Gerner T, Rasmussen Rinnov M, Halling AS, Haarup Ravn N, Hjorslev Knudgaard M, Ewertsen C et al. Differences in Occurrence, Risk Factors and Severity of Early-onset Atopic Dermatitis among Preterm and Term Children. Acta Derm Venereol. 2022;102:adv00737. doi: 10.2340/actadv.v102.575.
5. Egeberg A, Andersen YM, Gislason G, Skov L, Thyssen JP. Neonatal risk factors of atopic dermatitis in Denmark - Results from a nationwide register-based study. Pediatr Allergy Immunol. 2016;27(4):368-74. doi: 10.1111/pai.12560.
6. Barbarot S, Gras-Leguen C, Colas H, Garrot E, Darmaun D, Larroque B et al. Lower risk of atopic dermatitis among infants born extremely preterm compared with higher gestational age. Br J Dermatol. 2013;169(6):1257-64. doi: 10.1111/bjd.12581.
7. Godfrey KM, Barker DJ, Osmond C. Disproportionate fetal growth and raised IgE concentration in adult life. Clin Exp Allergy. 1994;24(7):641-8. doi: 10.1111/j.1365-2222.1994.tb00968.x.
8. Jutel M, Madejek K, Smolińska S. Th17 lymphocytes in allergy and asthma. Alerg

Astma Immunol. 2012;17:165–71.

9. Cuna A, Morowitz MJ, Ahmed I, Umar S, Sampath V. Dynamics of the preterm gut microbiome in health and disease. Am J Physiol Gastrointest Liver Physiol. 2021;320(4):G411-G419. doi: 10.1152/ajpgi.00399.2020.
10. Varga I, Uhrinova A, Toth F, Mistinova J. Assessment of the thymic morphometry using ultrasound in full-term newborns. Surg Radiol Anat. 2011;33(8):689-95. doi: 10.1007/s00276-011-0806-x.
11. Jeppesen DL, Ersbøll AK, Nielsen SD, Hoppe TU, Valerius NH. Low thymic size in preterm infants in the neonatal intensive care unit, a possible marker of infection? A prospective study from birth to 1 year of age. Acta Paediatr. 2011;100(10):1319-25. doi: 10.1111/j.1651-2227.2011.02329.x.
12. Magu S, Gathwala G, Agarwal S, Parihar A. Sonographic measurement of thymic size in preterm infants: prediction model for thymic size in the Indian subcontinent. Indian J Pediatr. 2012;79(6):764-8. doi: 10.1007/s12098-011-0594-7.
13. Decker ML, Gotta V, Wellmann S, Ritz N. Cytokine profiling in healthy children shows association of age with cytokine concentrations. Sci Rep. 2017;7(1):17842. doi: 10.1038/s41598-017-17865-2.
14. Manapat A, Umrao A, Purushothama H, Rao GA, Rao JA. Cytokine levels in Plasma and PHA Stimulated T Cells in Healthy Indian Subjects. J Cytokine Biol 2018;3:122. DOI: 10.4172/2576-3881.1000122.
15. BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit Instruction Manual. Available from: https://www.bdbiosciences.com/content/dam/bdb/marketing-documents/CBA_HumanTh1Th2Th17_Kit_Manual.pdf. Accessed on: 2nd Sep 2023.
16 . Hasselbalch H, Nielsen MB, Jeppesen D, Pedersen JF, Karkov J. Sonographic measurement of the thymus in infants. Eur Radiol. 1996;6(5):700-3. doi: 10.1007/BF00187675.
17. Jones DM, Read KA, Oestreich KJ. Dynamic Roles for IL-2-STAT5 Signaling in Effector and Regulatory CD4+ T Cell Populations. J Immunol. 2020;205(7):1721-30. doi: 10.4049/jimmunol.2000612.
18. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007;26(3):371-81. doi: 10.1016/j.immuni.2007.02.009.
19. Abbas AK, Trotta E, R Simeonov D, Marson A, Bluestone JA. Revisiting IL-2: Biology and therapeutic prospects. Sci Immunol. 2018;3(25):eaat1482. doi:

10.1126/sciimmunol.aat1482.

