Early childhood thymectomy - Impact on immune function

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Cover illustration: Thymus praecox ssp. arcticus (wild thyme) by Hörður Kristinsson at The Icelandic Institute of Natural History, with permission.

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Abstract

Introduction: The thymus is the site of T cell maturation. Children born with a congenital heart defect often endure surgery early in life, and during surgery their thymus is routinely removed, as it blocks the surgeons access to the heart. The overall aim of this study was to investigate the long-term immunologic and clinical effects of early childhood thymectomy.

Objectives: Investigation of the immunologic effects of early childhood thymectomy at 18 months and 18 years of age with regards to the subset composition of T cells, thymic output and the T cell receptor repertoire diversity (I, II). Investigation of the association between early childhood thymectomy and risks of autoimmune diseases, cancer, infectious diseases and atopic diseases (III).

Methods: Lymphocyte subsets were characterized with flow cytometry in eleven subjects preoperatively, at 18 months and 18-years follow-up. In addition, the T cell receptor repertoire was analyzed with TCR Vß flow cytometry, T cell receptor excision circles were quantified with PCR and telomere lengths of T and B cells were analyzed with PCR at 18-year follow-up (I). Also, the diversity of the T cell receptor and immunoglobulin heavy chain genes was determined using next generation sequencing (II). A nationwide population based cohort study was conducted using Swedish patient registers to identify subjects and controls and to analyze clinical outcome measures (III).

Results: Thymectomy was associated with a reduction in the number of T cells, especially the naive subset. The naive regulatory T cells and recent thymic emigrants ($CD31^+$ T cells) were also reduced. TRECs, indicative of thymic output, were below detection level in all but one thymectomized individual. Telomere lengths were shorter in $CD8^+$ T cells of thymectomized individuals (I). Disturbances were found in the TCR Vß repertoire (I), and sequencing of the T cell receptor confirmed reduced diversity (II). Compared with surgery controls, thymectomized individuals were at increased risk for hypothyroidism, type 1 diabetes and both viral and bacterial infections. Compared with the general population they were at increased risk for hypothyroidism, juvenile idiopathic arthritis, rheumatic diseases, celiac disease, cancer, infections and asthma (III).

Conclusion: Early childhood thymectomy is associated with immunologic aberrations as well as with increased risks of autoimmune diseases, cancer and infections. These observations stress that avoidance of total thymectomy during early cardiac surgery is advisable.

Keywords: Thymus, T lymphocyte, immunology, pediatric cardiac surgery, congenital cardiac defect

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SAMMANFATTNING PÅ SVENSKA

Nyfödda barn med hjärtfel genomgår ofta livräddande hjärtkirurgi tidigt i livet. I samband med hjärtoperationen blir vanligtvis brässen, eller thymus, borttagen på grund av att den ligger framför hjärtat och försvårar hjärtkirurgens åtkomst till hjärtat under operationen. I Sverige tas thymus bort hos ungefär 200 barn varje år, men eftersom hjärtoperationerna har möjliggjorts av senare tids medicinska framsteg inom hjärtkirurgi blev de vanliga först efter 1970. Det är fortfarande inte känt om borttagandet av thymus leder till någon ändrad sjukdomsrisk senare i livet. Tidigare forskning har påvisat mätbara immunologiska förändringar, t.ex. med ett lägre antal och minskat nybildning av T lymfocyter, men studierna är få och uppföljningstiden oftast kort. Ingen studie har hittills kunnat analysera möjliga kliniska konsekvenser.

Thymus är en del av vårt immunsystem och är mycket aktiv under fosterstadiet och tidigt i livet. Omogna blivande T lymfocyter vandrar från benmärgen till thymus där de genomgår en utmognad som bland annat sker genom genetisk modifiering av T cells receptorn (TCR) som blir unik för varje T cellsklon. T cellerna lär sig även att skilja på kroppsegna och kroppsfrämmande ämnen i thymus. Därmed kan de skydda oss mot främmande ämnen, som t.ex. kan uppvisas i kroppen vid infektioner eller malignitet men samtidigt inte angripa kroppsegna ämnen. Vid autoimmuna sjukdomar har toleransen mot kroppsegna ämnen brutits, och vid allergiska sjukdomar har kroppens immunsystem börjat reagera mot förhållandevis ofarliga ämnen, t.ex. olika födoämnen eller pollen. Dessa sjukdomar är således immunologiskt medierade.

De forskningsresultat som redovisas i denna avhandling är delvis från en långtidsuppföljning där T cellerna från 11 individer vars thymus blivit bortopererad före 6 mån ålder har analyserats avseende flera olika egenskaper, och delvis en registerstudie som innefattar hela Sveriges befolkning där vi analyserat kopplingen mellan borttagande av thymus tidigt i livet på grund av medfött hjärtfel och uppkomsten av olika immunologiskt medierade sjukdomar senare i livet.

Resultaten visade att borttagande av thymus leder till immunologiska förändringar såsom ett minskat antal T celler, med minskad nybildning av T celler och minskad diversitet. Detta kan leda till en nedsatt förmåga att känna igen och reagera mot de många olika ämnen immunsystemet blir utsatt för. Den registerbaserade epidemiologiska studien visade att det finns en association mellan borttagande av thymus och uppkomsten av infektioner och vissa autoimmuna sjukdomar senare i livet om man jämför med en grupp individer som blivit hjärtopererade tidigt utan att man tagit bort deras thymus. Detta har lett till konklusionen att det är önskvärt att undvika att ta bort thymus, delvis eller helt, vid hjärtoperationer på spädbarn.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals. Reprints were made with publishers' permission.

I. Judith Gudmundsdottir, Sólveig Óskarsdóttir, Gabriel Skogberg, Susanne Lindgren, Vanja Lundberg, Martin Berglund, Anna-Carin Lundell, Håkan Berggren, Anders Fasth, Esbjörn Telemo, and Olov Ekwall. Early thymectomy leads to premature immunological ageing; an 18-year follow-up. J Allergy Clin Immunol. 2016 Nov;138(5):1439-1443.e10. doi: 10.1016/j.jaci.2016.05.014.

II. Judith Gudmundsdottir, Christina Lundqvist, Hanna IJspeert, Eva van der Slik, Sólveig Óskarsdóttir, Susanne Lindgren, Vanja Lundberg, Martin Berglund, Jenny Lingman-Framme, Esbjörn Telemo, Mirjam van der Burg and Olov Ekwall. T cell receptor sequencing reveals reduced diversity 18 years after early thymectomy. Manuscript, submitted.

III. Judith Gudmundsdottir, Jonas Söderling, Håkan Berggren, Sólveig Óskarsdóttir, Martin Neovius, Olof Stephanson and Olov Ekwall. Long term effects of early thymectomy: associations with autoimmune diseases, cancer, infections and atopic diseases. Manuscript, submitted.

Publications not included in the thesis:

Lundberg V, Berglund M, Skogberg G, Lindgren S, Lundqvist C, Gudmundsdottir J, Thörn K, Telemo E, Ekwall O. Thymic exosomes promote the final maturation of thymocytes. Sci Rep. 2016 Nov 8;6:36479.

Skogberg G, Lundberg V, Berglund M, Gudmundsdottir J, Telemo E, Lindgren S, Ekwall O. Human thymic epithelial primary cells produce exosomes that carry tissue restricted antigens. Immunology & Cell Biology, 2015 Sep;93(8):727-34.

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CONTENT

ABBREVIATIONS

aHR	adjusted Hazard ratio
BCR	B cell receptor
CDR3	Complementarity determining region 3
CI	Confidence interval
CMV	Cytomegalovirus
cTEC	Cortical thymic epithelial cell
DC	Dendritic cell
DM1	Diabetes mellitus, type 1
DN	Double negative
DP	Double positive
Fup-yrs	Follow-up years
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HR	Hazard ratio
ICD	International Classification of Diseases
IGH	Immunoglobulin heavy chain
IMGT	The international immunogenetics information system
JIA	Juvenile idiopathic arthritis
mTEC	Medullary thymic epithelial cell
PBMC	Peripheral blood mononuclear cell
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0

RTE	recent thymic emigrant
SD	Standard deviation
sjTREC	signal joint T cell receptor excision circle
SLE	Systemic lupus erythematosus
tDC	Thymic dendritic cell
TCR	T cell receptor
TCR Vβ	T cell receptor variable beta chain
TRA	Tissue restricted antigen
TCRB	T cell receptor beta locus
TREC	T cell receptor excision circle
Treg	regulatory T cell
Tx	thymectomized individuals
18m	18 months follow-up
18y	18-years follow-up

1 INTRODUCTION

According to the dictionary a doctoral thesis is ”A long essay or dissertation involving personal research, written by a candidate for a college degree”. This means that those who write a thesis have never done it before, do not know how to do it and will never do it again. And now my time has come.

Curiosity is a strong driving force in research, and curiosity certainly is one of the things that actually has led to this doctoral thesis. As my residency in pediatrics at the Queen Silvia Children Hospital came close to an end in 2007 I contacted Prof. Anders Fasth who led the Dept. of Rheumatology and Immunology. To him I expressed my interest in the area, and he kindly accepted my informal application. Research was definitely on the to-do list and shortly thereafter a good friend and a colleague of mine, Sólveig Óskarsdóttir, introduced to me the concept of thymectomy that I had never heard of before. As it turns, the thymuses of small children born with a congenital heart defect are routinely removed during surgery due to the thymus relatively large size in infants, and its position right in front of the heart, blocking the surgeons access. I had a vague idea about the function of the thymus - but wanted to know more. Dr. Olov Ekwall, a pediatrician and a researcher within the field of immunology, especially the thymus, had recently been recruited to the Sahlgrenska Hospital and Academy. Fortunately, he shared our interest in thymectomy, recruited me as a Ph.D. student, and has supervised the projects presented in this thesis.

1.1 The thymus

The function of the thymus had been enigmatic for centuries when its importance for our immune system first began to emerge after 1960. An Australian physician and researcher named Jacques Miller observed that neonatal thymectomy of mice lead to infections and a failure to reject foreign skin grafts. Consequently the two major subsets of lymphocytes, the T and the B cells, were discovered(1). Thymectomy in older mice did not have such clear immunologic consequences, underlining the developmental importance of the thymus. A vast amount of our knowledge about the thymus and the immune system comes from animal studies, primarily on mice. Even though we share many similarities with mice there are important differences as well. In this thesis, the main focus is on the human thymus and its importance for the immune system.

The thymus is a primary lymphoid organ where T cell maturation takes place. Embryonic development of the thymus starts during the 5th week when thymic gland primordia appear bilaterally in the inferior pole of the third pharyngeal pouch. The endodermal cells that give rise to the thymus migrate caudally and medially to their final position in the thorax, in front of the heart, where the two halves of the primordial gland fuse with each other(2). The thymic epithelial cells differentiate to medullary and cortical thymic epithelial cells, mTECs and cTECs, respectively. By the 9th week of development lymphocyte progenitors from the bone marrow begin to home to and enter the thymus(3). Thereby the microenvironmental structure for thymic function has been set. The interaction between thymic stromal cells and precursor lymphoid cells is essential for normal growth and development of the thymus(4).

