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INTERNSHIP REPORT

Human thymus development and aging

By

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ABSTRACT

The thymus is a valuable factor in the immune system establishment and maintenance, and it is a priority to profoundly investigate the changes that take place in this organ during its development and involution and their possible impact on the overall immune system functioning. Human thymi are routinely removed from children with congenital cardiac defects as an obstacle disabling the heart treatment and discarded as medical waste. As thymus is one of the central organs of the immune system, these interventions might also have an impact on the health of the patients. An important fact about thymus that makes it distinctive among other organs is that it undergoes involution postnatally, the process that completely changes its molecular characteristics and functional properties. A number of studies have been and are currently being conducted, investigating a range of changes that might be affecting the thymus during different stages of its development, from postnatal time until puberty. In this study, seven different antigens were investigated immunohistochemically in six thymi of ascending ages. Among them, three specific epithelial cell markers (Cytokeratin 5, Cytokeratin 8/18, EpCAM) and four specific immature thymic cell antigens (FOXN1, Jagged1, CHD7, AIRE) were assessed using an appropriate immunohistology detection system. We show here a decrease in the expression intensity and the percentage of Cytokeratin 5, Cytokeratin 8/18 and EpCAM in thymi older than 24 months, indicating a decrease in the percentage of thymic epithelial cells (TEC). A similar pattern was noticed while investigating the expression of FOXN1 as a specific transcription factor mediating TEC development expressed in the thymic epithelium and Jagged1 as part of the Notch signaling pathway orchestrating T lymphocyte development. However, age-related changes in the expression of AIRE were not detectable in this system. There is a doubt that CHD7 expression might depend on age, but this must be confirmed in a more detailed study concerning a larger experimental group. Interestingly, CHD7 as an early development marker has not been previously studied in the human postnatal thymus, and we show here for the first time its presence in the thymus aged from 3 to 58 months.

Keywords: Thymus, thymic epithelial cells, immune system, T lymphocyte development

RÉSUMÉ

Le thymus est un facteur précieux dans l'établissement et le maintien du système immunitaire, et il est prioritaire d'étudier en profondeur les changements qui se produisent dans cet organe au cours de son développement et de son involution et leur impact possible sur le fonctionnement global du système immunitaire. Le thymus humain est régulièrement enlevé chez les enfants atteints de malformations cardiaques congénitales comme obstacle au traitement cardiaque et jeté comme déchet médical. Le thymus étant l'un des organes centraux du système immunitaire, ces interventions peuvent également avoir un impact sur la santé des patients. Un fait important à propos du thymus qui le distingue des autres organes est qu'il subit une involution postnatale, le processus qui modifie complètement ses caractéristiques moléculaires et ses propriétés fonctionnelles. Un certain nombre d'études ont été menées et sont actuellement menées, examinant une série de changements susceptibles d'affecter le thymus à différents stades de son développement, depuis la période postnatale jusqu'à la puberté. Dans cette étude, sept antigènes différents ont été étudiés immunohistochimiquement dans six thymus d'âges ascendants. Parmi eux, trois marqueurs spécifiques des cellules épithéliales (Cytokératine 5, Cytokératine 8/18, EpCAM) et quatre antigènes cellulaires immatures spécifiques (FOXN1, Jagged1, CHD7, AIRE) ont été évalués à l'aide d'un système de détection immunohistologique approprié. Nous montrons ici une diminution de l'intensité d'expression et du pourcentage de Cytokératine 5, Cytokératine 8/18 et EpCAM dans les thymus de plus de 24 mois, indiquant une diminution du pourcentage de cellules épithéliales thymiques (TEC). Une tendance similaire a été observée lors de l'étude de l'expression de FOXN1 en tant que facteur de transcription spécifique médiant le développement des TEC exprimés dans l'épithélium thymique et Jagged1 dans le cadre de la voie de signalisation Notch orchestrant le développement des lymphocytes T. Cependant, les changements liés à l'âge dans l'expression d'AIRE n'étaient pas détectables dans ce système. On soupçonne que l’expression de CHD7 pourrait être dépendante de l’âge, mais cela doit être confirmé dans une étude plus détaillée concernant un groupe expérimental plus large. Il est intéressant de noter que la CHD7 en tant que marqueur du développement précoce n'a pas encore été étudiée dans le thymus postnatal humain, et nous montrons ici pour la première fois sa présence dans le thymus âgé de 3 à 58 mois.

Mots clés: Thymus, cellules épithéliales du thymus, système immunitaire, développement des lymphocytes T

SAŽETAK

Timus je značajan faktor u uspostavi i održavanju imunosnog sustava te je analiza promjena koje se u njemu događaju tijekom razvoja i involucije, kao i njihov mogući učinak na sveopće funkcioniranje imunosnog sustava, prioritet. Ljudski timus se rutinski odstranjuje kod djece oboljele od urođenih srčanih mana kao prepreka koja onemogućuje liječenje srca i odbacuje kao medicinski otpad. Kako je timus jedan od središnjih organa imunosnog sustava, ovakvi zahvati mogu utjecati na zdravlje pacijenata. Važna činjenica o timusu koja ga razlikuje od drugih organa jest njegova postnatalna involucija, proces koji u potpunosti mijenja njegova molekularna svojstva i funkcionalne značajke. Veći broj istraživanja spektra promjena koje bi mogle utjecati na timus u različitim fazama njegovog razvoja, od postnatalne dobi do puberteta, je već proveden ili trenutačno u tijeku. U ovom istraživanju, sedam različitih antigena je imunohistokemijski analizirano u šest timusa rastuće dobi. Među njima, tri specifična markera epitelnih stanica (Citokeratin 5, Citokeratin 8/18 i EpCAM) i četiri antigena specifična za nezrele stanice timusa (FOXN1, Jagged1, CHD7, AIRE) ispitana su korištenjem prikladnog imunohistološkog detekcijskog sustava. Pokazali smo smanjenje intenziteta ekspresije i postotka Citokeratina 5, Citokeratina 8/18 i EpCAM-a u timusima starijima od 24 mjeseca, što ukazuje na smanjenje postotka timusnih epitelnih stanica (TEC). Sličan uzorak je uočen kod analize specifičnog transkripcijskog faktora FOXN1 eksprimiranog u timusnom epitelu, koji posreduje u razvoju timusnih epitelnih stanica, kao i antigena Jagged1, dijela signalnog puta Notch koji kao takav upravlja razvojem limfocita T. S druge strane, korištenjem ovog detekcijskog sustava nisu uočene o starosti timusa ovisne promjene u ekspresiji antigena AIRE. Postoji sumnja da bi ekspresija CHD7 mogla ovisiti o starosti, ali to mora biti potvrđeno detaljnijim istraživanjem unutar veće eksperimentalne grupe. Zanimljivo je da CHD7 kao marker ranog razvoja timusa nije prije proučavan u postnatalnom timusu čovjeka, a ovdje je po prvi put pokazana njegova prisutnost u timusu starosti od 3 do 58 mjeseci.

Ključne riječi: Timus, timusne epitelne stanice, imunosni sustav, razvoj limfocita T

PRESENTATION OF THE LABORATORY

The Ruđer Bošković Institute is considered as Croatia's leading scientific institute in the natural and biomedical sciences as well as marine and environmental research, owing to its size, scientific productivity, international reputation in research, quality of its scientific personnel and research facilities (https://www.irb.hr/eng/Divisions). The Antica group, as part of the Division of Molecular Biology at the Ruđer Bošković Institute, deals with research in the field of immunology at the cellular and molecular level. The laboratory has state of the art equipment including a sterile unit, molecular research unit and a facility for cell sorting. The group has been working in the field of lymphocyte development and thymus regeneration for many years, described the first lymphoid precursor cell from the mouse bone marrow, discovered the murine U2 snRNP-A' gene which is differentially expressed in lymphocyte development and found the expression of Aiolos, the transcription factor from the Ikaros gene family, in human malignancies. The Laboratory for Cellular and Molecular Immunology introduced flow cytometry in year 1998, and cell sorting in year 2017 at the Ruđer Bošković Institute and more recently introduced a new technology for detection of stem cells by producing 3D cell cultures from healthy tissues. It is currently developing a core facility for stem cells and organoids as part of a regenerative medicine approach in immunology. The group is part of the Scientific Center of Excellence for Reproductive and Regenerative Medicine (CERRM) from the Medical University Zagreb. As part of the FP7-HEALTH EU project Thymistem (Grant agreement ID: 602587), the Antica group has been working on thymus regeneration and discovered a new population of thymic epithelial stem cells from the neonatal human thymus.

The work described in this thesis is part of the Thyminnova research grant of the Croatian Science Foundation that includes collaboration with the Department of cardiac surgery in infants of the Cardiac Surgery Clinic of the University Hospital Center Zagreb, as the largest health institution in the Republic of Croatia, and the Department of Surgical Pathology of the Merkur Clinical Hospital, Zagreb. The experimental part of this thesis included the implementation of immunohistochemical methods performed at the Merkur Clinical Hospital on thymi specimens obtained from the University Hospital Center Zagreb, prepared, selected and fixed at the Ruđer Bošković Institute. The work has been supported by the Croatian Science Foundation Grant IP-2020-02-2431, The Terry Fox Foundation Zagreb Run and Croatian League against Cancer, and by the Scientific Centre of Excellence for Reproductive and Regenerative Medicine (Grant Agreement KK01.1.1.01.0008 which is funded by the European Union through the European Regional Development Fund).

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Table A2. Percentage of positive cells (%) and signal intensity (↑) in different thymi compartments upon labeling with three antibodies targeting epithelial cell markers. Signal intensity legend: / - no signal; + - visible at 400x magnification; ++ - visible at 100x magnification; +++ - visible at 40x magnification. SC – subcortical; IC – intracortical; CM – corticomedullar; CL – cortical lymphocytes; E – endothel; ML – medullar lymphocytes; HC – Hassall's corpuscles

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1. INTRODUCTION

1.1. Thymus - function, development and aging

Thymus is a primary lymphoid organ of the immune system, responsible for the development of different T-cell lineages. The mature thymus consists of two compartments - the cortex and the medulla. Multipotent hematopoietic progenitors originating from bone marrow enter the thymus through blood vessels at the border of these two compartments. Inside, they are restricted to a T-cell lineage (Shortman *et al.*, 1990; Wu *et al.*, 1991) and subjected to a process of positive and negative selection, until they exit the thymus and enter the periphery as mature T lymphocytes (for review see: Miller, 2020).