20. Silverberg JI, Rosmarin D, Chovatiya R, Bieber T, Schleicher S, Beck L et al. The regulatory T cell-selective interleukin-2 receptor agonist rezpegaldesleukin in the treatment of inflammatory skin diseases: two randomized, double-blind, placebo-controlled phase 1b trials. Nat Commun. 2024;15(1):9230. doi: 10.1038/s41467-024-53384-1.
21. León B. Understanding the development of Th2 cell-driven allergic airway disease in early life. Front Allergy. 2023;3:1080153. doi: 10.3389/falgy.2022.1080153.
22. Bachus H, McLaughlin E, Lewis C, Papillion AM, Benveniste EN, Hill DD et al. IL-6 prevents Th2 cell polarization by promoting SOCS3-dependent suppression of IL-2 signaling. Cell Mol Immunol. 2023;20(6):651-65. doi: 10.1038/s41423-023-01012-1.
23. Frezza S, Gallini F, Palazzo R, Carollo M, De Carolis MP, D'Andrea V et al. T-cell polarization: Potential serological markers in preterm and term infants. Early Hum Dev. 2016;101:69-71. doi: 10.1016/j.earlhumdev.2016.03.013.
24. Matoba N, Yu Y, Mestan K, Pearson C, Ortiz K, Porta N et al. Differential patterns of 27 cord blood immune biomarkers across gestational age. Pediatrics. 2009;123(5):1320-8. doi: 10.1542/peds.2008-1222.
25. Lusyati S, Hulzebos CV, Zandvoort J, Sauer PJ. Levels of 25 cytokines in the first seven days of life in newborn infants. BMC Res Notes. 2013;6:547. doi: 10.1186/1756-0500-6-547.
26. Wooldridge AL, McMillan M, Kaur M, Giles LC, Marshall HS, Gatford KL. Relationship between birth weight or fetal growth rate and postnatal allergy: A systematic review. J Allergy Clin Immunol. 2019;144(6):1703-1713. doi: 10.1016/j.jaci.2019.08.032.
27. Korhonen P, Haataja P, Ojala R, Hirvonen M, Korppi M, Paassilta M et al. Asthma and atopic dermatitis after early-, late-, and post-term birth. Pediatr Pulmonol. 2018;53(3):269-277. doi: 10.1002/ppul.23942.
28. Miller JFAP. The function of the thymus and its impact on modern medicine. Science. 2020;369(6503):eaba2429. doi: 10.1126/science.aba2429.
29. Moore SE, Prentice AM, Wagatsuma Y, Fulford AJ, Collinson AC, Raqib R, et al. Early-life nutritional and environmental determinants of thymic size in infants born in rural Bangladesh. Acta Paediatr. 2009;98(7):1168-75. doi: 10.1111/j.1651-2227.2009.01292.x.
30. de Felice C, Toti P, Musarò M, Peruzzi L, Paffetti P, Pasqui L et al. Early activation of

the hypothalamic-pituitary-adrenal-axis in very-low-birth-weight infants with small thymus at birth. J Matern Fetal Neonatal Med. 2008;21(4):251-4. doi: 10.1080/14767050801927871.

31. Toti P, De Felice C, Stumpo M, Schürfeld K, Di Leo L, Vatti R, et al. Acute thymic involution in fetuses and neonates with chorioamnionitis. Hum Pathol. 2000;31(9):1121-8. doi: 10.1053/hupa.2000.16676.
32. Ngom PT, Collinson AC, Pido-Lopez J, Henson SM, Prentice AM, Aspinall R. Improved thymic function in exclusively breastfed infants is associated with higher interleukin 7 concentrations in their mothers' breast milk. Am J Clin Nutr. 2004;80(3):722-8. doi: 10.1093/ajcn/80.3.722.
33. Thyssen JP, Andersen YMF, Zhang H, Gislason G, Skov L, Egeberg A. Incidence of pediatric atopic dermatitis following thymectomy: A Danish register study. Allergy. 2018;73(8):1741-1743. doi: 10.1111/all.13457.
34. Eriksen HB, Biering-Sørensen S, Lund N, Correia C, Rodrigues A, Andersen A et al. Factors associated with thymic size at birth among low and normal birth-weight infants. J Pediatr. 2014;165(4):713-21. doi: 10.1016/j.jpeds.2014.06.051.
35. Lee JK, Hern Tan LT, Ramadas A, Ab Mutalib NS, Lee LH. Exploring the Role of Gut Bacteria in Health and Disease in Preterm Neonates. Int J Environ Res Public Health. 2020;17(19):6963. doi: 10.3390/ijerph17196963.
36. Henderickx JGE, Zwittink RD, van Lingen RA, Knol J, Belzer C. The Preterm Gut Microbiota: An Inconspicuous Challenge in Nutritional Neonatal Care. Front Cell Infect Microbiol. 2019;9:85. doi: 10.3389/fcimb.2019.00085.
37. Tamburini S, Shen N, Wu HC, Clemente JC. The microbiome in early life: implications for health outcomes. Nat Med. 2016;22(7):713-22. doi: 10.1038/nm.4142.
38. Heida FH, Harmsen HJ, Timmer A, Kooi EM, Bos AF, Hulscher JB. Identification of bacterial invasion in necrotizing enterocolitis specimens using fluorescent in situ hybridization. J Perinatol. 2017;37(1):67-72. doi: 10.1038/jp.2016.165.
39. de la Cochetiere MF, Piloquet H, des Robert C, Darmaun D, Galmiche JP, Roze JC. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. Pediatr Res. 2004;56(3):366-70. doi: 10.1203/01.PDR.0000134251.45878.D5.
40. Mai V, Torrazza RM, Ukhanova M, Wang X, Sun Y, Li N et al. Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants. PLoS One. 2013;8(1):e52876. doi: 10.1371/journal.pone.0052876.