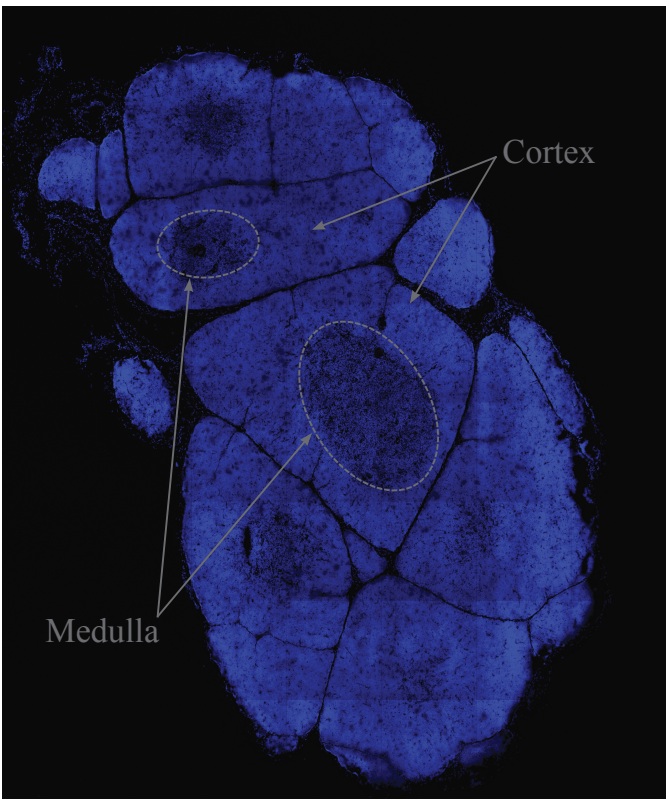

Figure 1. Thymic histology. A confocal microscopy of a part of the thymus showing the lobular structure and rich cellularity. The lighter cortical regions surround the darker medullary regions as indicated by arrows. Staining with Hoechst 33342 that binds nucleic DNA

The fundamental process of T cell maturation in the thymus begins with extensive proliferation of progenitor lymphoid cells, called thymocytes, accompanied by the generation of a huge T cell receptor (TCR) repertoire. In principle, each individual T cell will be equipped with a unique TCR due to the immense diversity achieved by the random recombination of the genetic elements coding for the TCR. Finally, a selection of T cells that carry functional TCRs takes place. Important concepts of this process are the TCR repertoire, positive and negative selection, and the generation of central tolerance to self.

1.1.1 Cortex

Immature lymphoid progenitor cells from the bone marrow enter the thymus at the corticomedullary junction, and then migrate to the subcortical regions. At this stage, they express neither CD4 nor CD8 and are depicted as double negative (DN) thymocytes. They pass through several maturational stages defined by differential surface expression of molecular markers. Early on maturational stages are defined by CD1, CD7 and CD34, and at later stages by CD3, a part of the TCR complex, and the lineage specific CD4 recognizing MHC class II, or CD8 recognizing MHC class I. As the maturation progresses the DN thymocytes successively become dedicated to develop into either αβ T cells or γδ T cells, depending on which type of the TCR heterodimer they express(5). The classic αβ T cells belong to our adaptive immune system and form the majority of the circulating human T cells. The majority of the γδ T cells belong to the innate immune system. Other subsets of unconventional or innate-like T cells have been described, such as invariant natural killer T cells (iNKT) and mucosal associated invariant T cells (MAIT). These T cells do not exhibit the classic αβ-MHC restricted repertoire, but rather have receptors of limited diversity recognizing a variety of antigens, sometimes presented by MHC-like molecules and sometimes not(6). Much less is known about unconventional T cells than the classic CD4 and CD8 positive αβ T cells. This research project, and the description of positive and negative selection in the thymus below focuses solely on the classic αβ T cell subset.

Positive selection

Positive selection is the process by which T cells expressing a TCR capable of recognizing peptides presented by self-MHC are rescued from deletion through apoptosis. Rearrangement of the TCR starts with the β-region on chromosome region 7q35 in the DN thymocytes. Successful rearrangement

leads to an allelic exclusion, and the expression of a pre-TCR complex on the T cell surface composed of the newly formed β chain, and an invariant pre-TCR α chain. The thymocyte proliferates and starts to express both CD4 and CD8, becoming a double positive (DP) thymocyte. Genetic recombination then occurs in a similar way in the TCR α locus on chromosome 14q11.2 and, if successful, a functional TCR which can bind to self MHC is expressed on the surface of the DP thymocyte(5).

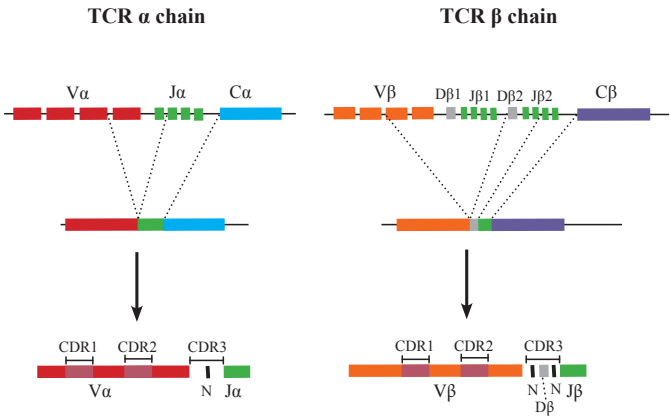

Figure 2. Simplified schematic presentation of the rearrangement of the TCR α (left) and β (right) loci. Several functional V, D, J and C genes exist, and are randomly combined to form the α and β chain of the TCR. First, the D and J segments of the TCR β locus are rearranged, followed by V-DJ combination, and finally a splicing of the VDJ to the C gene occurs and a TCR β chain is expressed. Similar rearrangement then takes place at the TCR α locus and a TCR αβ heterodimer is expressed by the thymocyte. In the lower part of the figure the hypervariable regions of the TCR are shown, the complementarity determining regions CDR1, CDR2 and CDR3. N indicates the addition of random nucleotides in the CDR3 region, a process that further increases TCR diversity. Adopted from (7).

The binding properties of the newly produced TCR dictates the fate of the thymocytes which are destined to apoptosis if not saved by signaling of intermediate strength through the TCR. Thymocytes that fail to produce either a pre-TCR or TCR that can bind to MHC will die from apoptosis. Thymocytes with a functional TCR interact with cTECs in the thymic cortex. cTECs have a key function in the T cell selection as they express self-peptides bound to MHC class I and II molecules. Thymocytes exhibiting intermediate affinity binding to MHC class I become single positive $CD8^+$ T cells, and in the same way those recognizing MHC class II become single positive $CD4^+$ T cells(3, 5, 8-10). Thymocytes carrying a TCR that either

cannot bind to MHC class I or II, or binds with high affinity, are directed towards apoptosis. MHC class II molecules on cTECs are loaded with self-peptides from late endosomes. This function of cTECs is highly specific, and cTECs have been shown to express specific lysosomal proteases, and to possess a high level of autophagy(11), presumably to facilitate the expression of self-peptides on MHC class II molecules. MHC class I molecules are, on the other hand, loaded with peptides derived from the cytosol. This function of the cTECs is also a highly specific process, where distinct proteolytic complexes (proteasomes) have been shown to be important(5). After the successful formation of single positive CD4 and CD8 T cells they migrate to the medulla where negative selection occurs.

1.1.2 Medulla

Central tolerance induction is the process by which potentially harmful T cells expressing a TCR recognizing self-structures with high affinity binding are identified and either directed towards apoptosis or directed towards the regulatory T cell (Treg) lineage. Even though some negative selection is present in the cortex, the induction of central tolerance is mainly taking place in the thymic medulla(5).

Negative selection and regulatory T cell formation

Positively selected T cells enter, and migrate within, the thymic medulla. They continue to be exposed to self-antigens presented on MHC class I and II molecules, now by specialized thymic dendritic cells (tDC) and mTECs. The mTECs are capable of expressing an array of self-antigens including many that are usually only expressed in peripheral tissues(12). These antigens are referred to as tissue restricted antigens (TRAs). Many, but not all, are expressed in mTECs under the influence of the transcription factor Autoimmune regulator, AIRE(5, 13-15). Mutations in AIRE cause a multiorgan autoimmune disease, underlining its important role in tolerance induction(16). The thymic dendritic cells are highly effective specialized antigen presenting cells of hematopoietic origin. In the thymus, there is an active transfer of antigens from the TRA-expressing mTECs to DCs increasing effective antigen presentation to maturating T cells. It is currently unknown exactly how this transfer is mediated(17). In addition to this promiscuous gene expression in thymic mTECs, antigens inducing tolerance have been shown to be imported into the thymus from the periphery in mice. Interestingly, this transport seems to be affected by the peripheral microenvironment where activation of danger signal mediating Toll-like receptors impedes this antigenic transfer through downregulation of thymus homing signal molecules(18).

Negative selection through apoptosis or anergy, which results in a functional deletion of the detrimental auto-reactive T cell clones, is due to high-affinity binding of the TCR to self-antigens presented in the thymus by mTECs or DCs. Another mechanism to induce tolerance is accomplished by the generation of Tregs. These cells can induce immunologic tolerance by suppressing the activation of effector T cells that have escaped negative selection. The affinity of the TCR has been shown to be an important modifier during T cell development, where T cells harboring intermediate affinity TCR seem to be preferably selected to become Tregs, but those binding with high affinity are more prone to apoptosis or anergy. Early Treg differentiation has also been shown to be epigenetically modified by strong transcription enhancers already in the DP stage, where SATB1 seems important(19, 20). SATB1 is a genome organizer, which regulates chromatin structure and gene expression. Nonetheless, exactly what mechanisms cause an autoreactive $CD4^+$ T cell in the thymus to become a Treg remain unknown(21-23).

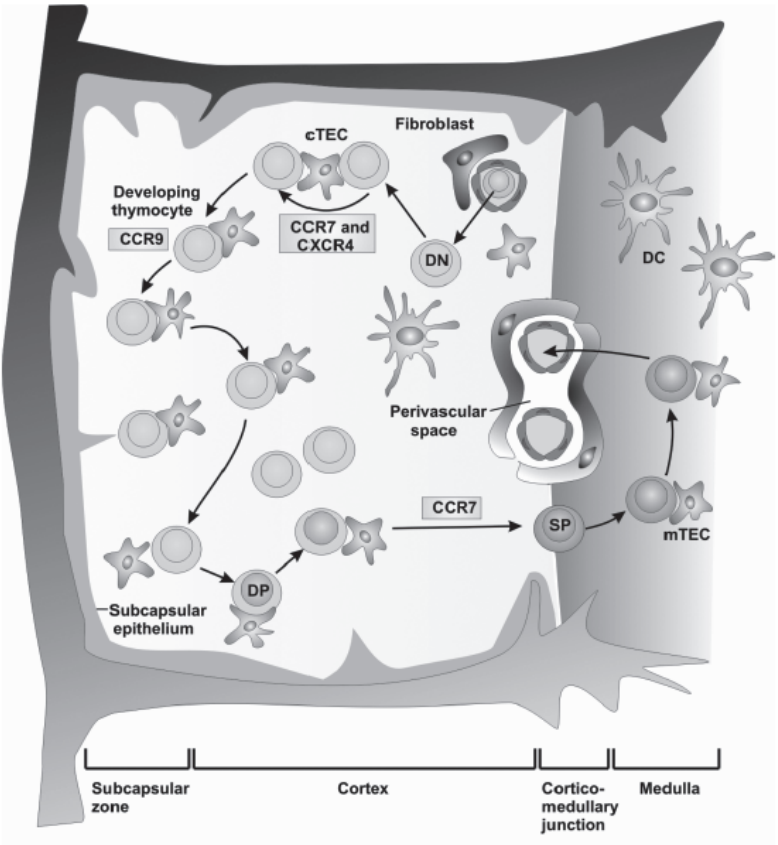

Figure 3. Overview of thymocyte migration, development and selection in the thymus. Lymphoid progenitor cells enter the thymus at the corticomedullary junction (upper right part of figure) as DN thymocytes, migrate to the cortex where they pass through the developmental stages of positive selection becoming DP (lower left side of figure) and finally SP thymocytes that enter the medulla for tolerance induction before departing the thymus as newly produced T cells. Migration is influenced by various chemokine signals such as CCR7 (C-C chemokine receptor type 7), CXCR4 (C-X-C chemokine receptor type 4) and CCR9 (C-C chemokine receptor type 9). Adopted from (24)

This process ensures that most potentially harmful auto-reactive T cells do not leave the thymus, and that some of the autoreactive T cells are turned into regulatory T cells that seed the periphery. The rest of the T cells that have escaped deletional negative selection exit the thymus as antigen-inexperienced naive T cells. In humans the expression of surface marker CD31 has been proposed as a marker of these recent thymic emigrants, especially for $CD4^+$ T cells(25, 26).

