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Morphological characteristics of microenvironment in the human thymus during fetal development

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Abstract

Background The thymus is a key organ for the development of T cells. T cell precursors first migrate from the bone marrow to the thymus. During maturation, these precursors require interactions with various types of cells that form the thymic microenvironment, such as epithelial, mesenchymal, and other immune cells not belonging to the T lineage. The aim of this study was to examine the changes in the number and diameter of Hassall's corpuscles, as well as the density and distribution of epithelial cells (p63+) and macrophages (CD68+).

Methods Twenty-five fetal thymus samples were examined, divided into five groups according to gestational age. The samples were processed using standard histological methods and immunohistochemical staining.

Results The study showed that the number and diameter of Hassall's corpuscles gradually increased during fetal development, with a significant increase from the 14th to the 38th gestational week. The average diameter of Hassall's corpuscles was largest in the age group of 34–38 weeks. The density of p63 + epithelial cells decreased in correlation with gestational week, while the density of CD68 + macrophages significantly increased, particularly in the thymic medulla, towards the end of the fetal period.

Conclusions An increase in the number and size of Hassall's corpuscles during fetal development was recorded, while the density of epithelial cells decreased and the density of macrophages increased.

Keywords Thymus, Hassall's corpuscles, Epithelial cells, Macrophages

Introduction

The thymus, a primary lymphoid organ, plays a critical role in pediatric immunology, though its embryology remains complex [1]. During childhood, the thymus is large but undergoes gradual fatty involution, with about 80% replaced by fatty tissue by age 20 and full involution by age 60 [2]. The lymphoid component decreases annually, faster before middle age [3]. Structurally, the thymus has two compartments: the epithelial space (cortex and medulla) and the perivascular space. The thymic microenvironment is a specialized cellular space comprised of thymic epithelial cells, myoid cells, and accessory cells derived from bone marrow [4]. This environment is essential for the differentiation, maturation, and selection of T lymphocytes. The histological attributes of the thymus are notably influenced by the age of the individual as well as various adverse stimuli (hormones, pesticides, certain diseases, poor nutrition, stress, and radiotherapy) [4].

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Thymus plays an important role in providing a suitable microenvironment for the proliferation, differentiation, TCR gene rearrangement and repertoire selection of T cells [4]. T cell development occurs in the cortex, while negative selection takes place in the medulla, where Hassall's corpuscles reside [5, 6]. Hassall's corpuscles (HC), unique and functionally active components of the thymic medulla's non-lymphocytic environment, emerge when lymphopoiesis is established and the thymic architecture, including the cortex, medulla, and cortico-medullary junction, is ready for T-lymphocyte selection [7]. They are thought to provide crucial signals for the differentiation of lymphocytes, highlighting their significant role in thymic education [8–11].

This study aims to analyze the thymus during fetal development, focusing on changes in the number and size of Hassall's corpuscles and the distribution of p63 + epithelial cells and CD68 + macrophages to better understand T cell maturation.

Materials and methods

This retrospective study was conducted at the Center for Pathology and Histology of the University Clinical Center of Vojvodina (UKCV). Samples were collected from October 1, 2019, to February 1, 2020, from both autopsies and archived materials. A total of 25 fetal thymus samples, from fetuses 14 to 38 weeks, were analyzed. Gestational week (GW) was confirmed through clinical documentation and fetal measurements (weight, crown-rump, femur, and foot lengths). Samples were divided into five groups based on GW: 14–18, 19–23, 24–28, 29–33 and 34–38 GW, with five samples per group.

Thymus samples were fixed in 4% formalin, processed through ethanol and xylene, and embedded in paraffin. Sections were cut at 5 µm and stained with hematoxylin and eosin (HE) for structural examination. Cylindrical samples, 5 mm in diameter, were extracted and re-embedded into paraffin molds for detailed histological examination. Immunohistochemical staining was performed using monoclonal antibodies to identify CD68 + macrophages and p63 + epithelial cells, with staining protocols adhering to manufacturer guidelines. Positive and negative controls ensured the specificity and reliability of the results.

Qualitative analysis of histological sections was performed using a DMLB100T light microscope to assess the morphological structure and distribution of CD68 + macrophages and p63 + epithelial cells in the thymus. Quantitative analysis was conducted using VisionTek TM Sakura, with images analyzed morphometrically using VisionTekLive2.6 software to measure HC and cell densities.

In HE-stained sections, the number and diameter of HC were measured, and their density per square millimeter was calculated. In immunohistochemically stained sections, the density of CD68 + macrophages and p63 + epithelial cells was determined over a 0.2 mm^2 area for both the cortex and medulla, and averages were calculated for each GW group.

Statistical analysis was conducted using Microsoft Excel and IBM SPSS Statistics (v27.0.2). Mean values, standard deviations, Pearson's correlation, and linear regression assessed relationships between GW and cell density or corpuscle diameter. Spearman's correlation evaluated the monotonic relationship with HC size, and one-way ANOVA tested differences in corpuscle diameters across GW groups. Statistical significance was set at $p < 0.05$.

The study adhered to the Declaration of Helsinki [12] and was approved by the UKCV Ethics Committee, Novi Sad (decision: 00-1212, December 31, 2019). Fetal samples were anonymized, obtained from legally compliant autopsies, and processed respecting privacy and confidentiality. Data were used solely for scientific purposes without disclosing personal information.

Results

Figure 1 shows an upward trend in the average number of HC from 14 to 38 GW. The lowest count is at 14–18, with the highest at 34–38 GW. A Pearson's correlation coefficient of 0.58 ($p = 0.007$) indicates a moderate positive correlation, suggesting that both the number and diameter of HC increase with GW. Additionally, a Spearman's rank correlation coefficient of 1.0 ($p < 0.001$) confirms a strong positive monotonic relationship between the average size of the corpuscles and GW.

Figure 2 shows linear regression results indicating a significant positive correlation between GW and HC count. Each additional GW increases the corpuscle count by 0.3355 ($p = 0.007$). The R^2 value of 0.336 means that approximately 33.6% of the variation in corpuscle numbers is explained by GW, highlighting a consistent increase in numbers with advancing pregnancy.