T-cell progenitors are double negative (DN) cells lacking specific T-cell coreceptors, namely CD4 and CD8. They pass through several stages of development, defined by the change in T-cell receptor (TcR), coreceptors and other T-cell specific surface proteins expression. The DN stage can be subdivided into four distinct phases. During the DN1 phase, the developing T lymphocytes called thymocytes, express CD44 adhesion molecules. In the DN2 phase they additionally express the CD25 adhesion molecules, while in the DN3 phase the expression of CD44 decreases. Rearrangement of the TcR starts in CD25+ CD44+ T-cells (DN2). Depending on the composition of the TcR, two distinct T-cell lineages can develop - the α:β and the γ:δ lineage. α:β T lymphocytes predominate, while γ:δ T lymphocytes are a minor, less known lineage, with still a lot of questions to answer regarding their development. TcR rearrangement of the α:β T lymphocytes starts by rearranging the β chain during the DN2 phase, and continues by its combination with the α chain precursor (pTα) during the DN3 phase, which results in the restriction of further β chain rearrangement, ligand independent dimerization and proliferation induction. Thymocytes in the DN4 phase no longer express CD25 nor CD44 and they intensively proliferate giving rise to double positive (DP) T-cells, expressing both CD4 and CD8 coreceptors. DP stage of development includes the first phase in which large DP cells proliferate and form smaller cells of the second phase characterized by the final α chain rearrangement and formation of a full, mature TcR. Positive selection of the thymocytes that successfully recognize and bind self major histocompatibility complex (MHC) molecules of cortical thymic epithelial cells (cTECs) starts in the DP stage along with the negative selection of only those that do not strongly interact with self tissue restricted antigens (TRAs) expressed by thymic antigen presenting cells (APCs) in the medulla. The process of negative selection continues through the SP (single positive) stage, characterized by the absence of one of the

coreceptors on the surface of mature T lymphocytes. These CD4+ helper and regulatory or CD8+ cytotoxic T-cell subsets can finally enter the periphery to drive and/or mediate immune responses (Figure 1) (Murphy and Weaver, 2017).

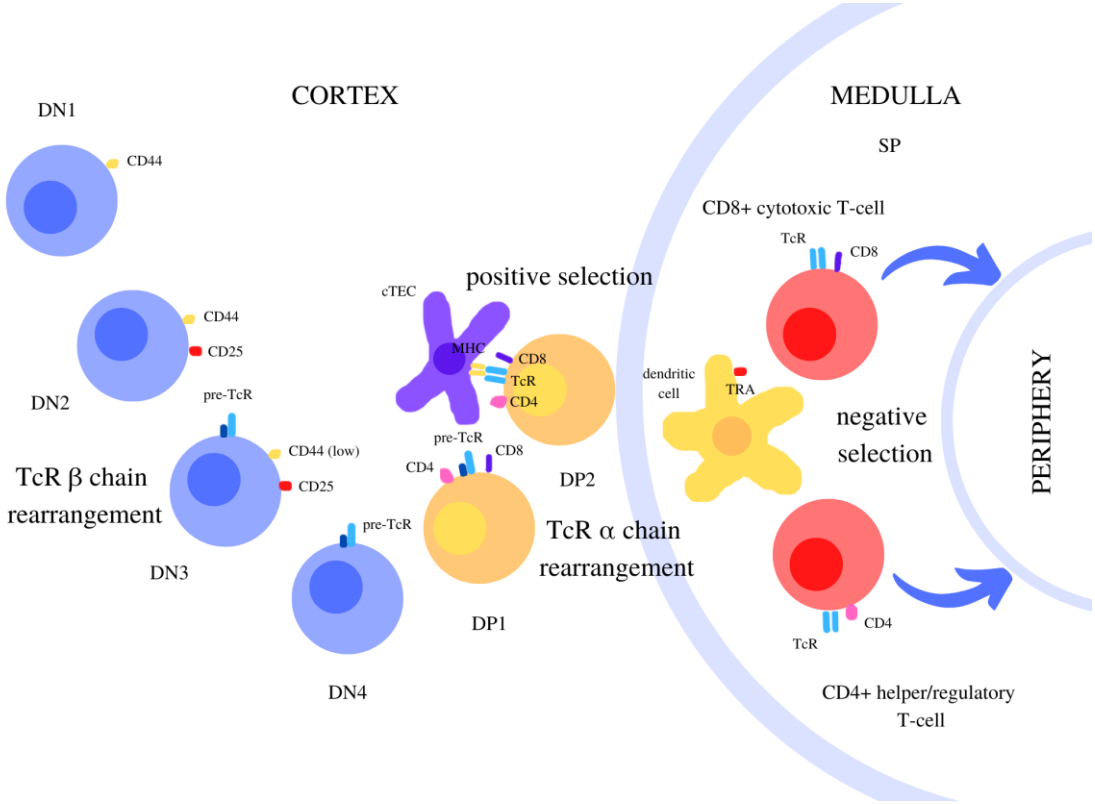

Figure 1. Schematic representation of different stages in the T-cell development.

Thymus is one of the organs originating from the pharynx, the most anterior part of the foregut. During the embryonic development, three (in mice) or four (in humans) transient pairs of outpockets form at lateral sides of this region, called the pharyngeal pouches. The third pharyngeal pouch gives rise to both thymus and parathyroid glands. First, the epithelial primordia surrounded by mesenchymal capsules form from each member of the pair. The primordial mesenchymal capsule is derived from the neural crest cells (NCC), which mediate the separation of the thymus from both pharynx and parathyroid glands, as well as thymus epithelial cells (TECs) proliferation and differentiation. During the 8th gestational week, the primordia separate from the pharynx into individual organs, and migrate to specific parts of the body (Figure 2). It is at this stage of the development that two lobes of the thymus fuse into a single organ situated above the heart (Nishino *et al.*, 2006; Gordon and Manley, 2011).

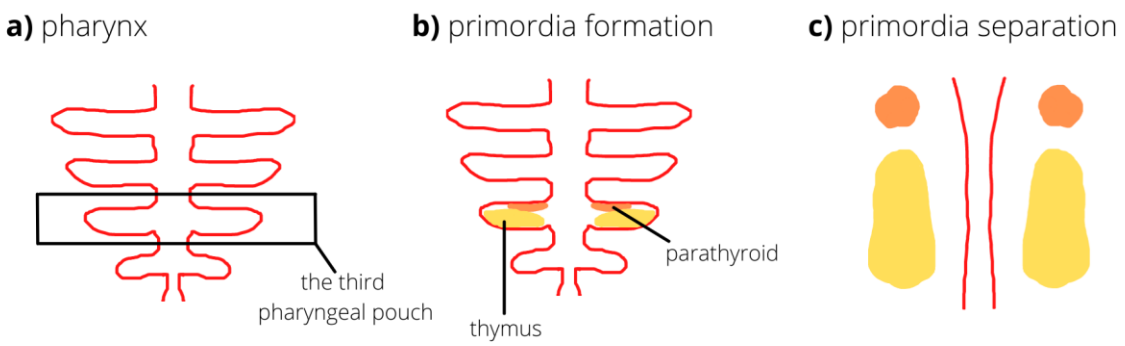

Figure 2. Schematic representation of the thymus development. The shown events are followed by organ migration and maturation.

Thymus reaches its maximum size in relation to the size and weight of the body before birth. In human it continues to grow until puberty when it reaches its maximum weight. Subsequently, it progressively decreases in size and volume, *i.e.*, undergoes involution, characterized by the epithelial compartment atrophy, and perivascular space and adipose tissue expansion. The adult thymus therefore usually consists only of a small number of epithelial cells or lymphocytes embedded in the frame made of adipose tissue (Nishino *et al.*, 2006). The lymphopoietic function of the thymus reduces accordingly in the older age (Kumar *et al.*, 2018). However, some residual lymphopoietic activity has been detected in the elderly, but at a significantly lower rate. The process of thymic involution seems to be inevitable, nonreversible, and distinct in that it starts at an early age as compared to other human organs. As it negatively influences lymphopoiesis, it may contribute to immunosenescence and potentially raise the risk of infections, autoimmune diseases, and tumor onsets at an advanced age. There are many known mechanisms underlying thymus involution, including reduced progenitor cell supply, their reduced proliferation and differentiation capability, functional changes of thymocytes, their microenvironment, etc. (Chaudry *et al.*, 2016).

1.1.1. Thymus organization

Thymus is enclosed into a capsule made of connective tissue. The extensions of this capsule (septa) penetrate the thymus, dividing it into many lobules, each consisting of two distinctive compartments: the cortex and the medulla. The heterogeneous thymic environment is composed of many cell types (for review see: Abramson and Anderson, 2017). These are predominantly T lymphocytes and epithelial cells, and smaller fractions of dendritic cells, B lymphocytes, macrophages, interdigitating reticulum cells, Langerhans cells, eosinophils, mast

cells, plasma cells, neuroendocrine cells, myoid cells, germ cells and sebaceous glands. Immature T lymphocytes or thymocytes are found mainly in the thymic cortex, where their differentiation commences. Parallel to their differentiation progress, they migrate towards the medulla, where they exist as either activated, immunocompetent, or inactivated SP T-cells (Bornstein *et al.*, 2018; Kalhor and Moran, 2019).

Thymus epithelial cells (TECs) can be divided into four distinct subtypes: subcapsular cortical, inner cortical or thymic nursing cells (TNCs), medullary and cells of Hassall's corpuscles (Kalhor and Moran, 2019). It has been shown that TECs are crucial in directing T-cell development towards the specific T-cell lineage with a distinct role in the immune system (Martínez-Ruíz *et al.*, 2022). TECs make up two major compartments that differ in their localization, molecular characteristics, and functions. They are known as cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs). The former are responsible for the commitment of progenitor cells to the T-cell lineage and positive selection of cells expressing functional T-cell receptors, while the latter promote negative selection of self-reacting T-cells and development of regulatory T-cells. As this process is crucial for normal functioning of the cellular immune response, it is not surprising that functional defects in either of these two groups of TECs lead to immunodeficiencies and autoimmune diseases (Kadouri *et al.*, 2020). It is generally accepted although still a matter of controversy that there is a single bipotent TEC progenitor (Blackburn *et al.*, 2002; Ucar *et al.*, 2014; Luan *et al.*, 2019; Kadouri *et al.*, 2020). In one study, sphere forming EpCAM+ cells with stemness characteristics were found to give rise to epithelial cells in the presence of FOXN1. These cells therefore have the potential to maintain functional and organizational properties of the thymus (for review see: Shichkin and Antica, 2020).

Dendritic cells play a key role in the establishment of the immune tolerance, as they present TRAs to developing thymocytes during their negative selection (Kurts *et al.*, 1997; Hawiger *et al.*, 2001; Kumar *et al.*, 2018). B lymphocytes are usually found in germinal centers of the thymus, where they act as antigen presenting cells and activate mature T lymphocytes. Thymic macrophages remove defective thymocytes at the cortico-medullary junction by phagocytosis. Other cell types are rarely found in the thymus, and their presence, as that of myoid cells, might sometimes point out a certain pathological condition (Kalhor and Moran, 2019).