1.1.3 Thymic involution

The thymus is highly active and proportionally large during late embryonic stages and in neonates. However, its function starts to diminish after puberty, with a steady reduction in thymic cellularity and a decreased thymic output with increasing age(27, 28). Naive T cells are produced at a much slower rate in humans than in mice, whereas their peripheral longevity by far exceeds their murine counterparts. The dynamics of thymic turnover therefore differ fundamentally between humans and mice. In humans, the peripheral naive T cell pool is primarily maintained through peripheral proliferation, and to a lesser extent dependent upon constant export of naive T cells from the thymus(29-31). Aging is accompanied by a proportional increase in memory T cells, a decrease in the naive $CD8^+$ T cell subset, a decrease in recent thymic emigrants, T cell telomere shortening and perturbations in the TCR repertoire diversity which is most prominent in the $CD8^+$ T cell subset(32). TREC concentration also successively decreases as the thymus involutes and the peripheral T cell number is maintained through peripheral proliferation(27).

1.2 Thymectomy

1.2.1 Pediatric cardiac surgery

Congenital heart defects are among the most common birth defects, and affects approximately 1% of all children. While some defects are minor, others are serious and carry a high mortality rate if left untreated. Surgical procedures to treat congenital heart defects started to evolve around and after 1950, after which the number and diversity of procedures slowly rose. In the 1970´s, aided by the development of extracorporeal membrane oxygenation during surgery, such surgeries increased markedly. As a result, children with previously fatal conditions now survive through adult life. This imposes new medical challenges with long-term complications related to the congenital heart defect, or its treatment(33, 34).

The thymus of children undergoing heart surgery is often removed due to its location in front of the heart, blocking the surgeon´s access. This procedure is known as thymectomy. Unfortunately, thymectomy is not necessarily noted in surgical reports, nor does it have a specific diagnostic code. Nonetheless, thymectomy is routinely performed during some surgical procedures. The annual numbers of cardiac surgery in Sweden between 1973-2009, with and without thymectomy, are shown in Figure 4 (Paper III). The gradually increasing number of heart surgeries performed is clear, rising from only a

handful in the early 1970´s to around 300 annually after 2000. The marked increase of surgery involving thymectomy observed in 1993-1997 is probably due to a combination of factors. The Norwood surgical procedure was introduced and cardiac surgery in Sweden was centralized, presumably leading to an increase in the number of surgeries as well as increased reporting to the National Patient Register.

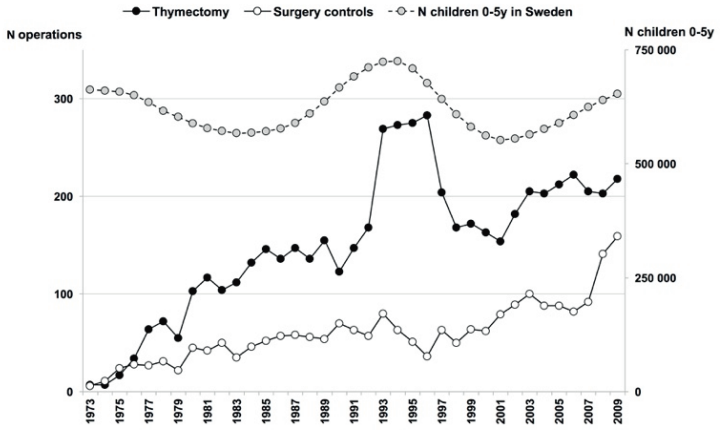

Figure 4. Number of heart surgeries per year in children before five years of age. Surgeries associated with thymectomy (black dots) and surgeries not associated with thymectomy (white dots), such as those using lateral approach, or catheterizations. The total number of children ≤ 5 years of age in Sweden (grey dots).

The number of early cardiac surgeries involving thymectomy in Sweden (~200/year) translates to ~21,000 individuals annually in Europe and USA assuming similar clinical practice. The number of individuals living without a thymus is accumulating in line with increased numbers of early cardiac surgery. This results in a growing population of older thymectomized individuals.

1.2.2 Studies on early childhood thymectomy

Thymectomy has traditionally been regarded as a safe procedure without known clinical consequences(35-37), although a number of studies have revealed a clear immunologic impact(38-54).

Early childhood thymectomy is associated with immunologic changes reminiscent of changes seen in normal aging(32, 55, 56). Lymphocyte

subsets are affected, and T cell lymphopenia characterized by a decrease of naive T cells with a concomitant increase in the memory T cell population has been reported (40, 42, 45, 46). The regulatory T cell (Treg) numbers have been shown to be unaffected in two studies (44, 49), whereas one study showed that although the absolute Treg number was lower, the Treg proportion was increased; albeit with a decrease in naive Tregs (52). Earlier qualitative analyses did not reveal a significant functional impairment of the different T cell populations (37-39, 42), but recent reports have described functional differences in thymectomized individuals, such as a decreased IL-7 mediated proliferation(57), and a decrease in IL-8 production by stimulated naive $CD4^+$ T cells as well as a distinguishing RNA transcription profile of recent thymic emigrants(53). A delayed response to tick-borne-encephalitis virus vaccination also supports a qualitative effect of early childhood thymectomy(43, 48). T cell receptor excision circles (TRECs) have been used to estimate thymic function and have consistently been found to be decreased in thymectomized individuals although to varying degrees (40, 42, 45, 58).

Previous studies have often included clinical parameters, but generally the groups have been small and often heterogeneous, information on the amount of removed thymic tissue has been lacking, and the follow-up time short. No study to date has been sufficiently large and adequately designed to address the clinical question of whether early childhood thymectomy is a safe procedure, or if it could lead to increased incidence of immune mediated diseases later in life. We therefore planned and conducted follow-up analyzes of the immune system of individuals thymectomized early in life (Paper I and II), and decided to investigate the association between thymectomy and long-term clinical consequences through a nationwide register-based cohort study in collaboration with the Karolinska Institute (Paper III).

2 AIM

The overall aim of this study was to investigate the immunologic and clinical long-term impact of early childhood thymectomy.

The specific objectives were:

I. Investigation of the immunological effects of early childhood thymectomy at both 18 months and 18-year follow-up with regards to the subset composition of T cells, the thymus output and an assessment the T cell clonality.

II. Assessment of the T and B cell receptor repertoire diversity using next generation DNA sequencing of the variable β chain of the TCR, and the immunoglobulin heavy chain.

III. Investigation of the association between early thymectomy and subsequent risks of infectious diseases, autoimmune diseases, allergies and cancer.

3 PATIENTS AND METHODS

3.1 Paper I

3.1.1 Study design and subjects

Paper I was a continuation of a hitherto unpublished research project initiated by Sólveig Óskarsdóttir and Anders Fasth in 1993. Individuals born between 1993-1995 with cardiac malformations demanding surgical correction at less than six months of age were identified pre-operatively at the Queen Silvia Children's Hospital, Sahlgrenska University Hospital in Gothenburg, Sweden. Included in the study group were patients in which the thymus removal was estimated by the surgeon to have exceeded 90%. Patients with syndromic cardiac malformations or known genetic disorders were not included. During that time period (1993-1995), 19 individuals agreed to participate. Blood samples for analysis of lymphocyte subsets were drawn pre-operatively and at 18 months of age. For comparison at 18 months of age, ten otherwise healthy children undergoing minor surgery (mainly urological) at the same hospital were recruited, but no comparison group was recruited for the pre-surgical analyses.

To investigate long-term immunologic effects of thymectomy the participants were contacted again through letters 18 years later (median age 18.7, range 17.2-19.9), in November 2011. Eleven of the originally included 19 (58%) agreed to participate in a follow-up study, whereas eight did not reply. At first, the intention was to use the same controls as at 18 months of age, but as only 2 of those agreed to participate, recruitment of a new control group was necessary. An equal number of age- and sex-matched controls were recruited (median age 18.4, range 17.1-19.9) all living in the Gothenburg area at the time of blood collection.

All participants and their caregivers answered a written questionnaire (provided in the appendix) regarding their history of general health, vaccinations, infections, allergies, autoimmune diseases and cancer.

Blood samples were collected from May 2012 until May 2013 from all participants, with samples from controls interspersed between samples from thymectomized. The samples taken at, or near, the Sahlgrenska University Hospital reached the laboratory for processing within 2 hours. However, if the participant was not located in or near Gothenburg, blood samples were collected at the participants nearest health care center with express delivery to

the laboratory within 24 hours. Participants did not have signs of infection at the time of blood collection. The blood samples were analyzed for lymphocyte subsets, helper and cytotoxic T cell receptor variable β chain (TCR Vβ) usage, T cell receptor excision circles (TRECs) and telomere lengths of T and B cells.

3.1.2 Methods

Cell preparation and flow cytometry

The information on cell preparation and flow cytometry pre-operatively and at 18 months of age was somewhat limited. Monoclonal antibodies towards CD3, CD4, CD8, CD19 and CD56 were used. In addition, several other markers were analyzed at that time, but at the 18-year follow-up, they were considered immunologically obsolete and therefore not included in the follow-up study.

At the 18-year follow-up, fresh peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque density gradient centrifugation. They were analyzed directly for $CD3^+$, $CD4^+$, $CD8^+$, $CD45RA^+$, $CD45RO^+$, $CD19^+$ and $CD16/56^+$ cell markers providing numbers and proportions of naive and memory helper and cytotoxic T cells, B cells and NK cells. The analyses were performed at the Dept. of Immunology, Sahlgrenska University Hospital.

All multicolor analyses were performed on a FACS Canto II flow cytometer and results were analyzed using FlowJo Data analysis software.

A second fresh sample was subjected to Ficoll-Paque density gradient centrifugation and further analyzed regarding TCR Vβ. The remaining cells were viably frozen using 15% DMSO (dimethyl sulphoxide) in fetal calf serum and stored in an -80°C freeze later used for cell sorting, naive, memory and regulatory T cell subset analyses, and telomere length analysis (Paper I), as well as sequencing of the T and B cell receptor (Paper II).

Recent thymic emigrants

Recent thymic emigrants defined as $CD45RA^+CD31^+$ cells were first described by Kimmig et al. in 2002 (25). The TREC concentration of the $CD31^+$ naive T cells was shown to be high, whereas the TREC concentration in the $CD31^-$ naive T cell population was very low, indicating extensive peripheral proliferation of the $CD31^-$ subset. The proportion of the $CD31^+$ naive T cells found to be high in neonates and children, 80-90%, but was

shown to decrease with age, and approached 50% at the age of 70 years. Later research has supported these findings(26, 59, 60).

Recent thymic emigrants were accordingly defined as $CD4^+CD45RA^+CD31^+$ in this study. Multicolor flow cytometry was performed on fresh cells using a panel of monoclonal antibodies to $CD4^+$, $CD45RO^+$, $CD45RA^+$ and $CD31^+$ at the Dept. of Immunology, Sahlgrenska University Hospital. The gating strategy is depicted in Figure 5.