Figure 1. Study design

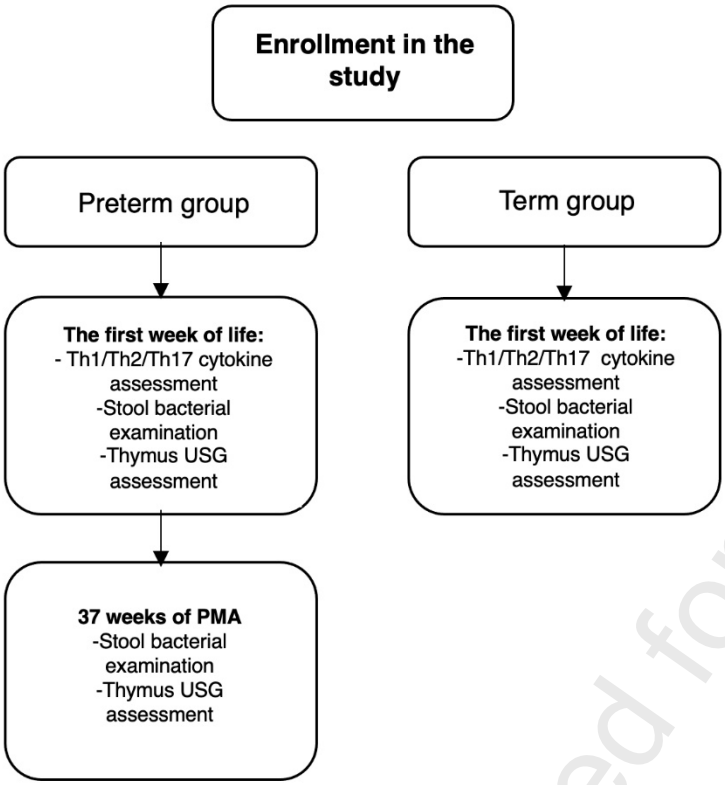
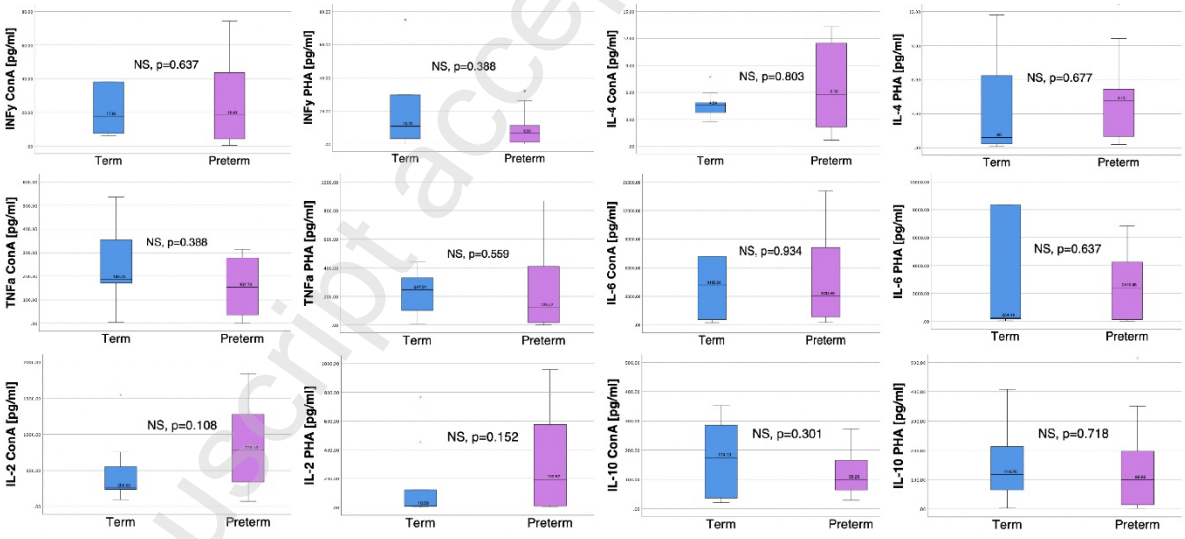
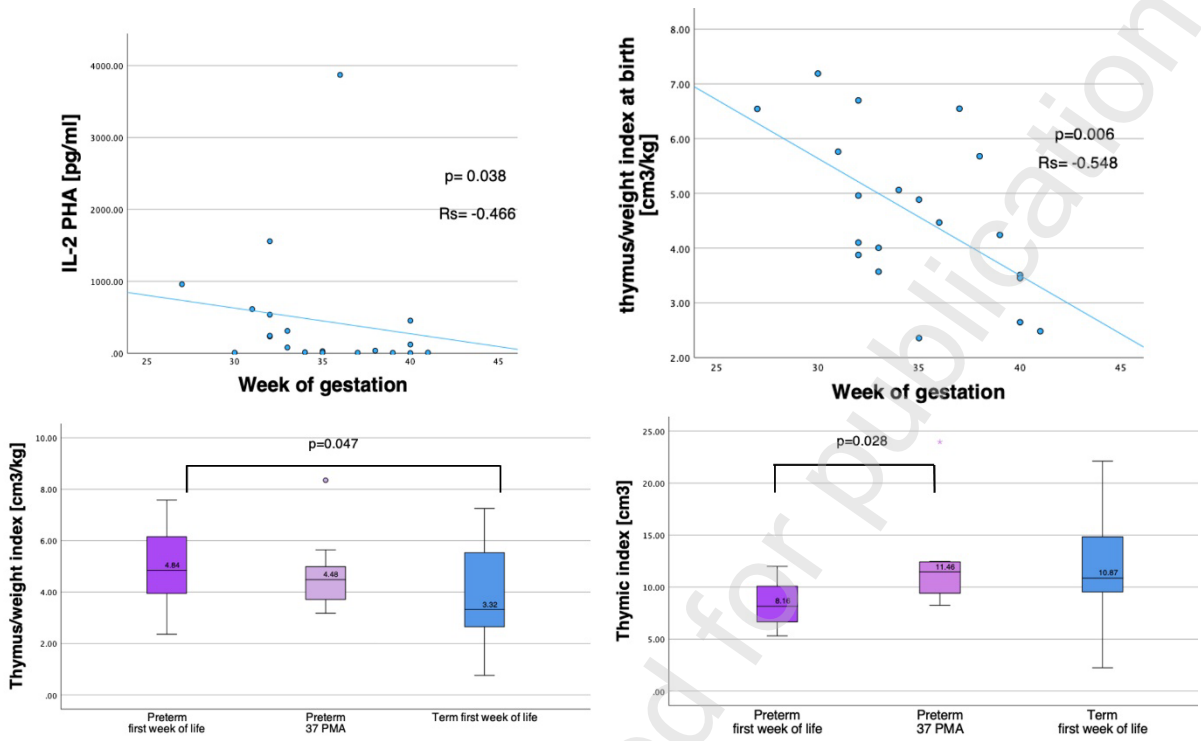


Figure 2. Levels of IFN-γ, TNF-α, IL-2, IL-4, IL-6 and IL-10 in the preterm and term groups



NS, non-significant

Figure 3. Correlation of IL-2 PHA, thymus/weight index and week of gestation. Comparison of the thymic indices and thymus weight indices between the study groups



Rs, Spearman's rank correlation coefficient

Table I. Characteristics of the studied population.