1.2. Notch signaling pathway

Notch signaling pathway is a key pathway responsible for communication between adjacent cells in complex organ formation processes. It has a central role in the development of most organs, including thymus (Radtke *et al.*, 1999; Meester *et al.*, 2019).

Notch pathway includes several single-pass transmembrane proteins: four receptors (Notch1-4) and five ligands (Jag1,2 and Dll1,3,4), belonging to two families (Serrate/Jagged and Delta). Prior to their transport to the cell surface, Notch receptors go through a series of posttranslational modifications important for their ligand specificity. Once in the membrane, they form a heterodimeric complex with a large extracellular domain and a Notch intracellular domain (NICD) that contains two nuclear localization signals (NLSs) and a transactivation region. Notch receptor on the signal-receiving cell is activated through specific binding of a ligand present on the signal-sending cell, a process followed by ligand endocytosis into the signal-sending cell, together with the extracellular domain of the receptor. A conformational change of the receptor NICD happens concurrently, resulting in its cleavage at two specific sites and release into the nucleus. Upon entering the nucleus, NICD binds transcription factor Rbpj and converts it from a repressor to an activator. The formed complex, along with some coactivators, binds to regulatory sequences of targeted genes, such as the genes encoding transcription factors of Hes and Hey families (Figure 3) (Wang *et al.*, 2021).

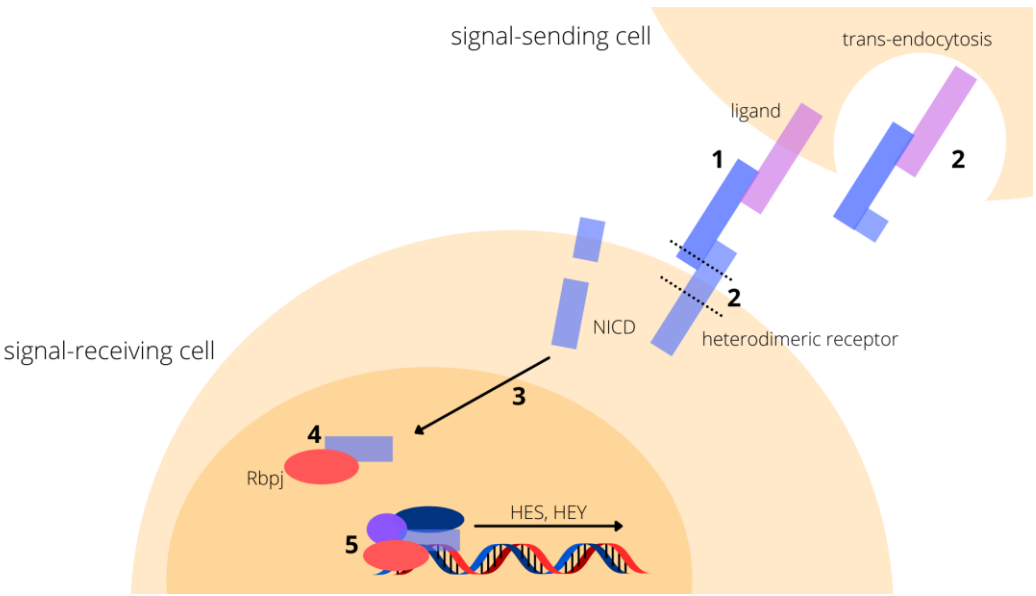

Figure 3. Schematic representation of the Notch signaling pathway.

1.2.1. Notch pathway involvement in thymus development and function

It is well established that the main role of the Notch signaling pathway in the thymus is the regulation of T-cell development. Binding of the Notch1 receptor expressed on the surface of the lymphoid progenitor to a Notch ligand DLL4 of the thymic epithelial cells restricts the progenitor into further T lymphocyte development. This induces the expression of some transcriptional factors crucial for the T-cell development as they promote the expression of T-cell specific genes (Murphy and Weaver, 2017).

Some findings suggest that, in the human postnatal thymus, the cells expressing Notch1 receptor are mainly located in the cortex, where the most of T-cell maturation and development process occurs. These cells are especially numerous in regions where the positive and negative selection occur. Differential expression of Notch ligands in distinct thymic compartments and their timely activation are crucial for the commitment of progenitors to the T-cell lineage. However, the expression of some of these ligands has been shown to decrease with increasing age, which might be another explanation for the reduced rate of thymopoiesis in adult humans (García-León *et al.*, 2018).

Notch pathway probably also works upstream of a transcription factor NF-κB, which has a central role in the expression of genes encoding TRAs, and therefore the immune tolerance establishment. It is additionally responsible for the regulation of the mTEC compartment specification (Martínez-Ruíz *et al*., 2022).

1.3. Some thymic markers related to the development of the thymus

Numerous transcription factors and molecular pathways contribute to the development and function of the thymus.

FOXN1 regulates TEC specific genes. It is a transcription factor crucial for DLL4 ligand expression in TECs, which is why it operates as an important T-cell development regulator. Its mutation or reduced expression lead to severe combined immunodeficiency, thymic atrophy and involution. It has previously been shown that its expression decreases with age (García-León *et al*., 2018; Martínez-Ruíz *et al*., 2022).

Chromodomain helicase DNA binding protein 7 (CHD7) is a chromatin-remodeling protein influencing the development of both NCCs and TECs and is therefore important in thymus organogenesis. It probably influences the expression of FOXN1. Deletions and variants in

CHD7 gene lead to CHARGE and sometimes DiGeorge syndrome, both characterized by thymus abnormalities (Liu *et al.*, 2018.). CHARGE syndrome stands for coloboma, heart defects, atresia choanae, growth and mental retardation, genital abnormalities, and ear abnormalities (Hall, 1979; Hittner *et al.*, 1979), and it is precisely these symptoms that characterize the syndrome. Patients with DiGeorge syndrome usually have specific facial features and suffer from different cardiac, endocrine, and immunological defects (Murphy and Weaver, 2017; Erhardt *et al.*, 2021).

AIRE (Autoimmune Regulator) is a transcription factor partly responsible for the promiscuous gene expression (PGE) in mTECs. This property of mTECs drives the negative selection and immune tolerance establishment in the thymus, as it enables the expression of a variety of genes encoding antigens found in the periphery. AIRE significantly induces the expression of some of these genes, known as AIRE-dependent and AIRE-enhanced (Derbinski *et al.*, 2001; Kyewski *et al.*, 2002; Kadouri et al., 2020).

1.4. Cytokeratin 5 (CK5), Cytokeratin 8 (CK8), Cytokeratin 18 (CK18) and EpCAM

Some common cytokeratin markers, such as cytokeratin 5 (CK5), cytokeratin 8 (CK8) and cytokeratin 18 (CK18) are also expressed in the thymus. CK5 has previously been shown to be more abundant in the medulla than in the cortex (Kalhor and Moran, 2019). On the other hand, CK8/18 markers are predominantly expressed in cTECs, but also in the medulla (Luan et al., 2019; Kadouri *et al.*, 2020). EpCAM (epithelial cell adhesion molecule, CD326) is a highly expressed glycoprotein originally found on rapidly proliferating tumors of epithelial origin (Koprowski *et al.*, 1979). It has been shown that it is a common non-hematopoietic, epithelial cell marker involved in signaling, cell migration, proliferation and differentiation, also found in the epithelial cells of the thymus. It is usually expressed by mTECs, while absent in cTECs (Luan *et al.*, 2019; Pathology Outlines).

1.5. Objectives

The aim of this project is to characterize the thymi of 3 to 69 months old children through the investigation of characteristic epithelial and T-cell antigens and transcription factors, in order to provide a more accurate mapping of thymic developmental pathways to better characterize thymus aging and possible regenerative potential of stem cell in human thymus.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Thymus tissue

Six thymi were obtained as medical waste from the University Hospital Center of Zagreb (Table 1) with informed consent of the parents/guardians, and ethics committee approval issued by the Ethical Committees of the University Hospital Center of Zagreb, Merkur Clinical Hospital, and the Rudjer Boskovic Institute Bioethical Committee. The study was conducted according to the ethical principles of medical research involving human subjects of the Helsinki Declaration of the World Medical Association (WMA). After they were collected, the thymi were grossed and left in 17% formalin prior to further processing of the specimens. Positive tissue controls were provided by the Merkur Clinical Hospital.

Table 1. Specifics of the analyzed thymi specimens

Specimen label	Age of the individual (Months)	Sex of the individual
T117	3	M
T122	16	M
T140	24	F
T143	44	F
T178	58	M
T190	69	M

2.1.2. Antibodies

Seven antibodies were used to characterize the specimens. Mouse anti-human EpCAM and rabbit anti-human CK8/18 antibodies were ready-to-use. Mouse anti-human CK5, rabbit anti-human FOXN1, rat anti-human AIRE, mouse anti-human CHD7 and goat anti-human Jagged1 were diluted as listed in Table 2 using Dako EnVision FLEX Antibody Diluent. Mouse or rabbit linkers and $CuSO_4$ were used for signal amplification with some antibodies. For Jagged1, anti-goat peroxidase-bound secondary antibody was used as a detection system.

Table 2. Antibodies used for characterization of the thymi

Target	Origin and clones	Source and reference/code number	Dilution	Target retrieval pH	Other
FOXN1	rabbit polyclonal	Abcam (Cambridge, UK), ab113235	1/150	high (9)	/
EpCAM	mouse monoclonal, clone BerEP4	Agilent Technologies, Inc. (Santa Clara, CA), GA637	ready-to-use	low (6.1)	/
AIRE	rat monoclonal, clone TM-724	eBioscience™ (San Diego, CA), 13-9534-82	1/50	low	/
CHD7	mouse monoclonal, clone F-11	Santa Cruz Biotechnology (Santa Cruz, CA), sc-390742	1/50	low	mouse linkers
CK5	mouse monoclonal, clone XM26	Abcam (Cambridge, UK), ab17130	1/50	high	mouse linkers
CK8/18	rabbit monoclonal, clone EP17/EP30	Dako (Denmark), IR/IS 094	ready-to-use	high	rabbit linkers, $CuSO_4$
Jagged1	goat polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA), sc-6011	1/50	high	anti-goat secondary antibody

2.1.3. Immunohistochemistry

EnVision FLEX set of reagents containing antibody diluent, peroxidase-blocking reagent, horseradish peroxidase (HRP) and goat secondary antibody bound dextran, DAB+ chromogen, substrate buffer, high pH target retrieval solution (50x, pH=9), low pH target retrieval solution (50x, pH=6.1), wash buffer (20x) and FLEX+ rabbit and mouse linkers were purchased from Dako (Denmark). ImmPRESS™ peroxidase reagent kit and ImmPACT™ DAB peroxidase substrate for Jagged1 signal detection were purchased from Vector Laboratories, Inc. (Burlingame, CA).