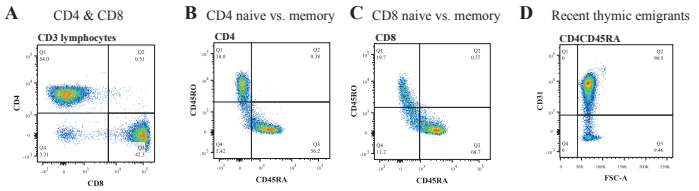

*Figure 5. Gating strategy of recent thymic emigrants ($CD4^+CD45RA^+CD31^+$). Representative flow cytometry plots. **A**, T helper ($CD4^+$) and cytotoxic ($CD8^+$) subsets from $CD3^+$ lymphocytes. **B**, $CD4^+$ T cells (Quadrant Q1 in A) separating naive $CD45RA^+$ (x-axis) and memory $CD45RO^+$ (y-axis) cell subsets. **C**, same as B for $CD8^+$ T cells (Q3 in A). **D**, Recent thymic emigrants as $CD31^+$, gated on $CD4^+CD45RA^+$ (Q3 in B).*

Naive, memory and regulatory T cells

Naive and memory T cells can be distinguished by their expression of different CD45 isoforms. CD45 is a tyrosine phosphatase, involved in signal regulation. The three different isoforms are generated through variations in splicing of three exons. Naive T cells express CD45RA, which contains all three extracellular domains encoded by these exons, while memory T cells express CD45RO, which is the shortest isoform where all three exons are absent(61).

Regulatory T cells are characterized by the expression of the marker combination $CD4^+CD25^+Foxp3^+$(21). The regulatory T cell marker Foxp3 is an intracellular transcription factor not as easily analyzed as surface cell markers. The cell surface markers $CD4^+CD25^+CD127^{low/-}$ have been described as an alternative method to correctly identify regulatory T cells in humans(62).

Analyses of naive ($CD3^+CD4^+CD45RA^+$ and $CD3^+CD8^+CD45RA^+$), memory ($CD3^+CD4^+CD45RO^+$ and $CD3^+CD8^+CD45RO^+$) and regulatory ($CD3^+CD4^+CD25^+CD127^{low}$) T cells were performed on thawed cryopreserved PBMCs incubated with a panel of monoclonal antibodies at the Dept. of Clinical Immunology, Sahlgrenska University Hospital. The

gating strategy of naive versus memory cells for both $CD4^+$ and $CD8^+$ T cells is shown in Figure 5, B and C, respectively. The gating strategy of Tregs is shown in Figure 6.

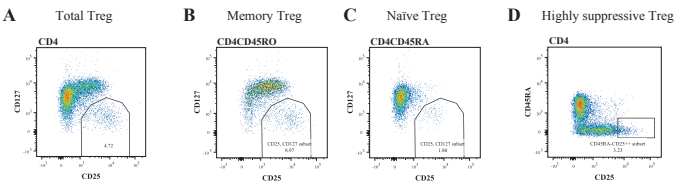

*Figure 6. Gating strategies of regulatory T cells **A**, Total Treg cells defined as $CD4^+CD25^+CD127^{low}$. **B**, Memory Treg cells $CD4^+CD45RO^+CD25^+CD127^{low}$ **C**, Naive Treg cells $CD4^+CD45RA^+CD25^+CD127^{low}$. **D**, Highly suppressive Treg cells defined as $CD4^+CD45RA^-CD25^{++}$.*

TCR Vβ repertoire analysis

An analysis of the TCR repertoire is challenging. Traditionally two main approaches have been used(63). One is a flow cytometry based method that utilizes monoclonal antibodies towards each of a total 24 different TCR Vβ families expressed on the cell surface. The other approach to analyze the TCR is based on PCR amplification, where the most common method is called CDR3 spectratyping. CDR3, or complementarity determining region 3, is one of three hypervariable regions of the TCR. CDR3 spectratyping analyses the variability in the lengths of each CDR3 region in 20 defined TCR Vβ gene families. The flow cytometry based method was preferred in Paper I, mainly because of availability of both equipment and experience. However, next generation sequencing of the TCR had started to emerge as an alternative to these earlier methods. Later, as presented in Paper II, this alternative method was used to analyze the T and B cell receptor repertoire.

T cell receptor variable chain β (TCR Vβ) repertoire was analyzed on fresh cells using IOTest Beta Mark from Beckman Coulter according to manufacturer's instructions. Cells were also stained with monoclonal antibodies to $CD4^+$ and $CD8^+$ to enable separate analysis of the helper and cytotoxic T cell populations. Approximately 200,000 cells were incubated in each well giving a minimum of 10,000 $CD4^+$ or $CD8^+$ cell count. Two individuals, one thymectomized and one control, had suboptimal $CD8^+$ cell counts and were thus excluded from further analysis.

Each member of the 24 different TCR Vβ families displays at least 75% sequence homology with any other member of the same family. There is

considerable variation in the size of the different families between individuals. During T cell stimulation and proliferation some clones can become more abundant than others, causing oligoclonality. As a result of this oligoclonality a larger proportion of T cells than expected are expressing a TCR from the same family. This is, however, a rather crude method to estimate diversity. For example, if oligoclonal peripheral T cell expansion is equally distributed between the different TCR Vβ families this method will fail to detect any abnormality(63-65). For detailed information and gating strategies see supplementary material, Paper I.

TREC PCR

The signal joint T cell receptor excision circles (sjTREC) are circular DNA strands excised from the chromosomal DNA during genetic rearrangement of the TCR α locus. The TCR δ segment, located between the variable and joining segments of the TCR α locus, is excised at two defined loci, δRec and ψJα, and the excised sequence forms the circular sjTREC. This circular DNA strand is not replicated during cell division, and thus the sjTREC concentration subsequently dilutes with each cell division(27, 66, 67). The TREC content of peripheral blood T cells thus depends on thymic output, the extent of peripheral cell division and cell death. Naive T cells, especially the proposed recent thymic emigrants expressing CD31(25), have a much higher TREC concentration than memory T cells. Also, the TREC content decreases with advancing age, although at a slow rate due to the longevity of human naive T cells(27, 30, 66).

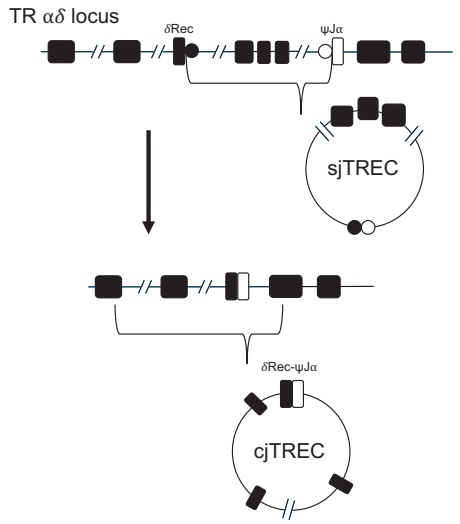

Figure 7. TREC formation. TCRδ locus is located within TCRα locus at 14q11.2. Splicing and rearrangement of constant (C), variable (V) and joining (J) regions during α chain formation generates stable circular DNA strands. Initially a signal joint (sj)TREC is excised and sequentially a coding joint (cj)TREC. Influenced by (27).

The method used to measure sjTRECs is described in detail in Paper I. In summary, genomic DNA from PBMCs was isolated and quantified, followed by a real-time PCR analysis using signal joint TREC primers as previously described(68). The reaction was done in triplicates, using *GAPDH* as a reference gene. TREC number was estimated by extrapolating sample quantities from a standard curve acquired by serial dilutions of a pCR2.1-human TREC and pCR2.1-*GAPDH* gene plasmids(69). The number of TRECs was estimated according to the formula: (Mean of TRECs quantity/(Mean of *GAPDH* quantity/2)) $x10^6$ = number of TREC molecules per 10^6 cells. The mean quantity of *GAPDH* was divided by two because of the biallelic occurrence of this gene.

Telomere length analysis and cell sorting

Telomeres are repetitive DNA sequences, $(TTAGGG)_n$, located at the end of each chromosome. Inherent to the process of DNA replication during cell division, a number of base-pairs are lost from the ends of the chromosomes, and the telomeres shorten with each round of division(70). The telomere length has been shown to decrease with advancing age, and to vary considerably between different cell types in adults. Decreased telomere

length is also associated with earlier onset of a number of chronic diseases(71).

Most of the previous telomere length analyses in lymphocyte subsets show that B cells have the longest telomeres, followed by $CD4^+$ T cells, and finally $CD8^+$ T cells(72-74). However, telomere length can be regulated, for example by the enzyme telomerase which elongates the telomere repeat sequences during cell proliferation(75, 76). The expression of telomerase has been shown to be low in resting lymphocytes, but to increase during antigen stimulation(77), thereby maintaining a proliferation potential essential for the proper function of lymphocytes. The telomere length of the naive T cell subset has been shown to exceed that of memory T cells from the same donor. In addition, the replicative potential of the naive T cell subset has been shown to be higher(78). Nonetheless, telomeres of both T and B cells shorten with time(71-73). The increased homeostatic peripheral T cell proliferation observed after thymectomy could have an overall negative impact on the telomere length, and thereby the replicative potential of T cells.

The method used to analyze telomere length is described in detail in Paper I. In summary, frozen PBMCs (-80°C) were thawed; the cells were pelleted, re-suspended and stained with monoclonal antibodies recognizing CD4, CD8, CD19, CD14 and CD56. Helper and cytotoxic T cells as well as B cells were then sorted with high purity (>95%). DNA was isolated from CD4, CD8 and CD19 cell subsets using QIAamp DNA mini kit. Telomere length was estimated using a qPCR method described by Cawthon(79) that allows an estimation of the relative telomere length from a ratio of the telomere repeat copy number to a single reference gene (RPLP0) copy number.

Statistics

For every thymectomized individual an age and gender matched control was recruited. All statistical analyses were done with Graphpad Prism. To assess quantitative differences in cell populations the Student's t-test for unpaired data was used for all variables with a Gaussian distribution whereas the Mann-Whitney test was used for a few variables ($CD8^+$, $CD19^+$ at 18 months and $CD56^+$; T regulatory cells and $CD8^+CD45RO^+$ absolute numbers) that differed from normality when tested using the D´Agostino & Pearson omnibus normality test. The unpaired t-test was used to compare TRECs and relative telomere lengths. TCR Vβ was analyzed by defining if each individuals Vβ-chain usage deviated more or less than 3 SD from the mean of the controls using Fisher's exact test. The comparison of TCR Vβ chain usage between the two groups was done using the Holm Sidak multiple comparison test.

Multivariate orthogonal projection to latent structures discriminant analysis (OPLS-DA) is a method based on partial least square regression analysis that examines the relationship between variables(80). OPLS-DA was used to evaluate whether thymectomized individuals and healthy controls could be discriminated based on the various immune variables assessed in the study. This method primarily aids visualization and thereby interpretation of analyzed variables. All data were scaled to unit variance so that all the variables were given equal weight regardless of their absolute value. The quality of the OPLS-DA model was based on the variable R2 (i.e. the goodness of the fit of the model) and Q2 (i.e. how well a variable can be predicted by a model).

3.2 Paper II

3.2.1 Study design and subjects

For a description on the studies design, subjects and methods of cell isolation please refer to Paper I, Chapter 3.1.1 and 3.1.2. In Paper II, DNA from sorted helper and cytotoxic T cells, and B cells was subjected to next generation sequencing of the T cell receptor ß chain (*TCRB*) and the immunoglobulin heavy chain (*IGH*).