Figure 3 illustrates the average dimensions of HC in micrometers across different GW groups from 14 to 38 weeks. There's a visible increase in corpuscle size as GW advances, with dimensions growing larger from the 14–18 to the 34–38 GW. The vertical lines on the graph indicate variability within each group. A one-way ANOVA analysis confirms this trend as statistically significant, with an F-value of 2.58 and a p-value of 0.001, suggesting that the size of HC consistently increases with GW.

Figure 4 reveals a statistically significant positive relationship between GW and the average diameter of HC, as evidenced by linear regression. The average diameter grows by about 0.996 µm for each additional GW ($p < 0.001$). With an R^2 of 0.55, approximately 55% of the

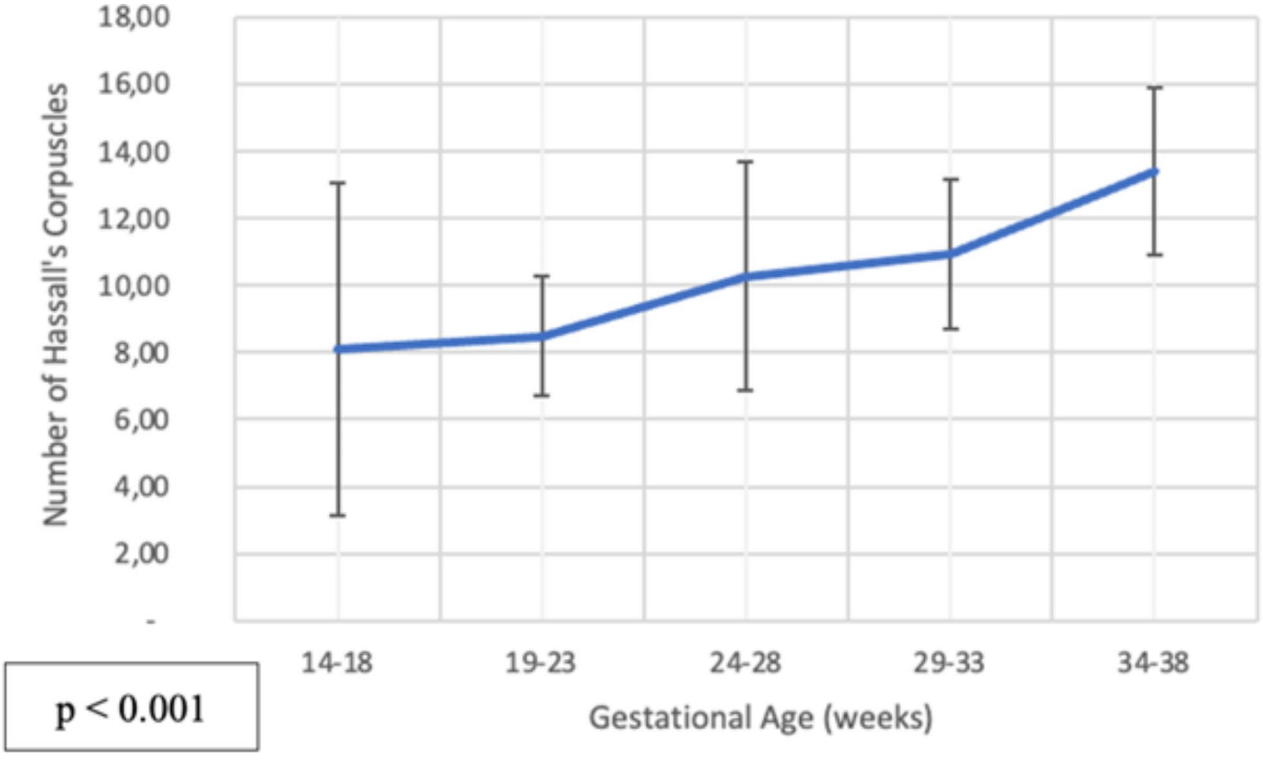

Fig. 1 Number of Hassall's corpuscles in the fetal thymus

variation in corpuscle diameter can be attributed to GW, indicating a clear trend of increasing corpuscle size as pregnancy advances.

Figure 5 shows a decreasing trend in the density of p63 + cells in the thymus cortex and medulla across GW from 14 to 38. Initially, the medulla displays a higher cell density that reduces more significantly by later stages compared to the cortex. Pearson's correlation analysis reveals a moderately strong negative correlation between GW and p63 + cell density in both regions: -0.53 for the cortex ($p = 0.042$) and − 0.51 for the medulla ($p = 0.051$), indicating that cell density decreases as GW progresses, particularly in the medulla.

Figure 6 presents regression analysis results for the density of p63 + cells in the thymus cortex (blue) and medulla (red) against GW, showing a negative trend in both areas. In the cortex, an R-squared value of 0.969 indicates that 97% of the variation in cell density is due to GW, with a decline of about 3.71 units per week ($p = 0.002$). In the medulla, 93% of the variation is explained by GW, with a steeper decline of approximately 5.98 units per week ($p = 0.008$). This analysis underscores a significant decrease in p63 + cell density with advancing gestation in both thymic regions.

Figure 7 shows histological sections of the thymus stained for p63 + cells. (A) In the cortex at 24 GW, p63 + cells, highlighted in blue-brown hues, are concentrated subcapsularly near the.

thymus capsule, with a more diffuse distribution elsewhere. (B) In the medulla at 18 GW, p63 + cells are evenly distributed throughout, with no significant clustering along the corticomedullary boundary.

Figure 8 shows that the number of CD68 + cells in the thymus increases with GW in both the cortex and medulla. Pearson's correlation analysis reveals a very strong positive association between GW and CD68 + cell density. In the cortex, the correlation coefficient is 0.993 ($p = 0.007$), and in the medulla, it is 0.99, although the latter is not statistically significant ($p > 0.05$). This highlights a pronounced increase in CD68 + cells with advancing gestational age.