2.2. Methods

Immunohistochemistry is a group of methods enabling the analysis of specific tissue biomarkers using antibodies, which form complexes with tissue antigens (Taylor, 2013). Herein, these methods were used to characterize some specific biomarkers of thymic cells and are described in detail below.

2.2.1. Processing and embedding of the specimens

All solvents used for processing of the specimens were purchased from BioGnost ® (Zagreb, Croatia). After proper formalin fixation of the grossed specimens, duration of which depended on their size and was approximately 24 h, they were moved into properly sized and labeled cassettes. This was followed by processing of specimens in the Tissue-Tek VIP ® 6 AI Tissue Processor from Sakura SI Co., Ltd. (Tokyo, Japan), following the protocol listed in Table 3. Processed specimens were embedded into paraffin using the Tissue-Tek TEC 5 Embedding Station from Sakura SI Co., Ltd. (Tokyo, Japan). After covering the specimens with paraffin melted at 62ºC, they were left to cool at -4ºC and stored in the fridge until cutting.

Table 3. Tissue processing protocol in Tissue-Tek VIP ® 6 AI Tissue Processor

Step	Solvent	Duration	Temperature
1	Formalin (10%)	5 minutes	35ºC
2	Alcohol (70%)	2 hours	35ºC
3	Alcohol (96%)	3 hours	35ºC
4	Alcohol (100%)	2 hours	35ºC
5	Xylene substitute	2 hours	35ºC
6	Paraffin	3 hours	60ºC

2.2.2. Microtomy

Formalin-fixed, paraffin-embedded, cold thymi specimens were further cut into sections using Leica microtome. Cold paraffin cubes were first trimmed at a 10 μm microtome setting to achieve a smooth, flat and shiny surface for whole sample layers. Once that was achieved, the microtome was set to a desirable layer thickness (1 μm) and several serial sections through the surface of the cube were done for each sample. The obtained sections were loaded onto the slides and left to dry in an oven set to 60ºC for an hour.

2.2.3. Immunohistochemical staining

Dry samples were subjected to pre-staining treatment at high temperature, the process known as heat induced epitope retrieval (HIER). This step was conducted to facilitate antigen-antibody complex formation in formalin-fixed tissue specimens consisting of a number of masked, non-immunoreactive epitopes (Taylor, 2013). The process was conducted in the Dako PT Link water bath. The containers in the bath were filled with Dako EnVision FLEX Target Retrieval Solution of high pH (pH=9), which was previously diluted by mixing 30 mL of the concentrated solution with distilled water to the final volume of 1,5 L. Racks containing thymi samples were then immersed into these containers preheated to 85ºC and the device was initiated. The samples were incubated at 97ºC for 20 minutes, cooled, and washed in Dako EnVision FLEX Wash Buffer (20x), which was previously diluted by mixing 750 mL of the buffer with distilled water to the final volume of 1,5 L. For antibodies that only interact with the samples pretreated at low pH, the samples were immersed into the previously diluted 50x concentrated Dako EnVision FLEX Target Retrieval Solution of low pH (pH=6.1) and heated in the pressure cooker to 112ºC.

After the pretreatment step, the racks were loaded onto the Autostainer Link instrument from Dako. Negative isotype controls were done by changing the primary antibody with a drop of dH_2O. The FLEX DAB+ substrate-chromogen solution was prepared by adding 25 drops of EnVision FLEX DAB+ Chromogen into 20 mL of the EnVision FLEX Substrate Buffer. Immunostained samples were counterstained with Gill's hematoxylin. Dehydration and deparaffinization of the samples were conducted by immersification in four different solvents (two 100% alcohols and two xylenes of different purity), one minute each. The slides were automatically covered using the Tissue Tek GLC from Sakura (Tokyo, Japan). Stained and covered samples were analyzed microscopically.

2.2.4. Microscopic analysis of the immunostained samples

Immunohistochemically labeled slides were analyzed using an Olympus 71 digital camera and an Olympus BX51 microscope. Digital images were saved as uncompressed 24-bit RGB .tiff files using the software program AnalySIS (Olympus Soft Imaging System GmbH, Munster, Germany). The photographs were taken at 100x, 200x and 400x magnifications. They were additionally modified using the Adobe Lightroom.

3. RESULTS

3.1. Specific thymic markers expression

To study the functional changes of the human thymus during its development and aging, a few characteristic thymic antigens were assessed through their interactions with different anti-human antibodies. Four thymus-specific molecules were chosen for this purpose: FOXN1, Jagged1, CHD7 and AIRE. The estimated values of percentage of positive cells and signal intensities in thymi labeled with anti-FOXN1, anti-Jagged1, anti-CHD7 and anti-AIRE are listed in Table A1 in Annex.

The most of FOXN1 positive cells were found in the medulla and subcortical region, where epithelial cells predominate. They were also present in the intracortical region and corticomedullar junction. Apart from the epithelial cells in the listed regions, lymphocytes in the cortex, but mostly those in the medulla, also expressed this marker. Positive signal was detected in nuclei and cytoplasms of the cells. The staining intensity was similar in thymi of all ages. A lower signal intensity and percentage of positive cells in older thymi was noticed among subcortical ECs, cortical lymphocytes, and partly among mECs, medullar lymphocytes, and in Hassall's corpuscles. Such a regular pattern of decreasing FOXN1 expression was not noticed in intracortical and corticomedullar regions, where the percentage of positive cells and signal intensity varied independently of the thymus age. A general decrease in FOXN1 expression in aged thymi is visible from microscopy photographs (Figure 4).

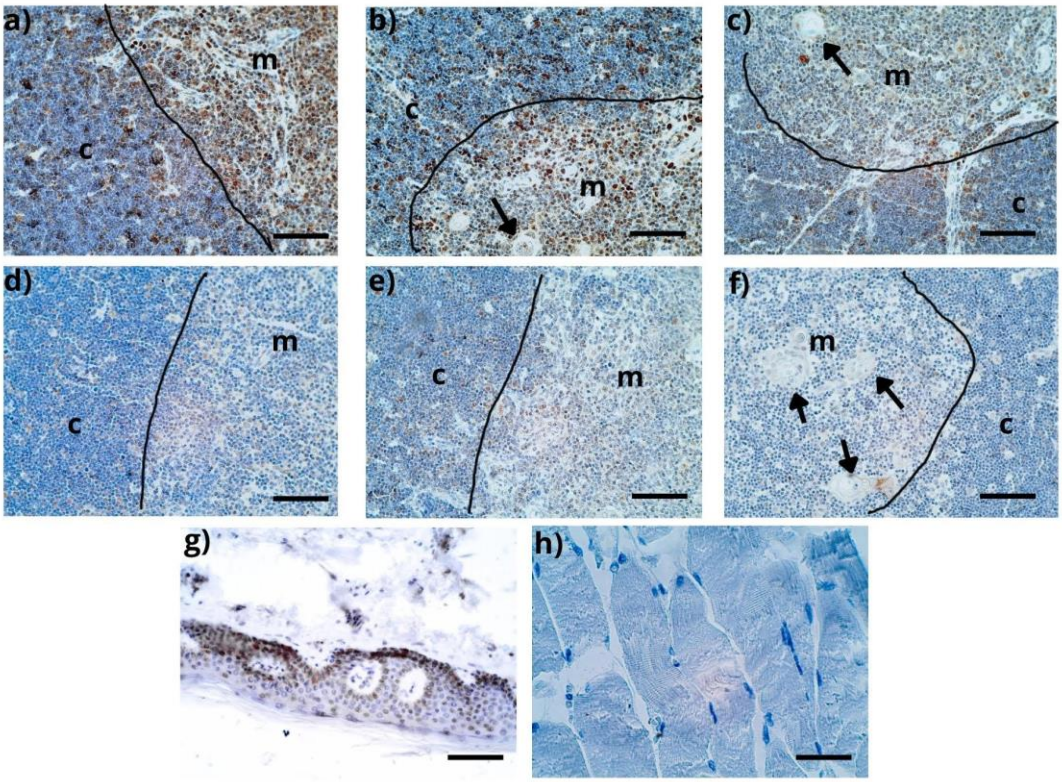

Figure 4. (a-f) FOXN1 expression in thymic cortex and medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 200x. Scale bars: 100 µm. (g-h) FOXN1 expression in a positive skin tissue control and a negative muscle tissue control, respectively. Magnification: 400x. Scale bars: 50 µm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

Jagged1 expression showed a similar pattern in dependance on thymic age as that of FOXN1, but with more drastic differences between younger and older thymi. The highest signal intensity and percentage of positive cells were detected in T117, and partly T122, moderate staining was present in some compartments of T122 and in T140, whereas in T143, T178 and T190 it was low or, in some compartments, completely absent. Mainly cytoplasms of epithelial cells and lymphocytes were stained, with the higher staining intensity and percentage in the medulla compared to the cortex (Figure 5).

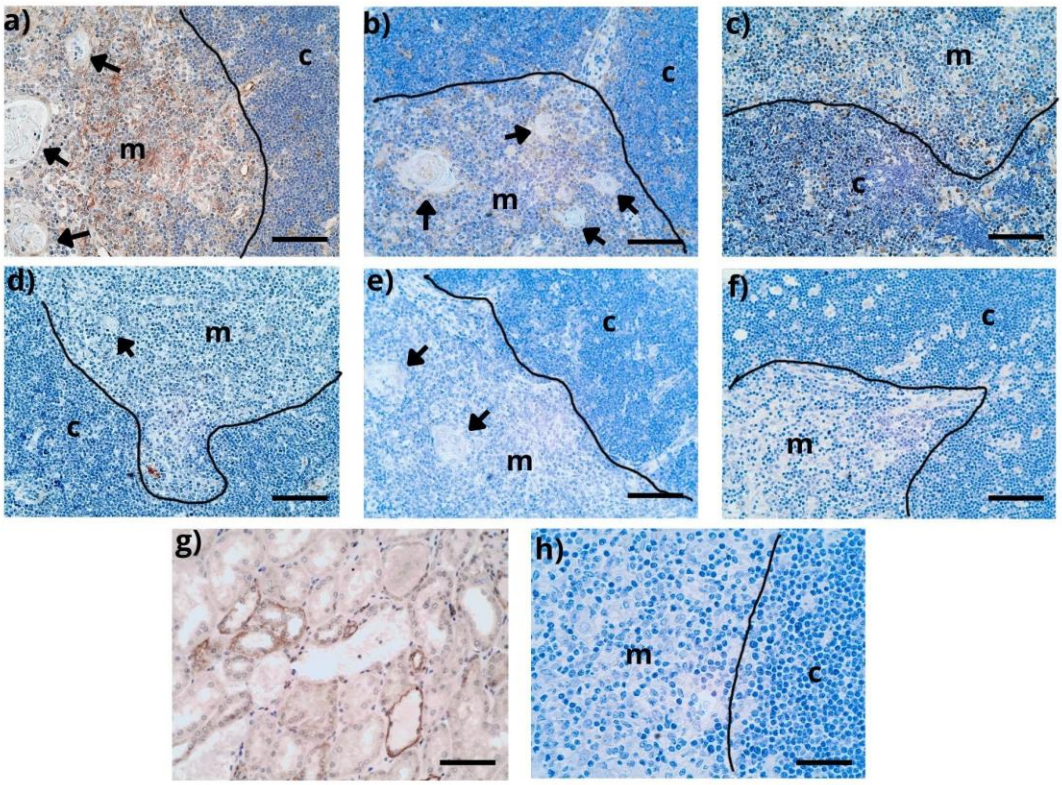

Figure 5. (a-f) Jagged1 expression in thymic cortex and medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 200x. Scale bars: 100 μm. (g-h) Jagged1 expression in a positive kidney tissue control and a negative thymic inner cortex tissue control, respectively. Magnification: 400x. Scale bars: 50 μm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

In Figure 6, the expression of CHD7 in the thymi is shown. Positive signal was detected in the nuclei of epithelial cells in the medulla of all positive thymi, and cortical regions of younger thymi (T117, T122 and T140). The staining intensity and percentage of positive cells were low and may depend on the thymic age. The highest percentage of positive cells was present in T140, while they were completely absent in T190.