3.2.2 Methods

Immune repertoire sequencing

The evolution of next generation DNA sequencing has made sequencing of the extremely diverse immune repertoire feasible, allowing a detailed analysis of the repertoire. The number of distinct sequences allows an estimation of the diversity. Sequencing also gives information on the V, D and J usage, the CDR3 length, the insertions of non-template nucleotides and deletions known to occur at the V-D-J junction sites, as well as amino acid usage(81, 82). The method used is based on the Illumina® sequencing by synthesis method. After a multiplex PCR reaction and purification of the DNA of interest, a single fluorescently labelled nucleotide is sequentially added in single cycles to the single strand DNA, with a read and washing of the label between rounds(83).

There are several challenges in immune repertoire sequencing(81, 82). In multiplex PCR, the different primer efficacy of multiple primers is a problem. Also, the sequencing of the highly homologous variants of the immune repertoire produces an enormous amount of data to be interpreted and analyzed necessitating the development and use of complex bioinformatics

tools. Furthermore, sequencing errors accumulate, and affect the results. The Miseq Illumina Sequencing platform has an estimated error frequency of 1-6%(84). To differentiate between true rare sequences and sequencing errors is sometimes impossible. However, the results of thymectomized and controls can be assumed to be comparably affected by methodological flaws, thus not compromising the comparison between the two groups.

In paper II BIOMED-2 consensus primers for *TCRB* and *IGH* were used(85). For the purpose of data interpretation and analysis, the IMGT High V-Quest database, developed by the International Immunogenetics Information system(86), and the web-based application IGGalaxy(87), were used.

The method is described in detail in Paper II, with additional details regarding *IGH* analysis published by Driessen et al in 2013(88). In summary, multiplex PCR reactions according to BIOMED-2 consensus guidelines(85) were performed with either the 23 Vβ forward and 13 Jβ reverse primers or the forward VH1-6 FR1 and JH consensus reverse primers, for *TCRB* and *IGH* analysis, respectively. The PCR products were purified with gel extraction and magnetic beads, the product concentrations were measured, and then sequenced. After sequencing the reads were uploaded to IMGT High V-Quest and analyzed with IGGalaxy tool extracting information about the V, D and J gene usage, the composition of the junctional regions, and the CDR3 region for both *TCRB* and *IGH*.

We analyzed 50 ng DNA from each individual. Assuming that the amount of DNA in a single cell is approximately 6 picograms, the population number of $CD4^+$, $CD8^+$ and $CD19^+$ cells analyzed was approximately 8.300 cells. For the flow cytometry TCR Vβ analysis in Paper I, the cell count was comparable, or a minimum of 10.000.

The clonality of the repertoire was evaluated using a method described by Boyd et al(89) in 2009. In summary, six aliquots from same individual were subjected to multiplex PCR and sequencing and the number of re-occurrences of identical *TCRB* rearrangements were counted. Increased numbers of re-occurrences, called coincidences, are indicative of oligoclonality as identical sequences are presumed to originate from the same T cell clone.

Statistical analysis

Statistical analyses were done with Graphpad Prism version 7.0b (Graphpad Software Inc., San Diego, CA). To assess differences between thymectomized individuals and controls the two-tailed Mann-Whitney test was used. The nonparametric Spearman correlation co-efficient of clonality

score and T cell numbers was calculated, and a simple linear regression analysis was performed.

3.3 Paper III.

3.3.1 Study design and subjects

To analyze the clinical long-term effects of thymectomy a large cohort, as well as a long follow-up time of subjects, was needed. The Swedish patient registers provide reliable information on both pediatric cardiac surgery as well as diagnoses within the hospital based in- and out-patient medical care(90, 91). A nationwide population-based cohort study was conducted, using the Medical Birth, Cause of Death and National Patient Registers in Sweden to identify subjects and controls. Subjects were individuals thymectomized before the age of five, and for each thymectomized individual ten control subjects from the general population were identified, and in addition a surgery control group, that had gone through cardiac surgery without thymectomy before the age of five, was included (Figure 8).

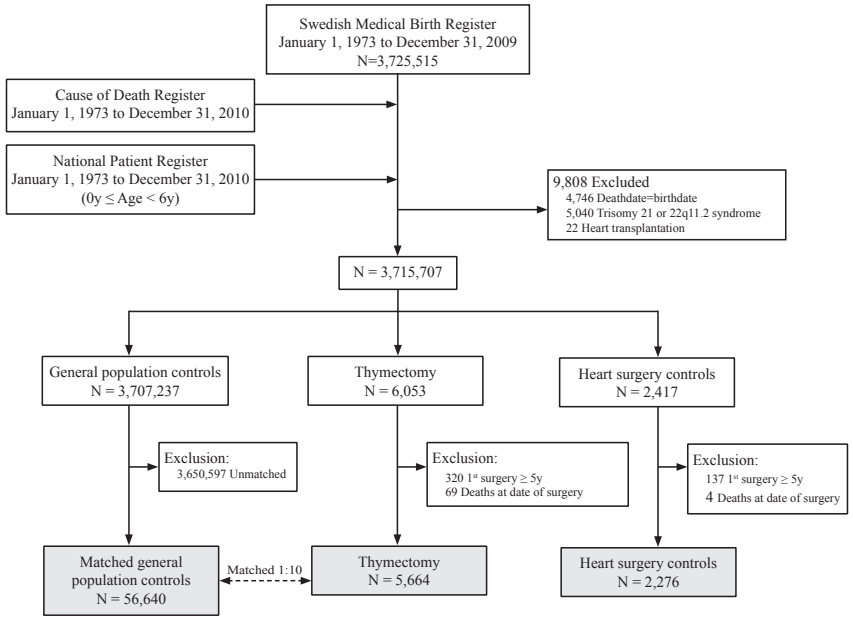

Figure 8. Study design overview. Thymectomized individuals and two control groups were identified, one surgery control group where individuals had undergone early cardiac surgery not including thymectomy, and an age and sex 1:10 matched control group from the general population.

3.3.2 Methods

Study population

To attain sufficient power, the inclusion period was long and thus the follow-up time for each subject was variable, ranging from only one to 37 years. All live births between 1973 through 2009 were identified the Swedish Medical Birth Register, including a total of 3,725,515 infants. They were linked with the Cause of Death, National Patient, and Prescribed Drug Registers using the Swedish unique personal identification number(91, 92). Infants whose death date was either the same as birth date (N=4746) or date of surgery (N=73) were excluded as well as individuals with trisomy 21, DiGeorge syndrome, or 22q11.2 deletion syndrome (N=5040), as these syndromes are known to cause immunologic abnormalities(93, 94). Also, infants who had undergone heart transplantation were excluded (n=22). This left a total of 3,715,707 infants.

Intervention Cohort

Cardiac surgery before the age of five years was used as a cut-off value. Information on cardiac surgery was retrieved from the inpatient care component of the National Patient Register using surgery codes from the Classification of Surgical Procedures (Nordic Medico-statistical Committee) for the years 1997-2010, and from the older Classification of Surgical Procedures, 6th version, published by the Swedish National Board of Health and Welfare for the years 1973-1996. Surgical codes are specified in Paper III.

The thymus, and the removal of the thymus, are rarely mentioned in surgical reports, and the medical registers contain no such individual data. Thymectomy does not have a specific surgery code, but is considered a standard surgical approach during certain types of cardiac surgery. The surgery codes were chosen during a thorough assessment of the Classification of Surgical Procedures under the supervision of an experienced surgeon in pediatric cardiac surgery. To assess the general degree of thymectomy, surgeons at one of the two pediatric cardiac surgery centers in Sweden (Gothenburg) documented the degree of thymectomy in all included consecutive surgeries (n=206) during a two-year period from the 1st of May 1997 until the 30th of April 1999.

After exclusion of individuals who died at the date of surgery (N=69) a total of 5664 cases were identified as having had an early surgery presumably involving thymectomy.

Controls

The presence of a congenital cardiac defect in the thymectomy group is a source of confounding. The effect of thymectomy cannot be isolated from the underlying cardiac defect, necessitating surgery and concurrent thymectomy. To address this issue two control cohorts were defined. One cohort consisted of infants born with congenital heart defects who underwent a cardiac surgery without thymectomy during the same time period. This comparative group was chosen to try to correct for any confounding impact on the outcomes originating from the congenital cardiac defect instead of thymectomy. Information on heart surgery before the age of five years was retrieved, as described for the thymectomized individuals, and surgery codes were selected in the same manner. The procedures were for example surgery of the aortic arch or ductus arteriosus through a lateral approach that do not involve thymectomy and miscellaneous transcutaneous approaches. No matching was performed for surgery controls. The second control cohort consisted of ten

general population controls per thymectomized individual, matched for birth year and month as well as sex.

Definition of outcomes and follow up

The possible long-term effects of early childhood thymectomy on disease occurrences later in life was unknown. Therefore, we selected a wide array of relatively common diagnoses within four main categories of diseases; autoimmune, cancer, infections and atopic diseases. The immune system has a primary role in all of these categories. Diagnoses were selected in line with the subjects ages, ranging from 7 to 43 years of age, with a much greater number in the younger ages.

- **Autoimmune:** hypo- and hyperthyroidism, type 1 diabetes, rheumatoid arthritis, juvenile idiopathic arthritis, systemic lupus erythematosus, Sjögren´s disease, ankylosing spondylitis, psoriatic arthritis, inflammatory bowel disease (Crohn´s disease, ulcerative colitis), multiple sclerosis, psoriasis, and celiac disease.
- **Cancer:** any cancer diagnosis.
- **Infections:** Pneumonia and certain infectious and parasitic diseases. The infections were roughly divided according to infectious agent into three groups consisting of bacterial, viral, and other (parasitic, fungal, protozoan) infections.
- **Atopic:** asthma, rhinitis, eczema, and anaphylaxis.

Information on outcomes was collected from the National Patient Register on inpatient care from 1973-2010 and on outpatient care at hospitals from 2001-2010. Individuals were followed from index-date until the first of an outcome event, death, or end of the study period at the 31st of December 2010. With the exception of infections, individuals diagnosed with any defined outcome before index-date were excluded from the study. International Classification of Diseases (ICD) codes from the eight to tenth revisions used for definitions of outcomes are specified in Paper III. The division of infections into three groups was relatively straightforward for the latest international classification of diseases, ICD-10, where emphasis is on etiology, whereas the differentiation for the older revisions (ICD-8 and ICD-9) was challenging due to a more anatomically based approach to disease classification. The approach used let the predominant etiology of the ICD8 and ICD9 diagnostic codes dictate the disease category. For example, a diagnosis of pneumonia was categorized as a bacterial disease, whereas an upper respiratory infection was considered a viral disease.

Statistics

The study population was compared to the two defined control populations. Between-group differences in time to first ever event of autoimmune, cancer and atopic disease outcomes were estimated by fitting a Cox proportional hazard regression model(95), adjusting for birth year and month, and sex. To take into account the risk of recurrent events of infections, and not only time to the first ever infection, we analyzed time to infection using the Andersen-Gill proportional intensity regression model(96), with the same included covariates as for the other outcomes.

Incidence rates, including 95% confidence intervals, per 10,000 person-years were computed by assuming that the number of incident cases followed a Poisson distribution. Between-group differences of mean age were analyzed using the student's t-test.

Data were analyzed using SAS software version 9.4. (SAS Institute Inc, Cary, NC)

4 RESULTS

The results are presented separately for each publication. In summary, the results of Paper I showed that early childhood thymectomy was associated with a decrease in T cells, predominantly the naive T cells. The Tregs seemed to be similarly affected, with a decrease in naive Treg number. The B cell number was unaffected. The TCR Vβ analysis showed signs of oligoclonality. The TRECs were severely reduced, and telomere length was decreased in the $CD8^+$ T cells. The results of Paper II showed a decreased diversity of the T cell receptor repertoire, whereas the B cell receptor repertoire was unaffected. The results of Paper III showed that early childhood thymectomy was associated with increased risks of certain autoimmune diseases, cancer as well as infectious diseases.