Figure 9 shows histological sections of the thymus stained for CD68+ cells in brown. (A) Cortex at 21 GW, with diffusely distributed CD68+ cells evenly spaced throughout the lobules. (B) Medulla at 19 GW, displaying a uniform distribution of CD68+ cells, evenly dispersed without clustering, against the blue-stained nuclei background.

Discussion

The thymic microenvironment plays a critical role in T cell development, ensuring proper differentiation and selection of T lymphocytes, which are vital for

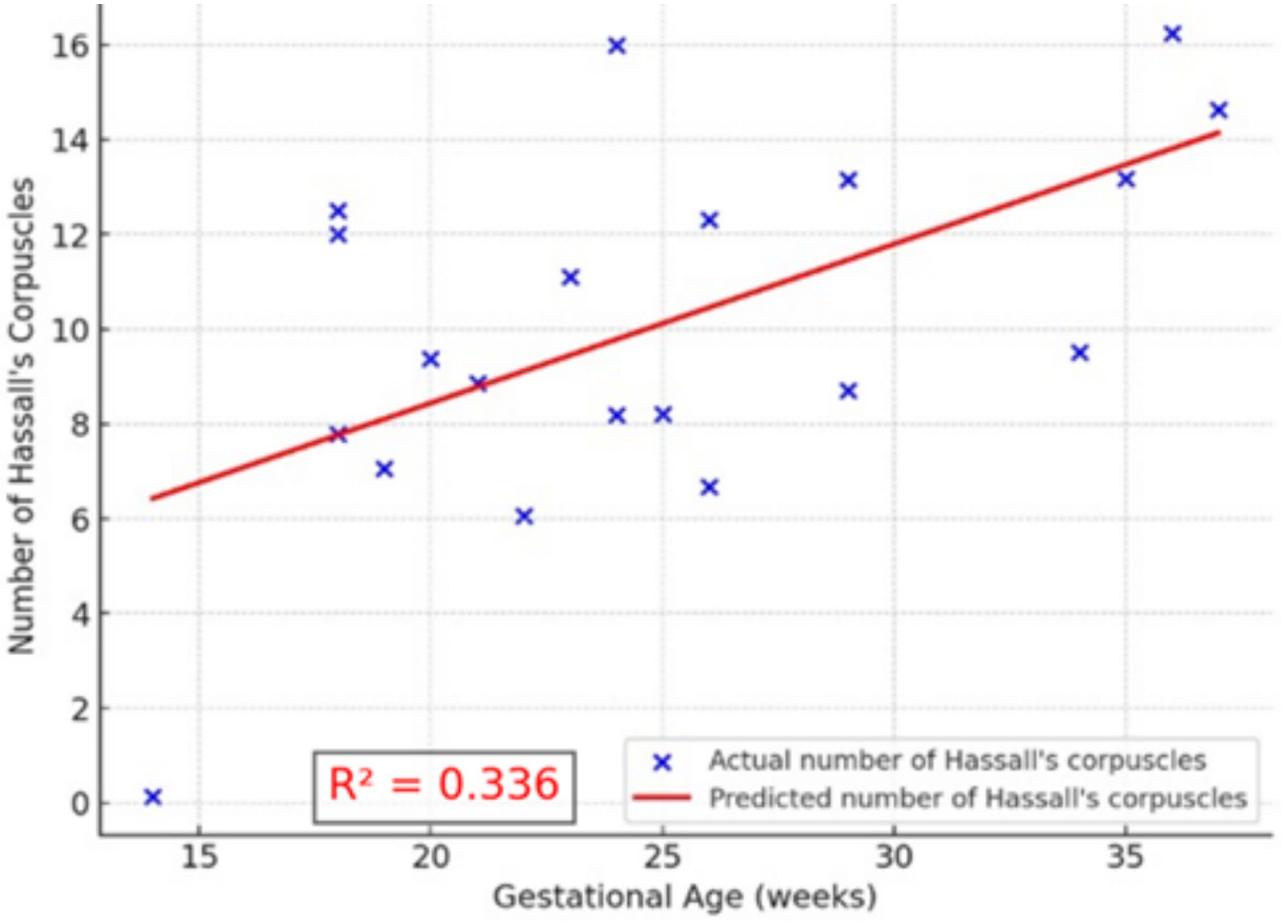

Fig. 2 Regression analysis: number of Hassall's corpuscles in relation to GW

maintaining immune function and preventing autoimmune diseases [13, 14]. This environment, however, is susceptible to damage from factors such as radiation and chemicals. Treatments like hematopoietic stem cell transplantation (HSCT) can significantly harm the thymus due to chemotherapy, radiotherapy, and antibody therapies [15]. Such damage is often exacerbated by infections or thymic graft-versus-host disease (GVHD) in cases of allogeneic HSCT [16, 17]. Nevertheless, the thymus has a regenerative capacity following acute damage, though this ability declines with age due to thymic involution.

Thymic epithelial cells (TECs) are essential for T cell development, forming a cytoreticulum that houses blast lymphocytes and supports their maturation [18]. TECs are classified into cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC) subtypes, each playing a vital role in shaping the T cell repertoire. TEC also produce thymic hormones, including thymulin and thymopoietin, essential for T lymphocyte maturation and immune response regulation [19, 20]. Notably, fetal and perinatal thymus research indicates the presence of a bipotent progenitor capable of generating both cTECs and mTECs [21].

In this study, the presence of epithelial cells was confirmed using Monoclonal Mouse p63, which predominantly marks thymic epithelial cells, particularly cTEC [22]. The postnatal thymic cortex comprises various microenvironments, including the subcapsular region, central cortex, and perimedullary cortex [23]. Staining with monoclonal antibodies, coupled with microscopic morphology and location analysis, identified at least four different subtypes of cTEC within the thymic cortex [24], although the specific functions of these subtypes are not yet fully understood.