Data are presented as medians (Q1-Q3) or means (SD) for continuous variables and % (n) for categorical variables. Statistical tests used for analysis are presented below:

[a] Chi-squared test

[b] Mann-Whitney U test

[c] Fisher's exact test

[d] Independent samples t-Test

Household distance from high-velocity road was standardized in grades 1-4, where 1: <10m, 2:10-200 m, 3: 200m-1km, 4: >1 km). Household dust mites presence was standardized in grades 1-6 accordingly to the frequency of household dust cleaning, where 1: every day, 2: every 2 days, 3: every 3 days, 4: once per week, 5: 2-3 times per month, 6: once per month or less. Pregnancy emotional stress intensity was assessed subjectively by the mother in grades 1-10, where 1: no stress, 10: the greatest imaginable stress.

Cohort characteristics	**Preterm n=28**	**Term n=19**	**p-value**
Female sex, % (n)	25.0 (7)	63.2 (12)	**0.009[a]**
Gestational age [weeks], median (Q1-Q3)	32 (30-33)	38 (37-41)	**<0.001[b]**
Cesarian section, % (n)	75 (21)	73 (14)	1.0[c]
Birth weight [g], mean, (SD)	1905 (639)	3321 (549)	**<0,001[d]**
Apgar score 1 min [points], median (Q1-Q3)	7 (5,25-7,75)	9 (7-10)	**0,001[b]**
Antibiotic therapy [days], median (Q1-Q3)	8.5 (4.25-12)	7 (4-9)	0,112[b]
Parenteral nutrition [days], median (Q1-Q3)	7 (4-12.5)	2 (0-6)	**<0,001[b]**
Breast milk feeding in NICU, % (n)	96 (27)	89 (17)	0.557[c]
Length of stay in NICU [days], median (Q1-Q3)	24 (18.25-50)	16 (9-20)	**<0,001[b]**
Respiratory failure, % (n)	85.7 (24)	52.6 (10)	**0.013[a]**
Sepsis, % (n)	21.4 (6)	0 (0)	0.068[c]
Pneumonia, % (n)	14.3 (4)	36.8 (7)	0.091[c]
Presence of Staphylococcus in microbiological culture, % (n)	39.3 (11)	15.8 (3)	0.084[a]
Blood eosinophils in the first week of life	0.4 (0.225-0.7)	0.6 (0.3-0.8)	0.316[b]

[10^3/ul], median (Q1-Q3)			
Blood hemoglobin in the first week of life [g/dl], mean (SD)	12.55 (2.39)	14.62 (2.49)	**0.006**[d]
Household distance from high-velocity road [grades 1-4], median (Q1-Q3)	3 (2-4)	3 (2-3)	0.400[b]
Household dust mites presence [grades 1-6], median (Q1-Q3)	4 (2.75-4)	3 (2-4)	0.104[b]
Any household furred pet presence, % (n)	42.9 (12)	36.8 (7)	0.767[a]
Smoking person present in the household, % (n)	35.7 (10)	15.8 (3)	0.134[a]
Parental atopic dermatitis, % (n)	3.6 (1)	5.3 (1)	1.0[c]
Parental allergic diseases other than AD, % (n)	42.9 (12)	26.3 (5)	0.247[a]
Siblings with AD, % (n)	3.6 (1)	10.5 (2)	0.557[c]
Siblings with allergic diseases other than AD, % (n)	7.1 (2)	5.3 (1)	1.0[c]
Pregnancy emotional stress intensity [grades 1-10], median (Q1-Q3)	5 (3-7)	6 (4-7)	0.821[b]

Table II. Comparison of the thymic index and thymus/weight index in both study groups

[a] Mann-Whitney U test
[b] Independent samples t-Test
[c] Wilcoxon signed-rank test
r_s – Spearman's rank correlation coefficient
* Between I and II
** Between I and III
*** Between II and III

	I. Preterm The first week of life n=20	**II. Preterm 37 weeks PMA n=10**	**III. Term The first week of life n=14**	**p-value**
Thymic index (cm^3) The first week of life median (Q1-Q3)	8.2 (6.5-10.5)	11.5 (9.3-12.4)	10.9 (9.4-14.9)	**0.028 *[c]** 0.086 **[b] 0.796 ***[a]
Thymus/weight index (cm^3/kg) The first week of life mean (SD)	4.9 (+/-1.4)	4.71 (+/-1.49)	3.8 (+/-1.8)	0.878 *[c] **0.047 **[b]** 0.122 ***[a]

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