Figure 7 shows AIRE-expressing epithelial cells in the medullas of the thymi. Primarily the nuclei of these cells were positively stained, with some cases of the cytoplasmic positivity. The signal was detected in all thymi, from the youngest to the oldest, with the highest percentage of positive cells in T122 and T140.

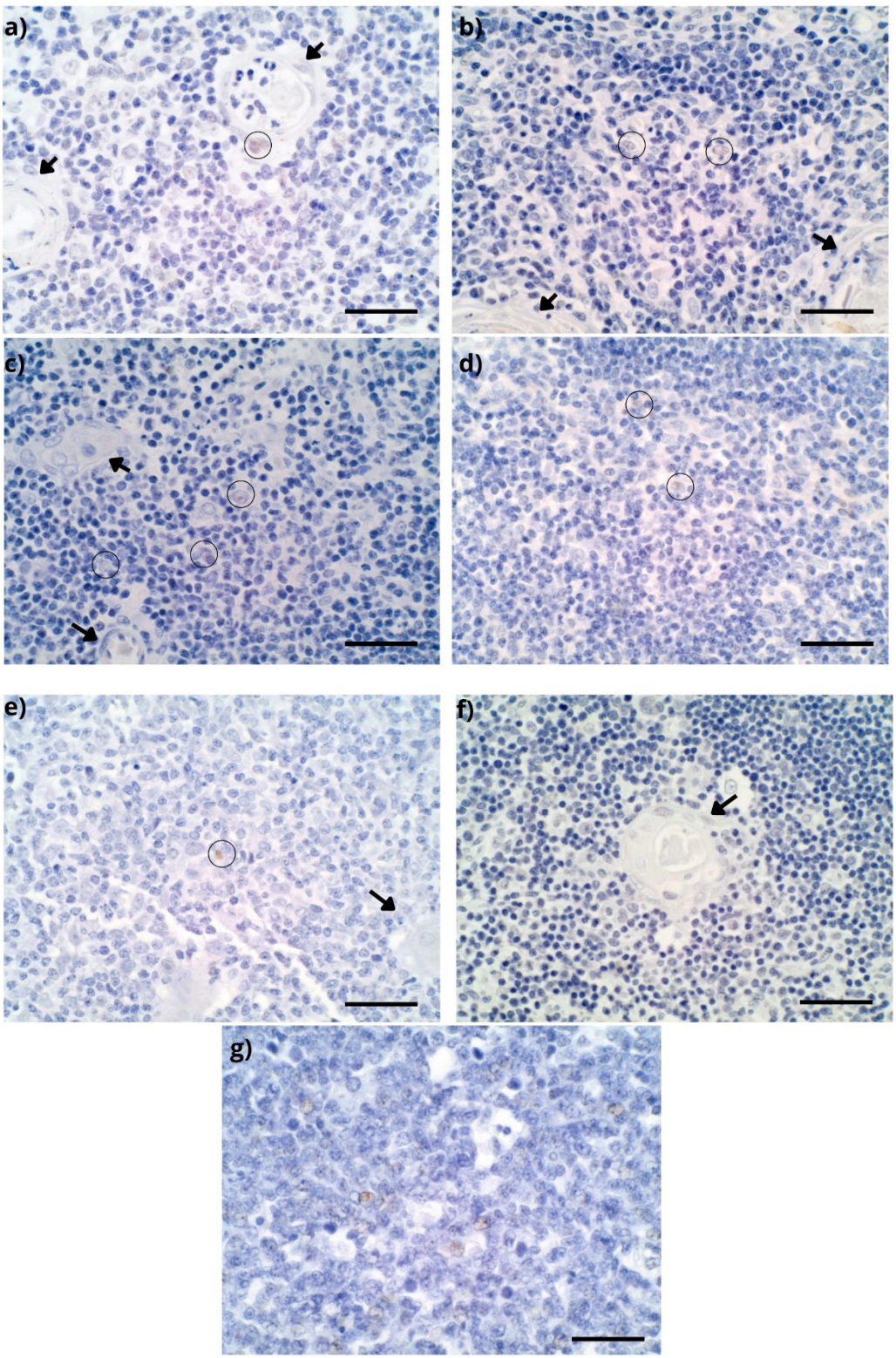

Figure 6. (a-f) CHD7 expression in the thymic medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 400x. Scale bars: 50 µm. (g) CHD7 expression in a positive tonsil tissue control. Magnification: 400x. Scale bar: 50 µm. The arrows are indicating Hassall's corpuscles. The circles are indicating positive cells.

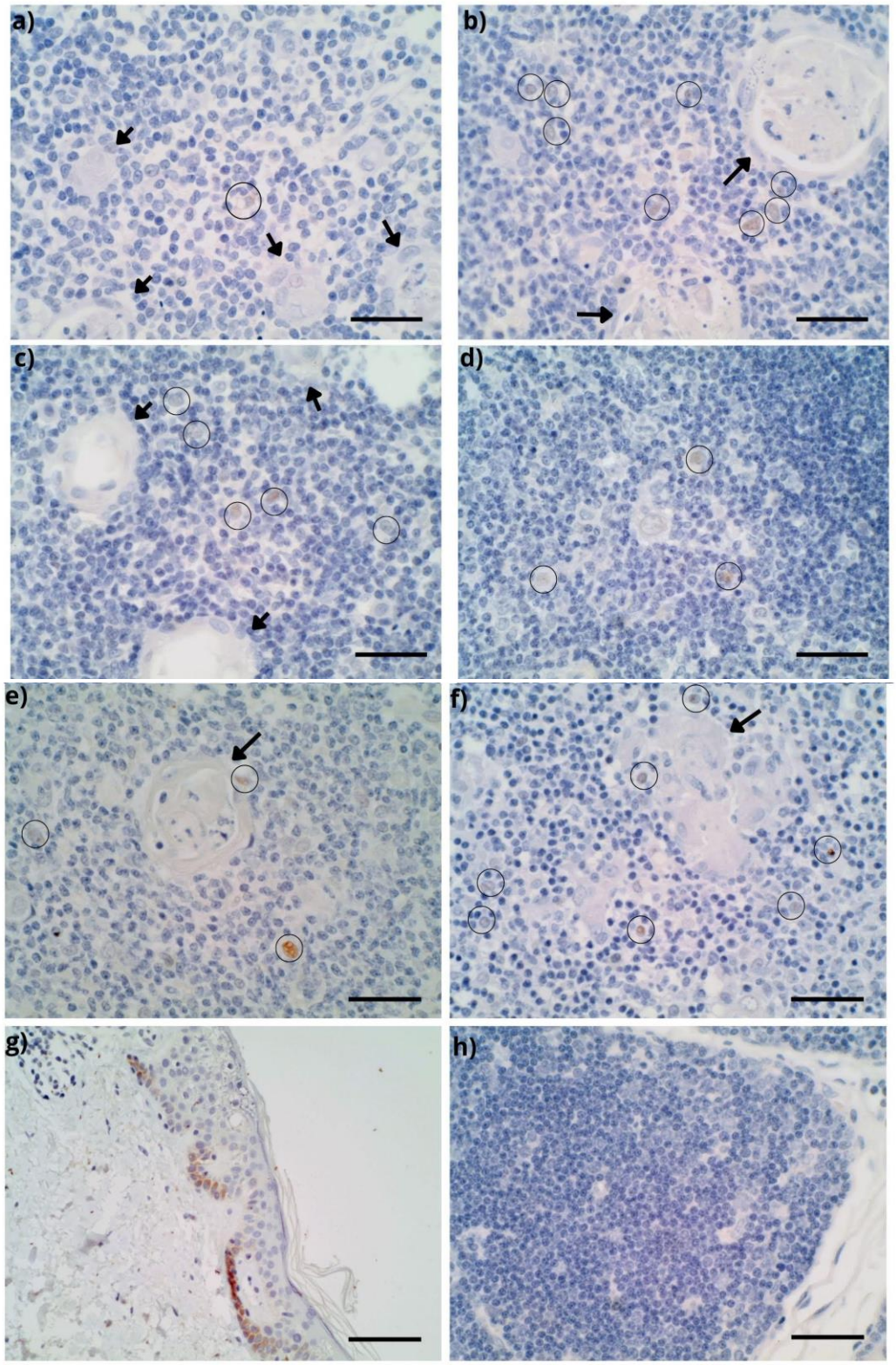

Figure 7. (a-f) AIRE expression in the thymic medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 400x. Scale bars: 50 µm. (g-h) AIRE expression in a positive skin tissue control and a negative thymic cortex tissue control, respectively. Magnification: 400x. Scale bars: 50 µm. The arrows are indicating Hassall's corpuscles. The circles are indicating positive cells.

3.2. Thymus epithelium characterization

Thymus epithelium is the center of functional changes in this organ, especially during the involution process. For this reason, the analysis of the thymic epithelium is of especially high importance when studying this organ. Here, three different epithelial cell markers known to be present in the thymus were analyzed: CK5, CK8/18 and EpCAM. The estimated values of percentage of positive cells and signal intensities in thymi labeled with anti-CK5, anti-CK8/18, and anti-EpCAM are listed in Table A2 in Annex.