Gene expression analysis across the subcapsular region, central cortex, and perimedullary cortex revealed diverse profiles, indicating functional variability [23, 25]. These cells are key in T cell maturation, with the p63 protein playing a crucial role in their development and maintenance. The ΔNp63 isoform is particularly essential for the proliferation and differentiation of thymic epithelial cells, ensuring thymic structure and function [26]. This isoform supports epithelial progenitor cells and facilitates the positive selection of T cells necessary for a robust immune response [22, 27]. cTECs also present

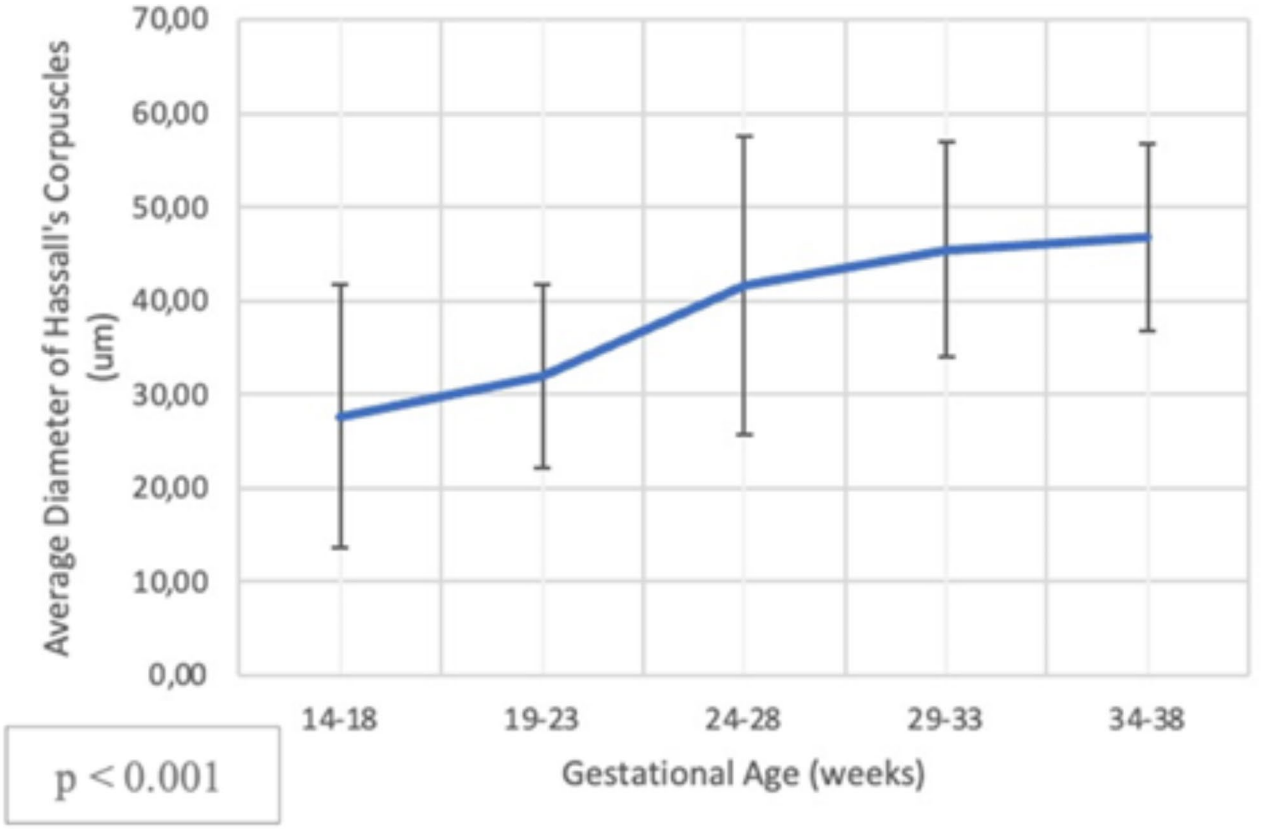

Fig. 3 Average diameter of Hassall's corpuscles in the thymus

self-antigens via MHC molecules, vital for positive selection and T cell diversity [28].

cTEC cells, derived from endodermal epithelium and distinct from lymphoid or hematopoietic cells, play a vital role in the adaptive immune system. They express specific molecules such as b5t and MHC class II, crucial for adaptive immunity and found only in vertebrates with this immune capability [23, 29].

While cTECs facilitate the positive selection of T lymphocytes, mTECs are crucial for negative selection during T lymphocyte development. mTECs are integral to the thymus's three-dimensional structure, which is essential for proper T lymphocyte selection, maintaining central immune tolerance, and preventing autoimmune diseases [30]. mTECs express a broad array of tissue-specific antigens (TRAs) that are pivotal for the negative selection of autoreactive T lymphocytes, eliminating those that bind strongly to self-antigens and thus establishing central tolerance [28, 31]. Furthermore, the self-antigens expressed by mTECs also help regulate the production of regulatory T cells (Tregs), which are essential for suppressing immune responses in peripheral tissues and ensuring peripheral tolerance [28, 32].

This study observed a decrease in epithelial cell density during fetal thymic development, especially in the thymic medulla and subcapsular regions. While Wang et al. reported a significant increase in epithelial cells during fetal thymic maturation [22], our findings suggest that the rapid growth of thymic tissue outpaces the proliferation of epithelial cells, leading to a relative decrease in their density.

Hassall's corpuscles (HC) are localized exclusively in the thymic medulla. Raica et al. developed a classification system for HC based on patient age, structural characteristics, and immunohistochemical features, identified four distinct groups: juvenile, immature, mature, and senescent [33]. Each category reflects specific stages in the development and aging of HC, indicating their varying appearances and functions within the thymus across different age groups [33]. These unique, antigen-specific structures play a functional role in both the prenatal and adult thymus, contributing to immune regulation and thymic function [34]. While their exact role is not fully understood, HC are known to produce cytokines that aid in dendritic cell maturation and the induction of regulatory T cells, which are essential for maintaining immune tolerance [35]. Recent studies suggest that they may be involved in the pathogenesis of autoimmune diseases like type 1 diabetes, rheumatoid arthritis, and multiple sclerosis [35, 36]. Additionally, they synthesise chemokines