4.1 Paper I

4.1.1 Study group characteristics and clinical data

The characteristics of the thymectomy group are presented in Table 1.

Patient	Gender	Type of heart defect	Age at thymectomy days
1	f	AS	107
2	f	CoA	10
3	f	TGA	64
4	m	Fallot	138
5	m	VSD	61
6	m	TGA	6
7	f	VSD	50
8	f	TGA	148
9	f	VSD	133
10	m	TGA	6
11	m	TGA	48

Table 1. Study group characteristics. Gender, type of congenital heart defect and age at operation. Abbreviations: AS Aorta stenosis, CoA Coarctation of the Aorta, TGA Transposition of the great arteries, Fallot Fallots tetralogy and VSD Ventricular septal defect.

The clinical data from the two groups are summarized in Table 2. Episodes of acute otitis media were reported by nine thymectomized individuals and seven controls but thymectomized were less likely to have undergone any surgery because of their otitis even though equal number reported more than

10 episodes of acute otitis media. Thymectomized individuals reported pneumonia, frequent respiratory infections, infections requiring hospital admission and allergies more frequently than controls. Only one individual in each group reported an autoimmune disease.

	Thymectomized (no. 11)	Controls (no. 10)
Acute otitis media:	9	7
< 10 per individual in total	6	4
≥ 10 per individual in total	3	3
Surgery related to recurrent otitis media	1	3
Pneumonia	5	2
Frequent respiratory infections	7	1
Severe infection (Hospital admission)	6	1
Autoimmune disease (any)	1	1
Allergy (any)	5	2

Table 2. Clinical data, reported from a questionnaire answered by participants and parents at 18-year follow-up.

4.1.2 Multivariate factor analysis

OPLS-DA demonstrated a clear distinction between thymectomized individuals and healthy controls based on the assessed immunological variables (Figure 9). All healthy controls appeared in the two left quadrants, while all but one thymectomized individuals were plotted in the two right quadrants (Figure 9 A). The immune variables that displayed the strongest association (positive or negative) with thymectomy are identified in the OPLS-DA loading column plot (Figure 9 B). Immune variables represented by a bar pointing in the same direction as *Thymectomized* (located far right) are positively associated and those with opposite direction are negatively associated. The larger the bar and smaller the error bar, the stronger and more certain is the contribution to the model. Thymectomy was associated with TCR Vβ oligoclonality and an increased proportion of memory Treg cells whereas it was negatively related to other cell counts such as $CD8^+$ and $CD4^+$ cells, Treg cells, naive Treg cells, memory $CD8^+$ cells as well as lower proportions of naive $CD4^+$ and $CD8^+$ cells and RTEs ($CD31^+$ cells). Thymectomy was also negatively related to telomere length in $CD4^+$ and $CD8^+$ cells and number of TRECs. Bars crossing the X-axis did not differentiate between the two groups, namely the telomere length of B-cells, the percentage of $CD4^+$ memory T cells ($CD4^+CD45RO^+$), proportion of Treg cells, memory Treg count, and the number and proportion of highly suppressive Treg cells ($CD4^+CD45RA^-CD25^{++}$).

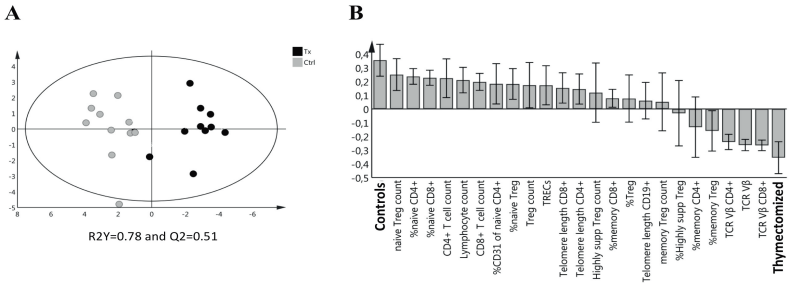

*Figure 9. Multivariate factor analysis. **A**, OPLS-DA scatter plot showing the distinction between thymectomized cases (black dots, n=11) and healthy controls (grey dots, n=11). **B**, OPLS-DA loading column plot correlating each X-variable of both thymectomized cases and healthy controls. X-variables pointing in the same direction as Thymectomized (far right bar) are positively associated, whereas variables in the opposite direction are inversely related.*

4.1.3 Lymphocyte subsets

T cells, B cells and NK cells.

A decrease in T cell number was apparent and consistent over time in the thymectomy group (Table 3, Figure 10). The mean number of total lymphocytes after thymectomy was lower at 18-year follow-up but the difference was not statistically significant at 18 months. Both $CD4^+$ and $CD8^+$ cells were decreased whereas no difference was detected in B cells or NK cells.

		Thymectomized	95% CI	Controls	95% CI	p-value
Pre-op	Lymphocytes	**3.67**	(2.75-4.60)	**n.a.**		
	$CD4^+$	**1.72**	(1.24-2.21)	-		
	$CD8^+$	**0.62**	(0.43-0.80)	-		
	$CD19^+$	**0.85**	(0.33-1.37)	-		
	$CD16^+/56^+$	**0.40**	(0.12-0.69)	-		
18 m	Lymphocytes	**2.99**	(1.80-4.18)	**4.25**	(3.15-5.35)	0.0925
	$CD4^+$	**0.79**	(0.43-1.15)	**1.90**	(1.39-2.41)	0.0014
	$CD8^+$	**0.52**	(0.27-0.77)	**1.00**	(0.67-1.33)	0.0144
	$CD19^+$	**0.94**	(0.52-1.36)	**0.98**	(0.65-1.31)	0.5870
	$CD16^+/56^+$	**0.37**	(0.08-0.66)	**0.20**	(0.11-0.30)	0.3717
18 y	Lymphocytes	**1.41**	(1.10-1.73)	**1.87**	(1.62-2.13)	0.0202
	$CD4^+$	**0.55**	(0.45-0.66)	**0.83**	(0.67-1.00)	0.0050
	$CD8^+$	**0.29**	(0.18-0.40)	**0.53**	(0.40-0.67)	0.0038
	$CD19^+$	**0.27**	(0.16-0.38)	**0.22**	(0.17-0.27)	0.3157
	$CD16^+/56^+$	**0.26**	(0.10-0.43)	**0.19**	(0.15-0.24)	0.9099

Table 3. Lymphocyte numbers in peripheral blood. Number of total lymphocytes, $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B-cells and $CD16^+/56^+$ NK cells analyzed pre-operatively, at 18 months of age (18 m) and 18-year follow-up (18 y) shown as mean cell number $x10^9$/L with 95% Confidence interval (CI) and p-value using either unpaired students t-test or Mann-Whitney test as appropriate.

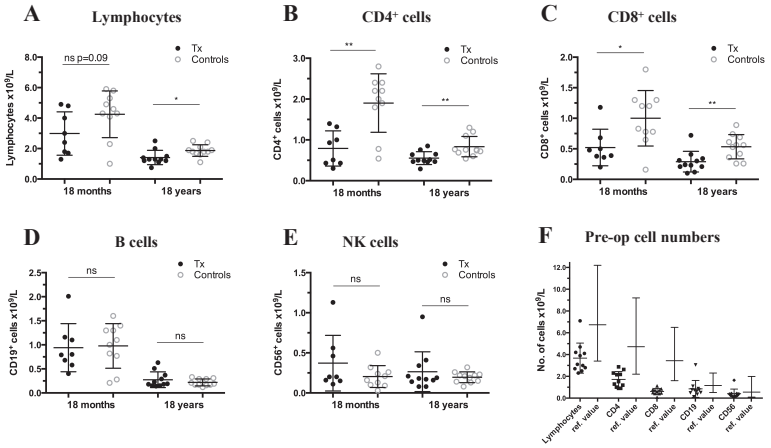

*Figure 10. Lymphocyte numbers and subsets in thymectomized patients and controls at 18 months and 18 years. **A-E**, Number of total lymphocytes, $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$ cells at 18 months and 18 years. **F**, Pre-operative absolute lymphocyte numbers in thymectomized patients, represented as individual values with mean and SD, reference values shown as bars indicating mean and 90% range from Schatorjé et al. (97)*

Before surgery the thymectomy group had decreased numbers of lymphocytes (Table 3, Figure 10), particularly T cells. Since no control group was recruited preoperatively the values were compared to previously published reference ranges (97-100). Figure 10 shows reference values adapted from Schatorjé et al. (97).

Naive and memory T cells

The number of naive helper T cells ($CD4^+CD45RA^+$) was lower in the thymectomized than in the controls at 18-year follow-up (0.15 vs. $0.49x10^9$/L, $p<0.0001$) whereas memory helper T cell ($CD4^+CD45RO^+$) number was unaffected (0.34 vs. $0.30x10^9$/L, p=0.48); thereby showing a proportional increase in memory T cell population (Figure 11, A-B). In cytotoxic T cells the same was true, absolute $CD8^+CD45RA^+$ naive T cell number was lower in cases (0.11 vs. $0.35x10^9$/L, p=0.0002) but the $CD8^+CD45RO^+$ memory T cell number was unaffected (0.11 vs. $0.13x10^9$/L, p=0.27) (Figure 11, C-D).

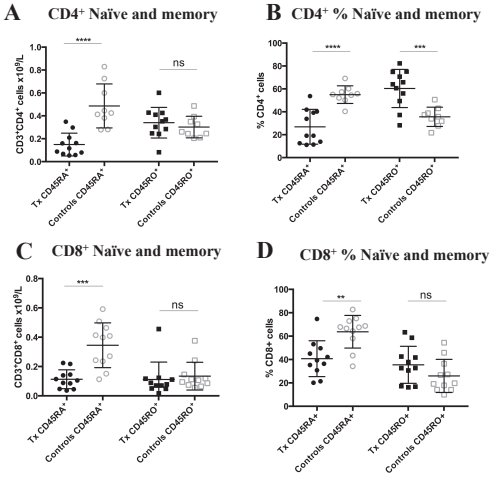

*Figure 11. Naive and memory T cells in thymectomized individuals and controls at 18 months and 18 years. **A**, Naive and memory CD4+ T cells at 18-year follow-up as absolute numbers and **B**, proportions. **C**, Naive and memory CD8+ T cells at 18-year follow-up as absolute numbers and **D**, proportions.*

Regulatory T cells

The total number of Tregs was lower in thymectomized compared to controls (0.035 vs. $0.053x10^9$/L, p=0.0417) whereas the proportions were unaffected (6.5% vs. 6.1%, p=0.54) (Figure 12, A-B). Further analysis of the Treg subset revealed that the thymectomized showed a decrease in naive Treg number but

not in the memory Treg subset, resulting in a higher percentage of memory Tregs in the thymectomy group (Figure 12, C-F). The Treg subset $CD4^{+}CD45RA^{-}CD25^{++}$ that has been shown to have the greatest suppressive potential (101) did not differ between the groups (Figure 12, G-H).