CK5 expression was detected in the cytoplasms of epithelial cells, some in the cortex, but predominantly in the medulla, including the epithelial cells of Hassall's corpuscles. As shown in Figure 8, high staining intensity and percentage of positive cells were present in the youngest thymi, and low to moderate signal was detected in T143, T178 and T190.

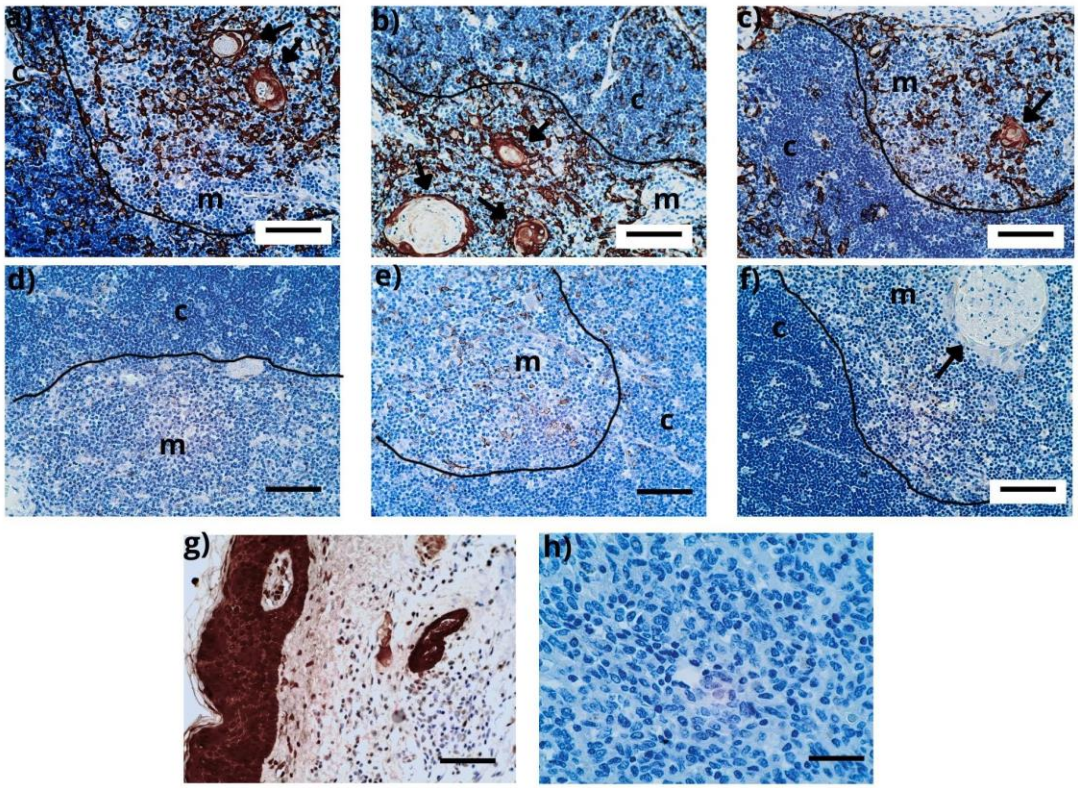

Figure 8. (a-f) CK5 expression in thymic cortex and medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 200x. Scale bars: 100 µm. (g-h) CK5 expression in a positive skin tissue control and a negative melanome tissue control, respectively. Magnification: 400x. Scale bars: 50 µm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

In figure 9, anti-CK5 labeled thymic medullas are shown at a higher magnification.

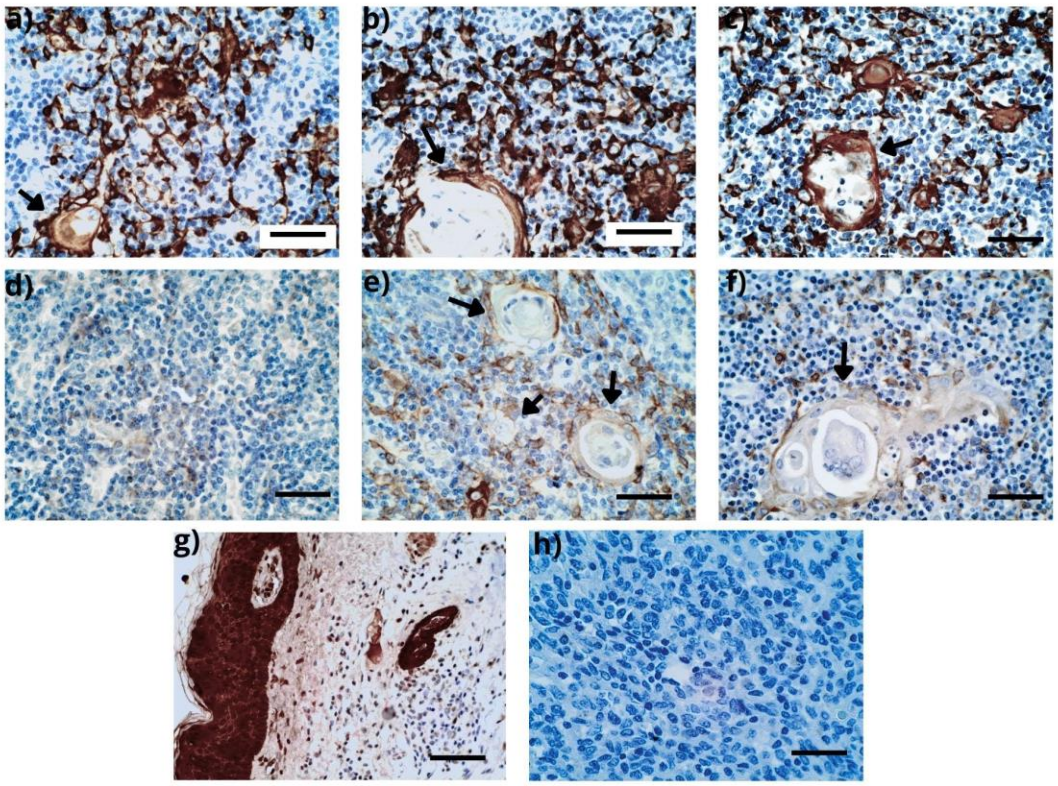

Figure 9. (a-f) CK5 expression in the thymic medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 400x. Scale bars: 50 μm. (g-h) CK5 expression in a positive skin tissue control and a negative melanome tissue control, respectively. Magnification: 400x. Scale bars: 50 μm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

In Figure 10, the comparison of CK5 expression in a young (T122) and an old (T178) thymus is shown in the less magnified thymi sections.

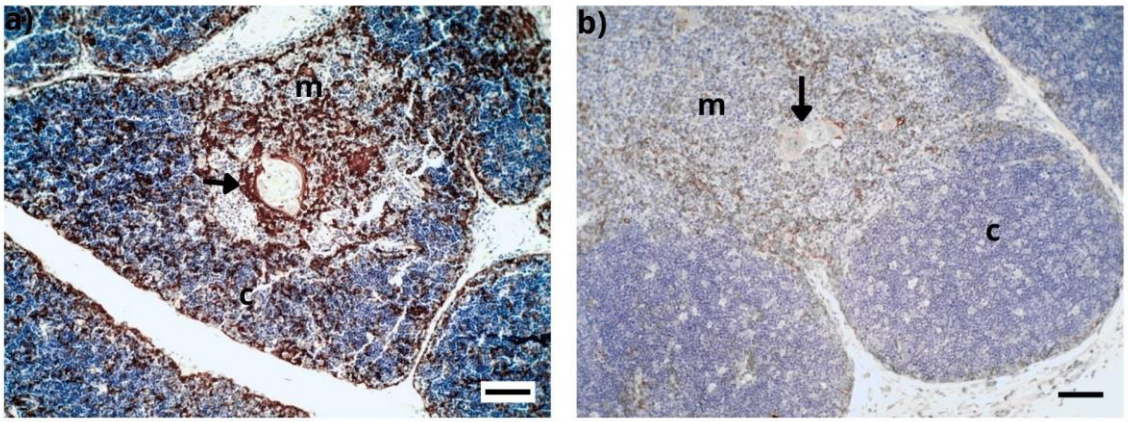

Figure 10. CK5 expression in a) T122, b) T178. Magnification: 100x. Scale bars: 100 μm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

CK8/18 was shown to be expressed in the thymus like CK5, only with less drastic signal intensity and positive cell percentage decrease in older thymi (Figure 11).

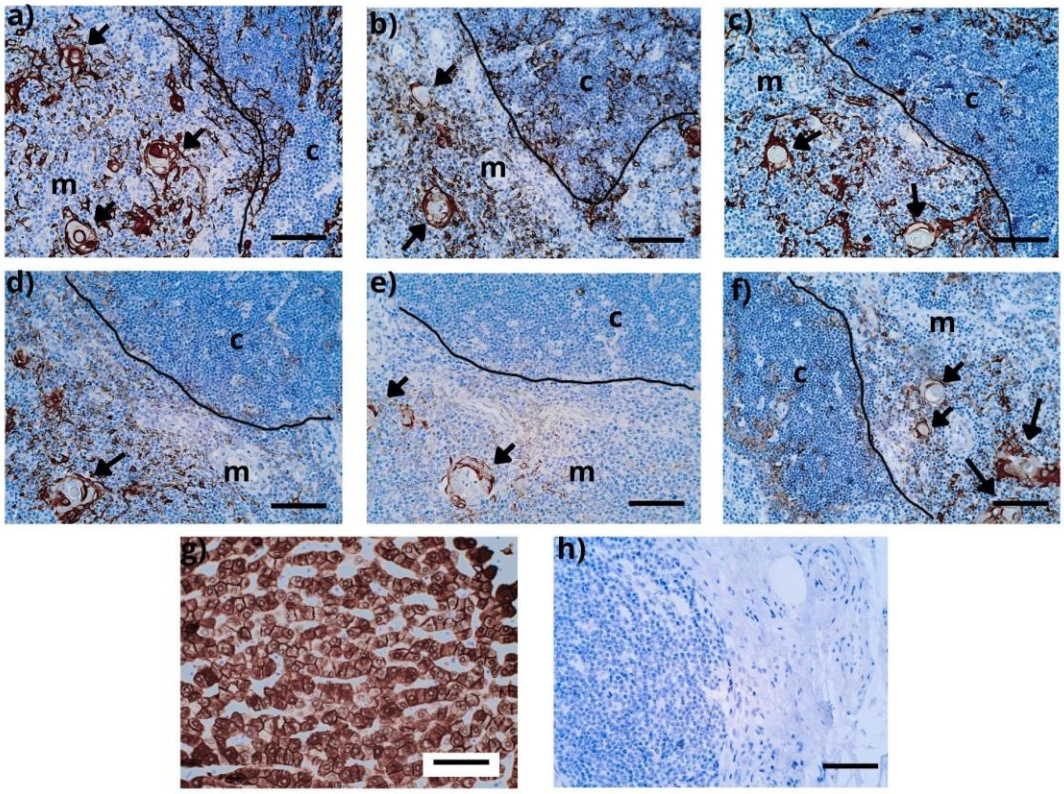

Figure 11. (a-f) CK8/18 expression in thymic cortex and medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 200x. Scale bars: 100 µm. (g) CK8/18 expression in a positive liver tissue control. Magnification: 400x. Scale bar: 50 µm. (h) CK8/18 expression in a negative appendix tissue control. Magnification: 200x. Scale bar: 100 µm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

Anti-CK8/18 labeled thymic medullas are shown at a higher magnification in Figure 12.