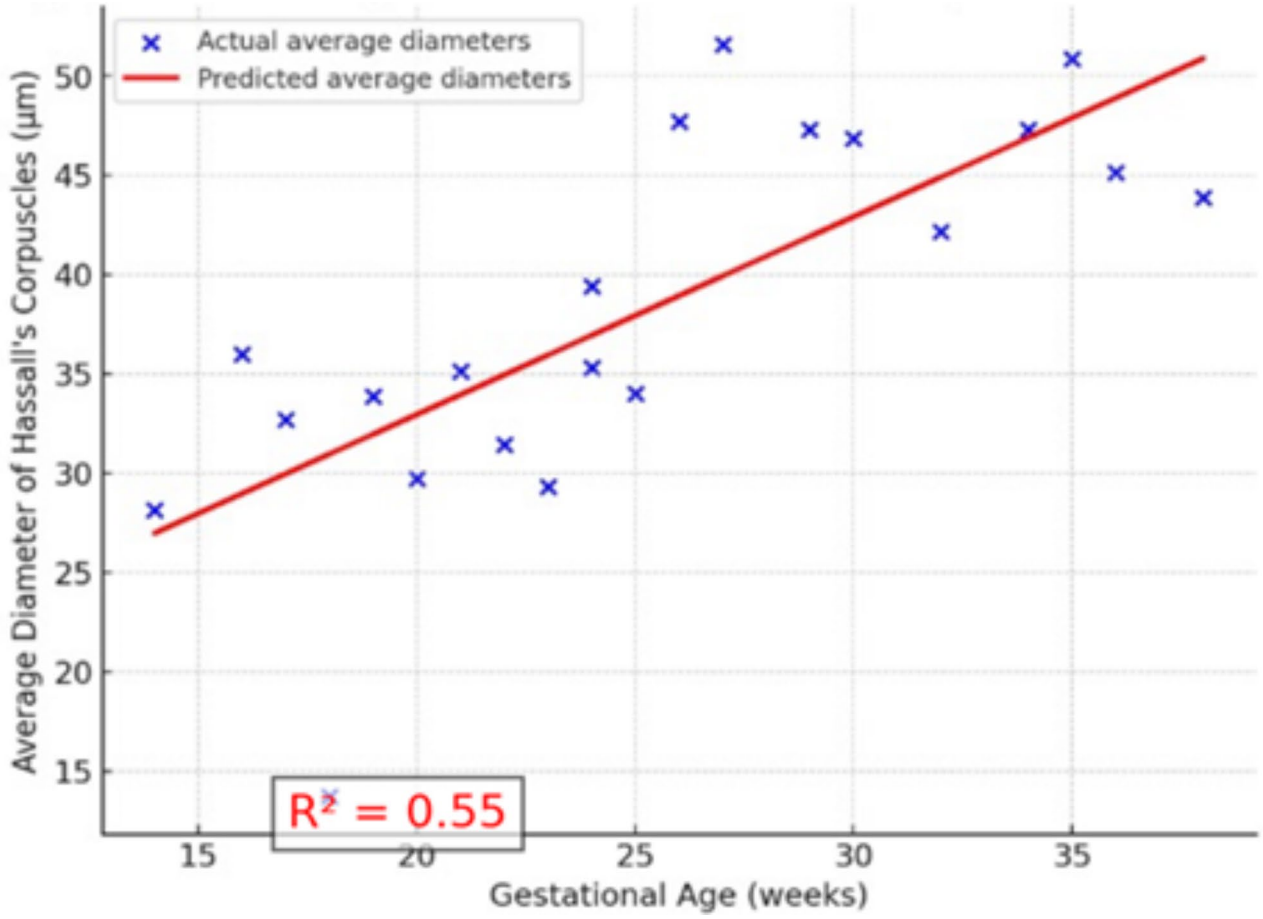

Fig. 4 Linear regression: average diameter of Hassall's corpuscles in relation to GW

affecting other thymic medulla cells, although their interactions with dendritic and neuroendocrine cells remain incompletely explored [33, 35]. Some research also indicates that HC participate in negative lymphocyte selection, the elimination of autoreactive T cells, and the storage of antibodies and antigens [6].

Variations in size, shape, and number of HC during development, particularly noticeable in children with various types of congenital heart defects, may be linked to disruptions in the formation, migration, or differentiation of cardiac neural crest cells [7]. These cells are crucial for the development of both the heart and thymus [7, 37].

HC form during thymic ontogeny once lymphopoiesis is established and the cortex, medulla, and cortico-medullary junction are capable of conducting T-lymphocyte selection [8, 9, 33, 34]. These corpuscles originate from reticular epithelial cells that undergo keratinization and hypertrophy before forming the outer layer of the corpuscle [38]. Varga et al. noted the first appearance of HC at 13 GW, while Liberti et al. described their emergence at 16 GW, and Bodey et al. reported their appearance between the 6th and 10th lunar months in humans [9, 34, 39]. In our study, the first HC was observed at 14 GW, which aligns with the findings of Gilhus et al., where HC were not detected before 14 GW [40].

Our study demonstrates that the number and diameter of HC increase as fetal development progresses, consistent with other studies [8, 9, 41]. Varga et al. showed a significant increase in the number of HC between the 16th and 18th GW, and between the 22nd and 25th GW [9], while our study recorded the largest growth in HC area observed between the 19th and 28th GW and the most significant increase in HC numbers between the 29th and 38th GWs, indicating continuous HC development throughout gestation [42, 43]. As the growth and development of the fetus are accompanied by the growth and development of the thymus and all its structures, it is natural to expect an increase in the number and diameter of HC as GW increases.