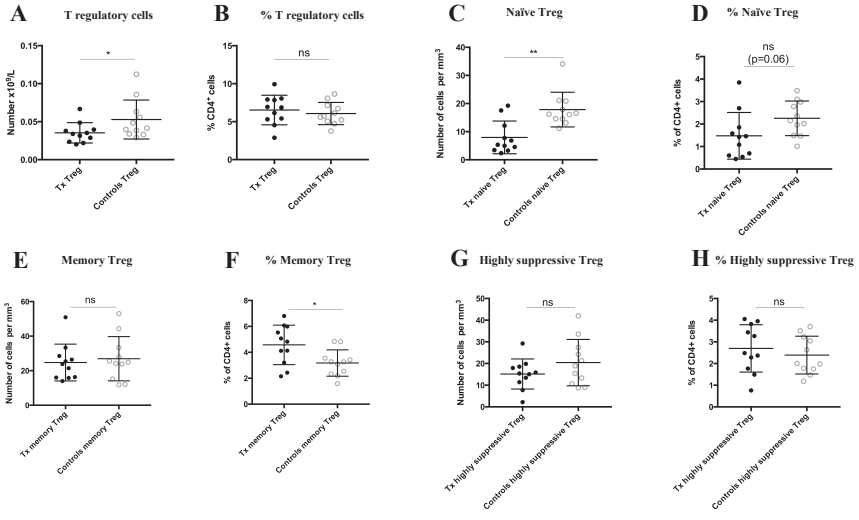

Figure 12. Regulatory T cells in thymectomized individuals and controls at 18-year follow-up. ***A****, absolute numbers and* ***B****, proportions.* ***C****, naive Treg ($CD4^{+}CD25^{+}CD127^{low}CD45RA^{+}$) cell number and* ***D****, proportion.* ***E****, memory Treg ($CD4^{+}CD25^{+}CD127^{low}CD45RO^{+}$) cell number and* ***F****, proportion.* ***G****, highly suppressive T regulatory ($CD4^{+}CD45RA^{-}CD25^{++}$) cell number and* ***H****, proportion. Results shown as individual values with mean and SD, p-value summary indicated on each graph. Tx = thymectomized.*

Recent thymic emigrants

The cell surface marker CD31 has been defined as a marker of recent thymic emigrants (RTEs) (25, 26, 59). Thymectomized individuals showed a lower proportion of recent thymic emigrants ($CD4^{+}CD31^{+}$), 55% versus 81% in the control group; p=0.034. Furthermore, the results were more wide-ranging in the thymectomy group, 25% to 89%, whereas the range for the control group was narrower, 76% to 90% (Figure 13, A).

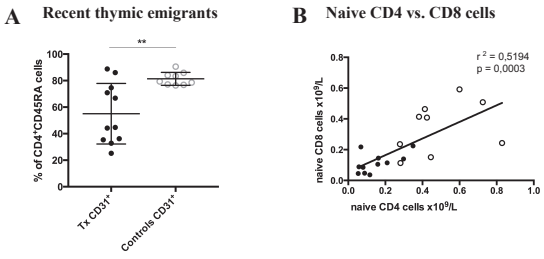

*Figure 13. **A**, CD31$^+$ recent thymic emigrants as a proportion of naive CD4$^+$ cells. Results shown as individual values with mean and SD, p-value summary indicated on graph. **B**, The correlation between naive CD4$^+$ and naive CD8$^+$ cells in thymectomized individuals and controls indicating r^2 and p value from linear regression analysis. Black circles = thymectomized, open circles = controls.*

There was a linear correlation between naive CD4$^+$ and CD8$^+$ cells, indicating that thymectomized individuals were similarly affected in both subsets (Figure 13, B). Furthermore, the figure emphasizes the lower numbers of both CD4$^+$ and CD8$^+$ naive T cells in thymectomized individuals (black circles).

4.1.4 TCR Vβ repertoire analysis

Analysis of TCR Vβ usage in CD4$^+$ and CD8$^+$ cells showed that thymectomized individuals had signs of oligoclonality that was particularly striking for the CD8$^+$ cells (Figure 14).

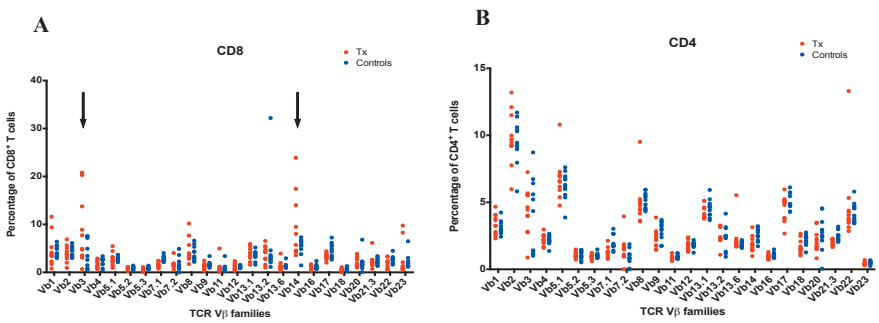

Figure 14. TCR Vβ chain usage in A, CD8$^+$ cells and B, CD4$^+$ cells presented as a scatter graph. Thymectomized (Tx) = red circles, controls = blue circles. Difference in usage of chains Vβ3 and Vβ14 in CD8$^+$ cells statistically significant, indicated by arrows (Holm-Sidak method for multiple t test comparison, α=0.05). Nomenclature according to Wei et al (102).

Perturbations in thymectomized individuals defined as a TCR Vβ chain usage deviating more than +/- 3SD from the mean value of the control group was noted in 8 out of 10 individuals regarding $CD8^+$ cells and in 6 out of 11 regarding $CD4^+$ cells whereas only a single chain in one control was affected (Table 4).

Tx	**$CD8^+$ Oligoclonality**	**Vβ chain**	**$CD4^+$ Oligoclonality**	**Vβ chain**
1	**yes**	Vβ21.3	no	
2	**yes**	Vβ2,3,5.1,5.3,12,14	**yes**	Vβ7.2
3	**yes**	Vβ3,5.1,12,14	no	
4	**yes**	Vβ13.2	no	
5	no		no	
6	**yes**	Vβ1,12,14,23	**yes**	Vβ1
7	**yes**	Vβ5.1	**yes**	Vβ13.6
8	**yes**	Vβ1,3,5.1,5.3,8,11,136,23	**yes**	Vβ8
9	no		no	
10	**yes**	Vβ1	**yes**	Vβ22
11	n.a.		**yes**	Vβ5.1,17
Controls	**$CD8^+$ Oligoclonality**	**Vβ chain**	**$CD4^+$ Oligoclonality**	**Vβ chain**
1	no		no	
2	n.a.		no	
3	no		no	
4	no		no	
5	no		no	
6	**yes**	Vβ13.2	no	
7	no		no	
8	no		no	
9	no		no	
10	no		no	
11	no		no	

Table 4. TCR Vβ usage in $CD8^+$ and $CD4^+$ cells in thymectomized and controls. Oligoclonality is defined as Vβ usage exceeding +/- 3SD from the mean of the controls. Nomenclature according to Wei et al.(102) N.a. = not analyzed.

4.1.5 TREC

TREC PCR analysis showed non-detectable values in ten out of eleven thymectomized individuals (Figure 15) whereas the controls had a wide distribution of TREC quantity. Surprisingly, no correlation was found

between number of TRECs and expression of CD31 in the naive T cell population in the healthy controls.

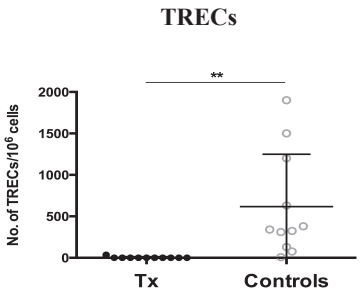

Figure 15. T cell receptor excision circles, number of TREC molecules per 10^6 cells. Data shown as individual values in thymectomized (black circles) and controls (open circles) with mean and SD, p-value summary indicated on graph. Tx=thymectomized.

4.1.6 Telomere length

The thymectomized individuals had shorter telomere lengths in their $CD8^+$ T cell populations compared to the controls, 2.66 vs. 3.32, p=0.036 (Figure 16) as measured by a telomere/single gene ratio according to a method originally described by Cawthon in 2002(79). For the $CD4^+$ T cell populations the numbers were 2.73 vs. 3.32, p=0.068 and thus the difference did not reach statistical significance. The telomere length in $CD19^+$ B cells was not different between the thymectomized and the controls (3.03 vs. 3.45, p=0.2335).

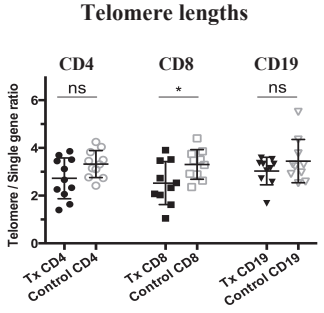

Figure 16. Telomere length analyses. Telomere/single gene ratio in $CD4^+$, $CD8^+$ T cells and $CD19^+$ B cells. Data shown as individual values with mean and SD, p-value summary indicated on graph. Tx=thymectomized.

4.2 Paper II

4.2.1 *TCRB* Clonality index

Coincidences tables (Figure 17) show re-occurrences of identical *TCRB* rearrangements analyzed in six different reactions from the same individual. Increased number of coincidences is indicative of increased clonality as they are presumed to originate from the same T cell clone.

A $CD4^+$ Coincidences table

	Coincidences						*Clonality Score*
Thymectomy	0	2	3	4	5	6	
1	10177	1327	94	28	8	6	2.84×10^{-5}
2	6106	853	109	29	7	9	5.47×10^{-5}
3	6873	1103	119	20	9	6	4.68×10^{-5}
4	9638	682	151	42	17	13	3.04×10^{-5}
5	10574	1304	111	17	6	4	2.60×10^{-5}
6	9232	994	106	34	9	2	3.03×10^{-5}
7	5558	239	35	7	9	12	4.00×10^{-5}
8	7364	1104	137	39	16	10	4.81×10^{-5}
9	10886	529	80	16	3		1.44×10^{-5}
10	8283	935	184	68	31	23	5.12×10^{-5}
11	6790	474	96	29	7	3	3.77×10^{-5}
Controls							
1	11008	425	41	8	3		1.10×10^{-5}
2	10019	1369	47	10	2	2	2.36×10^{-5}
3	10891	535	99	36	10	13	2.06×10^{-5}
4	10608	1526	170	33	12	8	3.12×10^{-5}
5	11905	533	103	20	7	4	1.46×10^{-5}
6	11405	579	83	14	5	4	1.49×10^{-5}
7	9386	477	76	17	7	5	2.00×10^{-5}
8	13181	1918	144	27	5	4	2.20×10^{-5}
9	8686	1391	126	20	9	3	3.45×10^{-5}
10	15847	1907	149	24	6		1.60×10^{-5}
11	11146	540	83	22	7	3	1.57×10^{-5}

B $CD8^+$ Coincidences table

	Coincidences						*Clonality Score*
Thymectomy	0	2	3	4	5	6	
1	7410	1074	130	43	32	44	$5{,}97 \times 10^{-5}$
2	1918	204	62	52	31	47	4.16×10^{-4}
3	2497	549	112	59	28	35	2.45×10^{-4}
4	4431	811	164	95	50	69	1.46×10^{-4}
5							
6							
7	4655	412	90	46	27	20	1.14×10^{-4}
8	2076	237	71	43	43	47	3.79×10^{-4}
9	9050	1119	124	46	19	17	$3{,}86 \times 10^{-5}$
10	4644	1161	312	134	80	80	1.38×10^{-4}
11	2799	477	97	40	27	17	1.94×10^{-4}
Controls							
1	10049	714	150	47	21	23	$3{,}16 \times 10^{-5}$
2	4462	272	71	38	41	44	1.26×10^{-4}
3	7032	837	146	85	29	40	$6{,}86 \times 10^{-5}$
4	3213	379	62	15	16	14	1.30×10^{-4}
5	6746	629	208	123	56	81	$9{,}28 \times 10^{-5}$
6	9171	1644	314	118	43	43	$5{,}19 \times 10^{-5}$
7	8165	1129	116	69	39	27	$5{,}10 \times 10^{-5}$
8	7740	1268	166	50	41	22	$5{,}35 \times 10^{-5}$
9	9180	453	77	29	19	26	$3{,}04 \times 10^{-5}$
10	3630	586	48	18	6	7	$9{,}62 \times 10^{-5}$
11	8064	1330	128	30	12	20	$4{,}44 \times 10^{-5}$

*Figure 17. Coincidences tables for **A**, $CD4^+$, and **B**, $CD8^+$ T cells as described by Boyd et al(89) where increased sequence recurrences indicate increased clonality. Clonality score results in far-right column.*

The clonality index was increased in thymectomized individuals compared to healthy controls in the $CD4^+$ (Thymectomized = $3.71x10^{-5}$, controls = $2.04x10^{-5}$) and even more so in the $CD8^+$ T cell subset (Thymectomized = $1.92x10^{-4}$, controls = $7.06x10^{-5}$) (Figure 18, A and B, respectively)

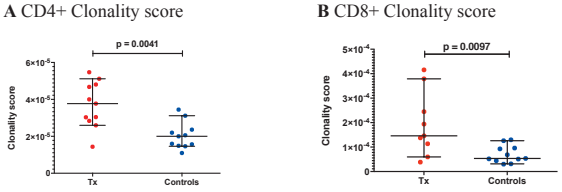

*Figure 18. The Clonality score for **A**, $CD4^+$ and **B**, $CD8^+$ T cells, Tx=thymectomized.*

There was a negative correlation between the number of both $CD4^+$ and $CD8^+$ T cells in peripheral blood and the clonality index; T cell lymphopenia was associated with increased clonality index (Figure 19, A and B). Separate analyses revealed that the oligoclonality only correlated with the number of naive T cells, and not with the number of memory T cells (Figure 19, C-D and E-F, respectively).