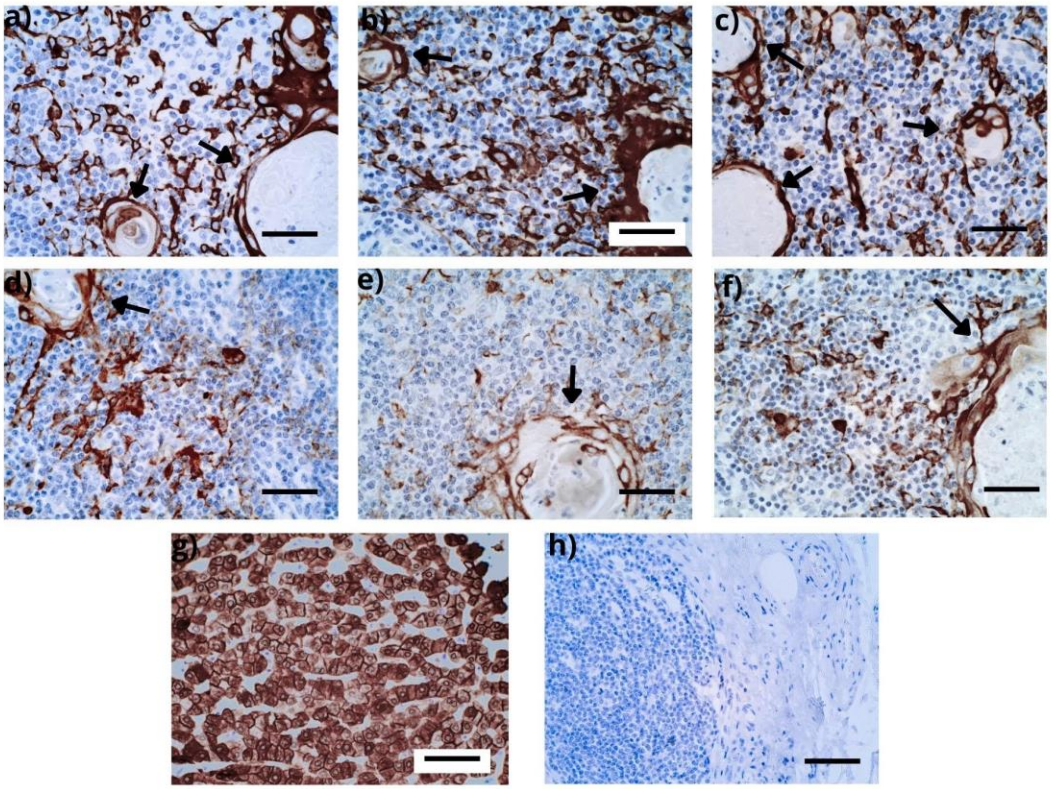

Figure 12. (a-f) CK8/18 expression in the thymic medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 400x. Scale bars: 50 μm. (g) CK8/18 expression in a positive liver tissue control. Magnification: 400x. Scale bar: 50 μm. (h) CK8/18 expression in a negative appendix tissue control. Magnification: 200x. Scale bar: 100 μm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

In Figure 13, the comparison of CK8/18 expression in a young (T122) and an old (T178) thymus is shown in the less magnified thymi sections.

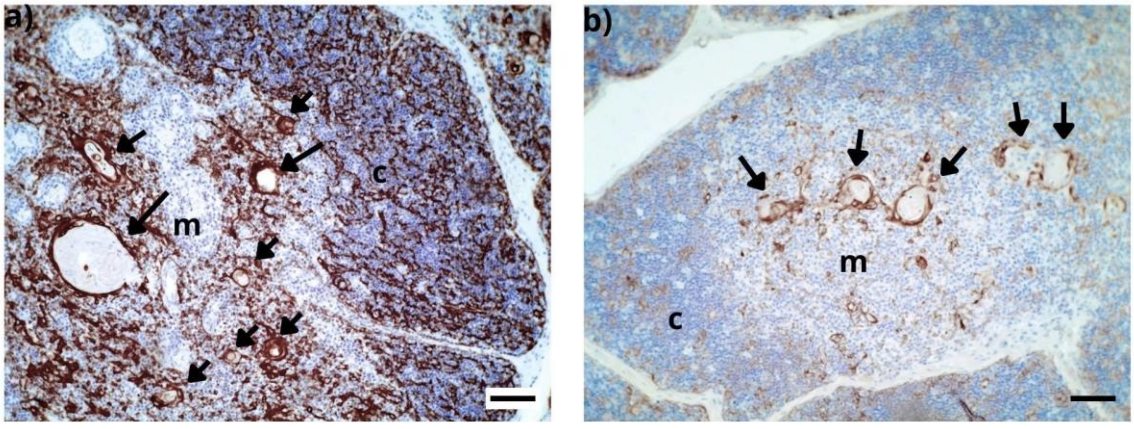

Figure 13. CK8/18 expression in a) T122, b) T178. Magnification: 100x. Scale bars: 100 μm. c-cortex, m-medulla. The arrows are indicating Hassall's corpuscles.

In Figure 14, the expression of EpCAM detected in cytoplasms of epithelial cells in the medulla, including those of Hassall's corpuscles, is shown. Again, a pattern of decreasing expression in older thymi was observed. As expected, positive cells were not present in the thymic cortex, except in the cortico-medullar junction. No signal was detected in T178.

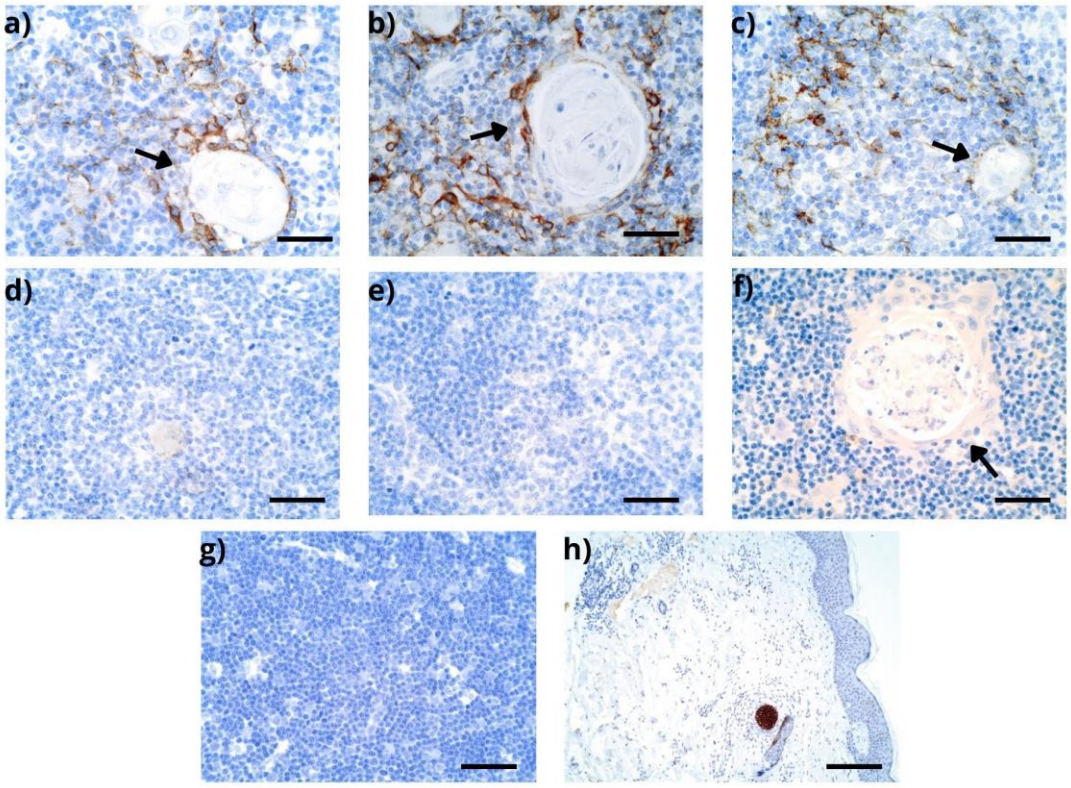

Figure 14. (a-f) EpCAM expression in the thymic medulla of T117, T122, T140, T143, T178, T190, respectively. Magnification: 400x. Scale bars: 50 μm. (g-h) EpCAM expression in a negative thymic cortex tissue control and a positive skin tissue control, respectively. Magnification: 200x. Scale bars: 100 μm. The arrows are indicating Hassall's corpuscles.

4. DISCUSSION

In order to investigate the changes in molecular and functional properties of the thymus during its aging and involution in the postnatal period, we have chosen a range of thymus specific and characteristic epithelial cell antigens, and microscopically examined six thymi of different age, immunohistochemically stained using appropriate antibodies and detection systems. We have used previously published scientific papers, the Human Protein Atlas and Pathology Outlines as a guideline of previous findings in the expression of the examined markers in the thymus, as well as their subcellular localization. Some of our results are in accordance with the results of the previous studies, while some of them slightly differ or bring some new information about the biomarkers that have not been intensely studied before.

4.1. Expression of specific biomarkers in the thymi of different age

FOXN1 is a crucial regulator of the TEC development, maintenance, and a transcription factor responsible for epithelial organizational properties of the thymus. Herein, we have detected the protein in nuclei and cytoplasms of thymic epithelial cells and lymphocytes. The general pattern of decreasing FOXN1 expression during the thymus involution in the postnatal period, which was observed in previous studies (for review see: Vaidya *et al*., 2016) was confirmed once again, but restricted to the subcortical region, Hassall's corpuscles, and lymphocytes in the cortex. The percentage of positive cells did not have such a regular pattern in other cortical regions, medullar ECs and medullar lymphocytes. All in all, 3 and 16 months old thymi showed a high anti-FOXN1 staining intensity, indicating its major role as a TEC regulator during this period. In a 24 months old thymus its expression is slightly lower, and additionally decreases in the older thymi (44, 58 and 69 months old).