Nearly all thymocytes (95–97%) undergo apoptosis during T cell development in the thymus [44]. **Macrophages** play a crucial role in clearing these apoptotic thymocytes, particularly those that fail positive selection or are eliminated during negative selection [45]. They are essential for phagocytosing dead thymocytes

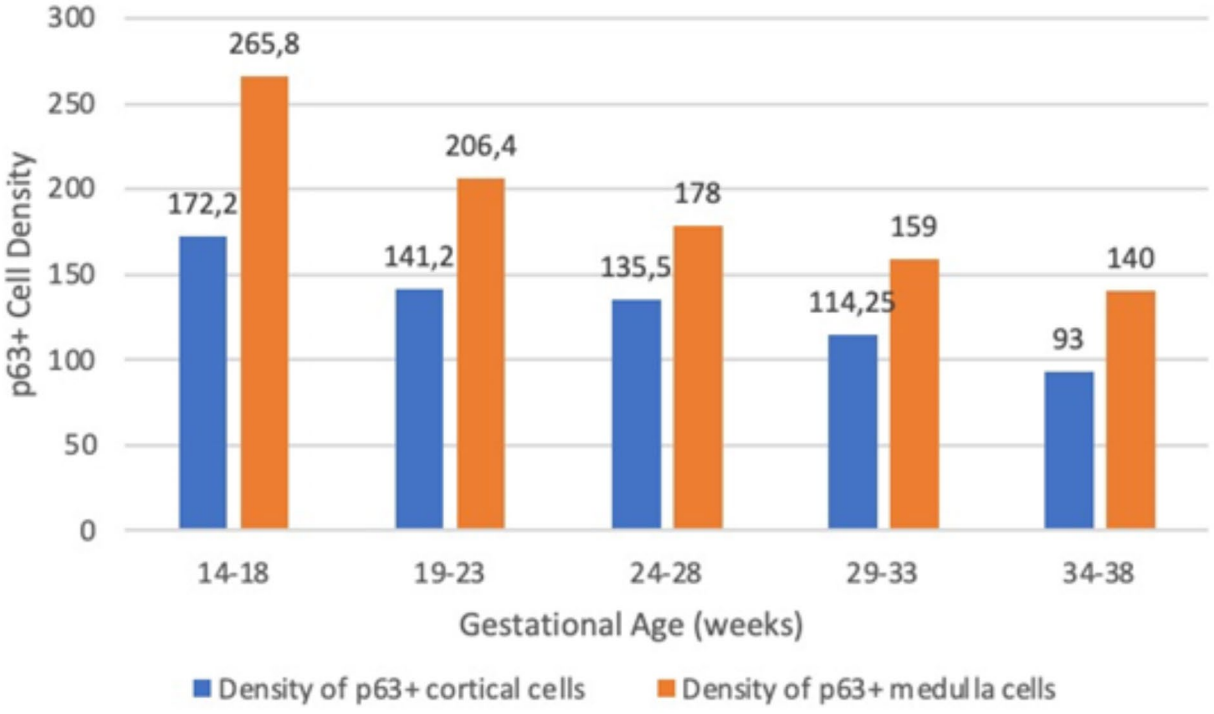

Fig. 5 Density of p63 + cells in the thymus by GW

and maintaining the immune microenvironment necessary for T cell development [11, 46]. Additionally, thymic macrophages contribute to tissue repair, particularly after injuries like radiation, which is used in some oncology treatments. They secrete cytokines, growth factors, and other molecules that promote epithelial regeneration and help maintain thymic structure following damage [11, 47].

For many years, thymic macrophages were poorly understood due to technical limitations in analyzing these cells and conducting functional studies. Only a few macrophage markers (e.g., ED1 and ED2 in rats, CD68, F4/80, and CD11b in mice) have been identified, making it difficult to study their origin and heterogeneity in the thymus [48, 49]. As the thymus grows during fetal development and the number of apoptotic thymocytes rises, macrophage density also increases, especially in the medulla. Our study corroborates these findings, showing a continuous increase in macrophage density throughout fetal development, supporting their role in clearing apoptotic cells and aiding thymic immune function.

Further research is needed to better understand the thymus's role in cellular immunity, as disruptions in this microenvironment can impair T cell maturation and increase the risk of autoimmune diseases. Although thymic involution is commonly associated with aging, emerging data suggest that sex hormones, obesity, infections, and oxidative stress also contribute to thymic atrophy [50]. Studies have shown that thymectomy in adults increases the risk of cancer and weakens immunity, underscoring the importance of preserving thymic function [51, 52].

Limitations

The limitations of this study include a relatively small sample size, which may limit the generalizability of the results to a broader population. The study is retrospective in nature and depends on the quality and availability of previously collected samples and documentation, which may introduce certain biases. The samples were collected exclusively during standard fetal autopsies, so the quality of the samples may vary. Additionally, we were unable to determine whether the examined thymuses were physiological or had been exposed to long-term negative stress. Genetic testing of the fetuses could not be performed, and the study did not account for potential additional factors such as fetal sex or genetic mutations, which could have influenced the results.

Conclusion

During fetal development, the thymus undergoes significant morphological changes. The number and diameter of Hassall's corpuscles gradually increase, while the density of epithelial cells (p63+) decreases. In contrast, the

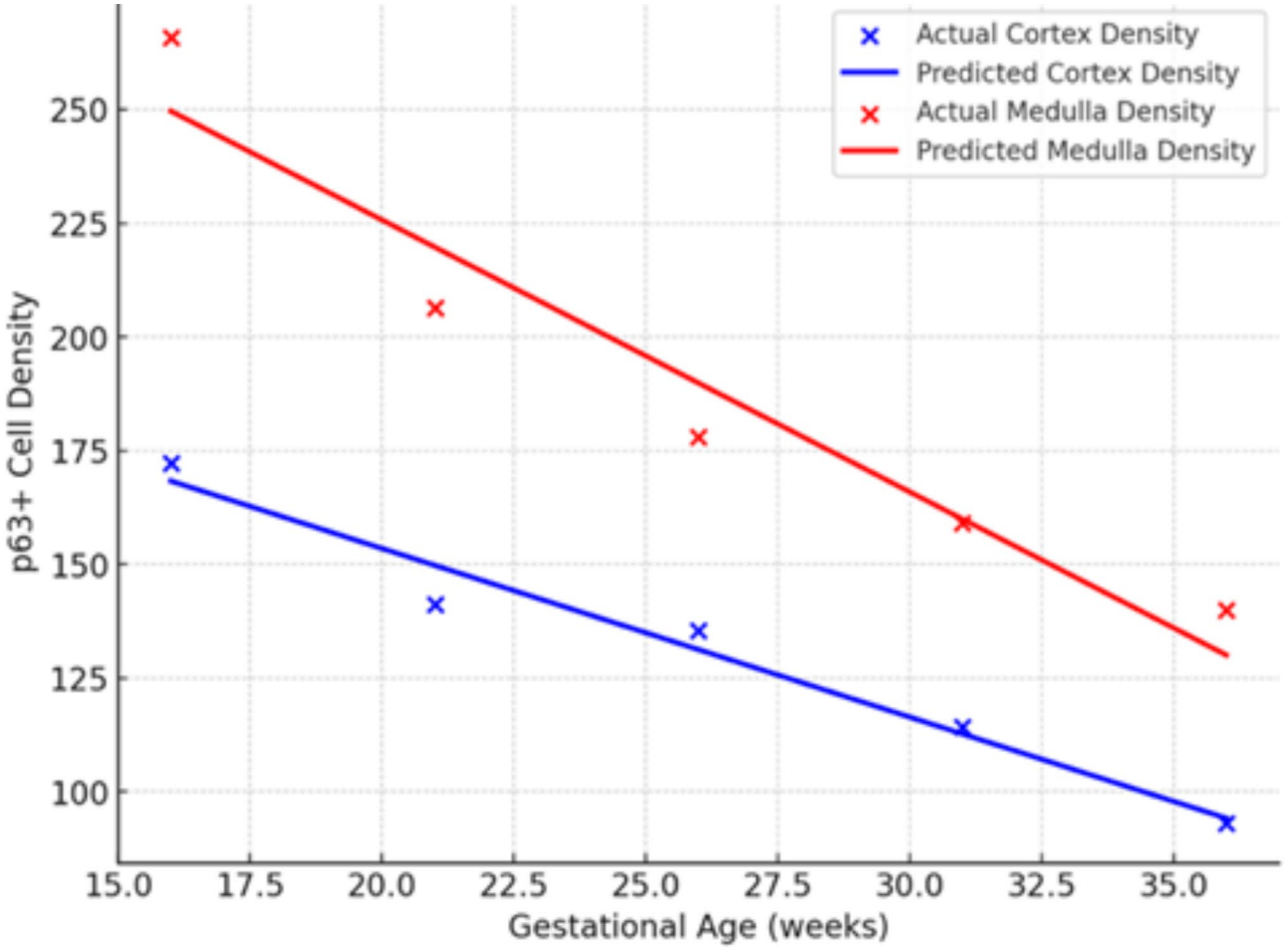

Fig. 6 Regression analysis: density of p63 + cells in relation to GW

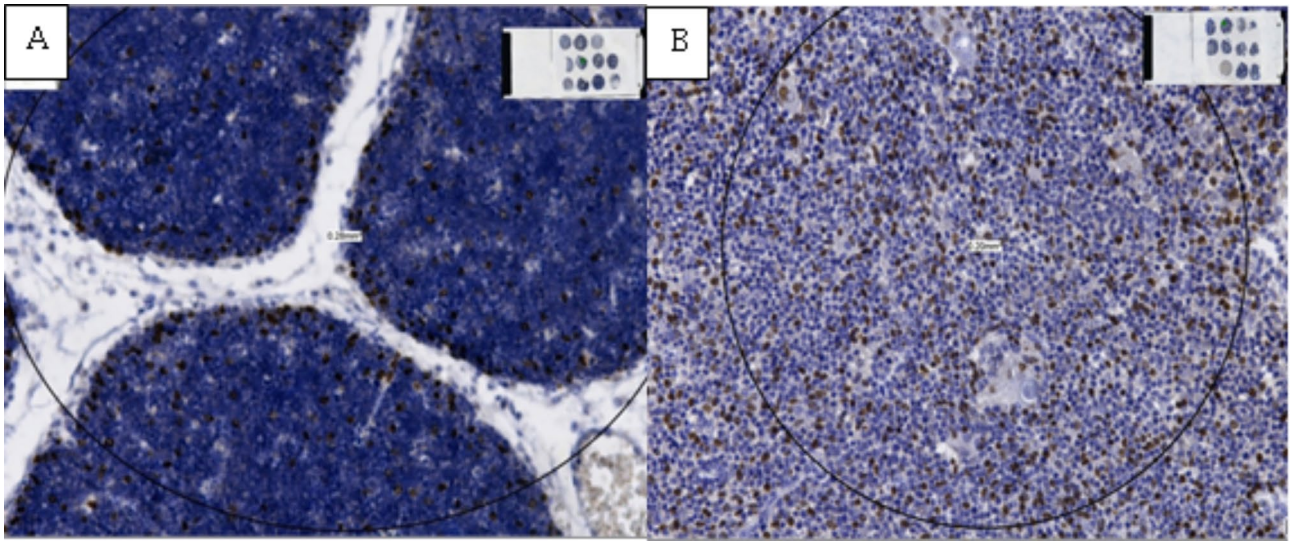

Fig. 7 Histological sections of the thymus showing p63+ immunohistochemistry at 20x magnification: (**A**) cortex at 24 GW and (**B**) medulla at 18 GW