Jagged1 is one of the ligands of the Notch signaling pathway. Unlike the Delta-like ligands of this pathway, Jagged1 has previously been shown not to be crucial for the commitment to the T-cell line or early T lymphocytes development, but instead inhibit B-cell development and promote the T-cell development in the later stages. It has also been found that it is expressed mainly by mTECs, dendritic and endothelial cells of the thymus, predominating in the medulla and showing a low expression in the cortex (Lehar *et al*., 2005). In our experiment, the highest percentage of positive cells was observed among mTECs and cortical lymphocytes, whereas a smaller fraction of other cortical ECs, medullar lymphocytes and ECs of Hassall's corpuscles was stained. An age-dependent decrease in Jagged1 expression was also observed, more drastic

compared to that of FOXN1. What differs in our results compared to what has previously been known about Jagged1, apart from the cell types expressing this antigen, is its subcellular localization. It has previously been detected mostly in the membranes, and diffusely in the cytoplasms of the cells (Karamchandani *et al.*, 2016), while we have also detected it in the nuclei. This might point out some possible nonspecific Jagged1 staining in our case, making the interpretation of the results, and drawing conclusions regarding the real Jagged1 expression in the postnatal thymus harder.

CHD7 is an early thymus organogenesis and development regulator which might have an impact on FOXN1 expression. No studies of this type have been conducted in the postnatal thymus until now, which is why we have chosen to examine the presence of this protein in our specimens. We have indeed detected CHD7 in the nuclei, mostly in mTECs of the analyzed thymi, and subcortical, intracortical and corticomedullar ECs of younger ones, raising questions about its potential role in the postnatal period, apart from the possible FOXN1 expression regulation. However, a more detailed investigation should be conducted to assess this, as we have not managed to find a correlation between the CHD7 and FOXN1 expression. CHD7 expression in all positive thymi was low. The highest percentage of positive epithelial cells was detected in the medulla of the 3 months old thymus, but, in general, the highest percentage of positive cells was present in the middle-aged thymus T140 (24 months old). Small amount of positive cells were detected in the 16 months old thymus, almost none of them were present in the 58 months old thymus, and none of them in the 69 months old thymus. Some of these facts might point out a dependance of CHD7 expression on the thymic age, but a more detailed study in a larger experimental group would be necessary to confirm this doubt.

AIRE is a major regulator of autoimmunity, working by promoting the expression of a range of antigens from the entire human body on the surface of medullary epithelial cells. In our experiment, this protein was found to be expressed in the nuclei of epithelial cells in the medulla. This is in accordance with other studies about AIRE location in the thymus (for review see: Anderson and Su, 2011). Unexpectedly, some probably unspecific signal was also present in the cortical regions. An interesting observation from the previous studies is a higher AIRE expression in male thymi (Zhu *et al.*, 2016). Conclusions on this could not be drawn from our experiment as the experimental group was relatively small and the highest expression was detected in mTECs of the 16 months old male, as well as the 24 months old female thymus. The age-dependent change in expression has also not been found.

4.2. Age-dependent changes in the epithelial compartment of the human thymus

Thymic epithelium is an especially interesting compartment in studies of the human thymus involution, as it enables a clear general view of the degradation in the thymic epithelial network. We have observed this through the expression of three common epithelial cell markers expressed in the thymus: CK5, CK8/18 and EpCAM.

Available information about localization of CK5, CK8/18 and EpCAM in the mouse and/or human thymus suggests that CK5 and CK8/18 can be found throughout the whole epithelial network of the thymus, with CK5 being expressed predominantly in the mTECs, CK8 in the cTECs, and CK18 in the Hassall's corpuscles, whereas EpCAM, according to Pathology Outlines, is restricted to the thymic medulla. The reported subcellular localization of these proteins is in the cell cytoplasm (Lee *et al.*, 2011; Kalhor and Moran, 2019; Luan *et al.*, 2019; Kadouri *et al.*, 2020; Menz *et al.*, 2021).

These findings have been confirmed in our experiments, with EpCAM showing a low expression in the corticomedullar junction of three younger thymi, as well. Hence, CK5 and CK8/18 were expressed both in the cortex and in the medulla of the analyzed thymi, being concentrated mostly in the subcortical region of the cortex and the medulla, where the most epithelial cells of the thymus are situated. These proteins showed a decrease in expression in older thymi, but in a different quantity. This can be observed in the microscopy photographs at different magnifications, where the intensity of anti-CK8/18 staining is stronger in old thymi compared to that of anti-CK5, especially in the cells lining Hassall’s corpuscles. This suggests that CK5 expression in the thymus might cease earlier than CK8/18 expression. An early expression restriction of EpCAM was also observed, as this marker was only hardly or not at all detected in the 44, 58 and 69 months old thymi. As for the whole epithelial network of the analyzed thymi, a visible degradation was detected parallel with the increasing age.

5. CONCLUSIONS

This study was conducted in order to provide a more accurate mapping of some common specific and more general markers found in the human thymus as an insight into the change of its molecular and functional properties during the aging and involution process. We have confirmed some of the findings stated in similar studies conducted before and gained some new insights into the expression of antigens, which might serve as a starting point for further studies. All in all, an age-dependent decrease in the expression of most analyzed proteins was observed, once again pointing out the degeneration of the thymic cell network parallel to its involution. AIRE and CHD7 have not shown such a regular pattern in our experiments, requiring a more detailed investigation in a larger experimental group. However, the study of expression of these two proteins in the human postnatal thymus, especially CHD7, was very significant as little or no such studies have been conducted. Our results might have raised some new questions about the role of CHD7 in the postnatal thymus and remain to be answered in the future.

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ANNEX

Table A1. Percentage of positive cells (%) and signal intensity (↑) in different thymi compartments upon labeling with four antibodies targeting specific thymus antigens. Signal intensity legend: / - no signal; + - visible at 400x magnification; ++ - visible at 100x magnification; +++ - visible at 40x magnification. SC – subcortical; IC – intracortical; CM – corticomedullar; CL – cortical lymphocytes; E – endothel; ML – medullar lymphocytes; HC – Hassall's corpuscles

		SC ECs		IC ECs		CM ECs		CL		mECs		E		ML		HC	
		%	↑	%	↑	%	↑	%	↑	%	↑	%	↑	%	↑	%	↑
FOXN1	T117	70	++	30	++	20	++	40	++	70	++	0	/	90	+++	20	++
	T122	70	+	30	++	15	++	60	+	5	+	0	/	80	++	20	++
	T140	40	++	30	++	30	++	60	++	70	++	0	/	90	+++	5	+
	T143	30	++	20	++	20	++	10	+	60	++	0	/	80	++	15	+
	T178	30	++	40	++	30	++	10	+	70	+	0	/	80	++	5	+
	T190	15	+	30	+	15	+	10	+	20	+	0	/	40	+	5	++
Jagged1	T117	30	++	10	++	15	++	80	++	80	++	0	/	20	+	30	++
	T122	20	++	20	+	15	++	60	+	10	+	0	/	10	+	10	+
	T140	5	+	15	+	15	+	40	+	40	+	0	/	10	+	5	+
	T143	1	+	1	+	1	+	0	/	5	+	0	/	0	/	1	+
	T178	1	+	1	+	1	+	0	/	5	+	0	/	0	/	0	/
	T190	5	+	0	/	1	+	0	/	1	+	0	/	0	/	5	+
CHD7	T117	1	+	1	+	0	/	0	/	10	+	0	/	0	/	0	/
	T122	1	+	1	+	1	+	0	/	1	+	0	/	0	/	0	/
	T140	5	+	5	+	0	/	0	/	5	+	0	/	0	/	0	/
	T143	0	/	1	+	0	/	0	/	1	+	0	/	0	/	0	/
	T178	0	/	0	/	0	/	0	/	1	+	0	/	0	/	0	/
	T190	0	/	0	/	0	/	0	/	0	/	0	/	0	/	0	/
AIRE	T117	1	+	1	+	1	+	0	/	1	+	0	/	0	/	0	/
	T122	1	+	1	+	1	+	0	/	5	+	0	/	0	/	0	/
	T140	2	+	2	+	0	/	0	/	5	+	0	/	0	/	0	/
	T143	1	+	1	+	1	+	0	/	1	+	0	/	0	/	0	/
	T178	0	/	1	+	0	/	0	/	3	+	0	/	0	/	0	/
	T190	1	+	1	+	1	+	0	/	1	+	0	/	0	/	0	/

Table A2. Percentage of positive cells (%) and signal intensity (↑) in different thymi compartments upon labeling with three antibodies targeting epithelial cell markers. Signal intensity legend: / - no signal; + - visible at 400x magnification; ++ - visible at 100x magnification; +++ - visible at 40x magnification. SC – subcortical; IC – intracortical; CM – corticomedullar; CL – cortical lymphocytes; E – endothel; ML – medullar lymphocytes; HC – Hassall's corpuscles

		SC ECs		IC ECs		CM ECs		CL		mECs		E		ML		HC	
		%	↑	%	↑	%	↑	%	↑	%	↑	%	↑	%	↑	%	↑
CK5	T117	20	+++	20	+++	5	+++	0	/	80	+++	0	/	0	/	95	+++
	T122	60	+++	30	+++	60	+++	0	/	90	+++	0	/	0	/	95	+++
	T140	60	+++	2	+++	15	+++	0	/	80	+++	0	/	0	/	95	+++
	T143	5	++	5	++	5	++	0	/	70	++	0	/	0	/	20	++
	T178	10	+	2	+	15	+	0	/	40	++	0	/	0	/	10	+
	T190	10	++	1	++	5	++	0	/	60	++	0	/	0	/	20	++
CK8/18	T117	60	+++	65	+++	20	+++	0	/	50	+++	0	/	0	/	95	+++
	T122	60	+++	65	+++	40	+++	0	/	95	+++	0	/	0	/	95	+++
	T140	40	+++	15	+++	30	+++	0	/	70	+++	0	/	0	/	95	+++
	T143	15	++	15	++	30	++	0	/	50	++	0	/	0	/	80	++
	T178	15	++	30	+++	30	+++	0	/	80	+++	0	/	0	/	80	+++
	T190	15	++	30	++	30	++	0	/	40	++	0	/	0	/	80	+++
EpCAM	T117	0	/	0	/	5	++	0	/	60	++	0	/	0	/	5	+
	T122	0	/	0	/	1	+	0	/	60	++	0	/	0	/	5	+
	T140	0	/	0	/	1	+	0	/	60	++	0	/	0	/	5	+
	T143	0	/	0	/	0	/	0	/	5	+	0	/	0	/	1	+
	T178	0	/	0	/	0	/	0	/	0	/	0	/	0	/	0	/
	T190	0	/	0	/	0	/	0	/	1	+	0	/	0	/	0	/