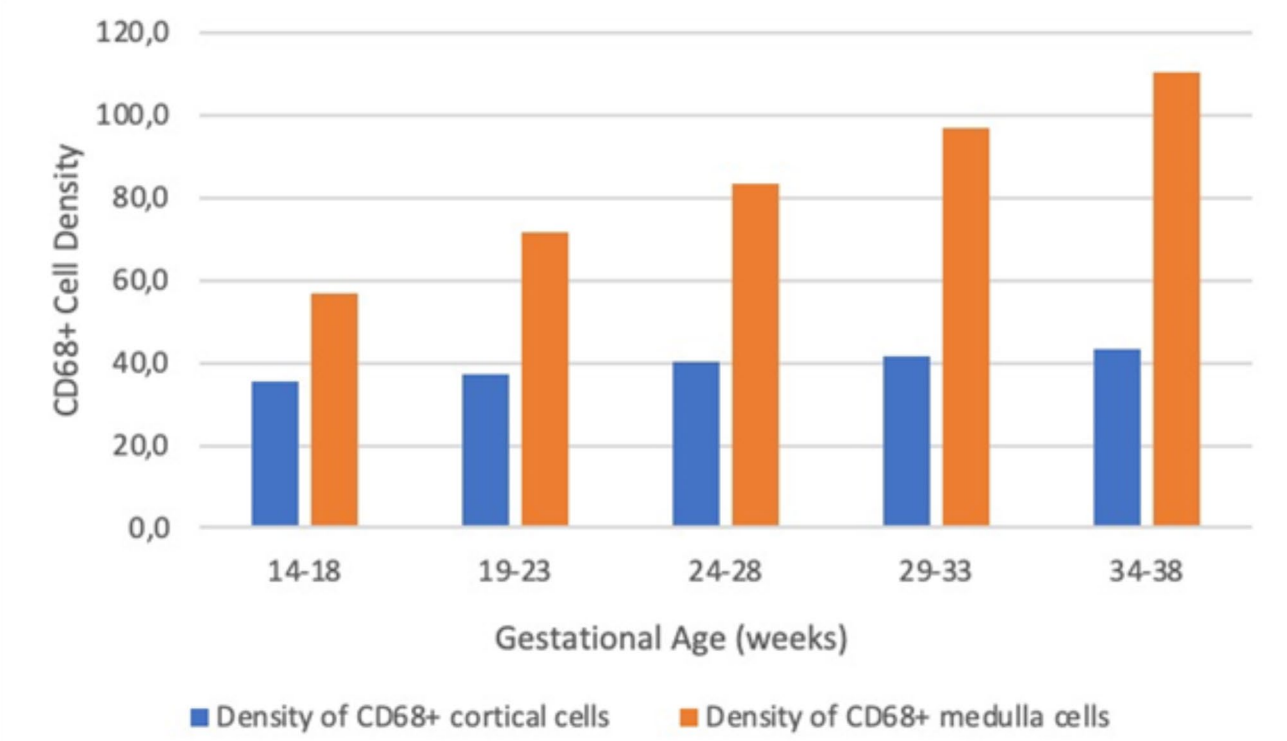

Fig. 8 Density of CD68 + cells in the thymus by gestational age

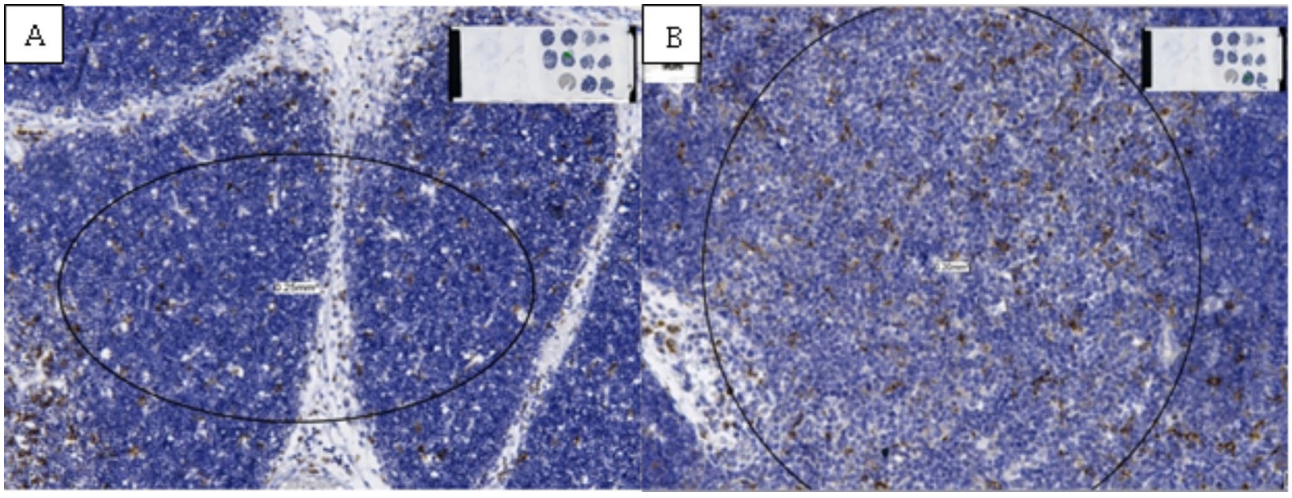

Fig. 9 Histological sections of the thymus stained for CD68 + immunohistochemistry at 20x magnification: (**A**) cortex at 21 GW and (**B**) medulla at 19 GW

density of macrophages (CD68+) increases in correlation with gestational age. These results provide important insight into the development of the thymus, which plays a key role in the maturation of T lymphocytes and immune development.

Abbreviations

HC	Hassall's corpuscles
GW	Gestational week
UKCV	University Clinical Center of Vojvodina
HE	Hematoxylin-eosin
ANOVA	One-way analysis of variance
HSCT	Hematopoietic stem cell transplantation
GVHD	Graft-versus-host disease
TECs	Thymic epithelial cells
cTECs	Cortical thymic epithelial cells
mTECs	Medullary thymic epithelial cells
MHC	Major histocompatibility complex
TRAs	Tissue-specific antigens
Tregs	Gulatory T cells

Author contributions

N.M., A.F.L. and J.A. contributed to the conception and design of the study. J.A., A.F.L., S.B., B.A.V., N.D. and N.M. contributed to collecting the data. N.M., J.A.

and N.D. participated in the statistical analysis and interpretation of data. N.M., N.D., S.B., A.F.L., Z.G., A.R., B.A.V. and J.A. contributed to drafting and revising the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All steps of the study were conducted in accordance with the Declaration of Helsinki [9]. The study protocol was reviewed and approved by the Ethics Committee of the University Clinical Center of Vojvodina, Novi Sad (decision from December 31, 2019, decision number: 00-1212). All samples were processed anonymously, respecting the privacy and confidentiality of information about the fetuses and their families. The material used in the study was collected exclusively from the archives of the Center for Pathology and Histology and from autopsies conducted in accordance with legal and ethical guidelines. All tissue analysis procedures were performed under laboratory conditions with strict adherence to ethical and professional standards. No aspect of the study compromised the physical or psychological well-being of the families, and all data were used solely for scientific purposes, without identifying personal information.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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