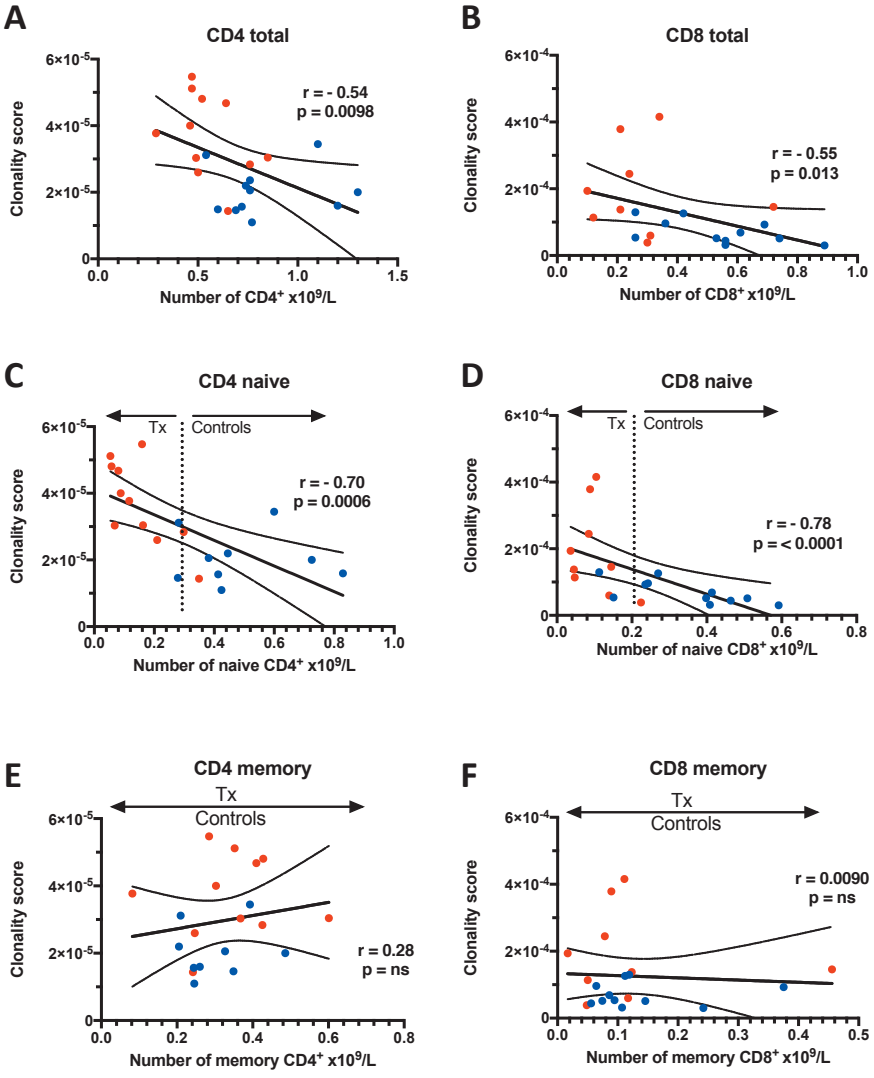

*Figure 19. Linear regression analysis showing the correlation between TCRB clonality score and T cell numbers. **A**, Clonality score vs number of total CD4$^+$ cells. **B**, Clonality score vs number of total CD8$^+$ cells. **C**, Clonality score vs number of naive CD4$^+$ cells (CD4$^+$CD45RA$^+$) and **D**, Clonality score vs number of naive CD8$^+$ cells (CD8$^+$CD45RA$^+$); dotted vertical line and arrow on top of graph indicates clear distinction between thymectomized and controls. **E**, Clonality score vs number of memory CD4$^+$ cells (CD4$^+$CD45RO$^+$) and **F**, Clonality score vs number of memory CD8$^+$ cells (CD8$^+$CD45RO$^+$); double headed arrow on top of graph indicates lack of distinction between thymectomized and controls. Red dots thymectomized individuals = Tx, blue dots controls; individual results, linear regression with 95% confidence interval, the Spearman correlation coefficient, r, and p value indicated on graphs.*

4.2.2 Qualitative aspects of the *TCRB*

Results for qualitative differences in the *TCRB* are presented in Figure 20. For $CD4^+$ T cells no significant changes were found in the number of junctional deletions, N- or P- nucleotides, CDR3 lengths or amino acid composition. For $CD8^+$ T cells only minor changes were detected in the thymectomized with a slight increase in the number of junctional deletions in productive rearrangements, but no difference was detected in N- and P-nucleotides, and no effect was found on CDR3 lengths or amino acid composition. The VB-JB recombination usage was analyzed, and $CD8^+$ T cells from thymectomized individuals showed dominant VB-JB combinations (Figure 20, H), whereas more subtle differences were observed for their $CD4^+$ T cells (Figure 20, D). Results shown are Circos plots, in which the band width is proportional to the usage frequency. The different VB regions are marked, and their combination with the different JB regions is shown. Examples in Figure 20 are from one thymectomized individual and one control. Complete results for VB-JB, as well as VB-DB and DB-JB recombinations for all subjects are presented in Paper II, supplementary material.

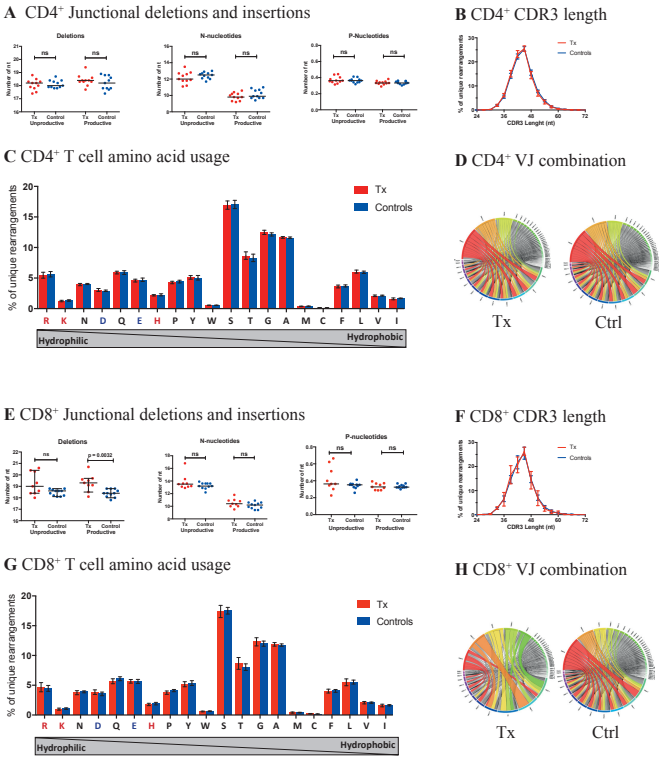

Figure 20. TCRB junction characteristics. A-D CD4$^+$ T cells, E-H CD8$^+$ T cells. ***A****, junctional deletions, N- and P-nucleotides (average number, individual values with median, p value),* ***B****, CDR3 length distribution (group values, mean with SEM, nt = nucleotides),* ***C****, amino acid usage in CDR3,* ***D****, Frequency of TCRB V-J chain usage (representative results from a single thymectomized individual, and a single control). Results shown as circos plots where the width of the band is proportional to the frequency. The different V regions are marked, and their combination with the different J regions shown. Corresponding results for CD8$^+$ T cells in* ***E****,* ***F****,* ***G*** *and* ***H****. (ns = not significant; Tx= thymectomized, red dots and line thymectomized; blue dots and line controls; positively charged amino acids indicated in red and negatively charged in blue).*

4.2.3 Results of *IGH* B cell analysis

The CD19$^+$ B cell *IGH* rearrangements were correspondingly analyzed but no differences were detected between thymectomized and controls. Complete results are presented as supplementary figure in Paper II.

4.3 Paper III

4.3.1 Participant characteristics

There was a predominance of boys in the thymectomy group, 55%, whereas the opposite was true for the surgery control group with 44% boys (Paper III, supplementary material). Age at surgery was similar between the thymectomy and the surgery control groups.

4.3.2 Degree of thymectomy

The degree of thymectomy was estimated in 170 consecutive surgeries and the results are presented in Paper III, supplementary material. Individuals thymectomized before 1 year of age were more likely to have had a total or near-total thymectomy. Overall the degree of thymectomy was ≥90% in 77 out of the 170 surgeries (46%) and ≤50% in only six (3%).

4.3.3 Thymectomy compared to surgery controls

Incidence rates and hazard ratios

Incidence rates (IR) per 10,000 person-years (95% CI) including number of study subjects, events and sum of follow-up years for the thymectomy group as well as both controls groups are presented in Paper III, table 1. Hazard ratios are presented as tables in supplementary material in Paper III, as well as in forest plots below (Figure 21).

During follow-up in total 141 thymectomized individuals (IR, 19.7, per 10,000 person-years; 95% CI, 16.5-23.0) and 42 surgery controls (IR, 13.9; CI, 9.7-18.1) developed any kind of autoimmune disease (aHR, 1.57; 95% CI, 1.11-2.22). Type 1 diabetes was diagnosed in 27 thymectomized individuals (IR, 3.7; 95% CI, 2.3-5.1) and in four surgery controls (IR, 1.3; CI, 0-2.6) which gave aHR 3.16 with 95% CI 1.08-9.21. Hypothyroidism was diagnosed in 32 thymectomized individuals (IR, 4.4; 95% CI, 2.9-5.9) and five surgery controls (IR, 1.6; 95% CI, 0.2-3.1)(aHR, 3.03; 95% CI, 1.17-7.83). The aHR for the risk of hematologic cancer was 3.74 when compared to the surgery control group although with a very wide confidence interval, 95% CI 0.48-29.44. The aHR for all cancer risk was 1.07 (95% CI, 0.52-2.19), and for solid cancer 0.84 (95% CI, 0.38-1.86). Infections were diagnosed at 3573 separate occasions in the thymectomized (IR, 490; 95% CI, 474-506) and at 1042 for the surgery controls (IR, 339; 95% CI, 318-360) (aHR, 1.42; 95% CI, 1.33-1.52) with similarly increased aHR 1.40 and 1.26 for viral and bacterial infections respectively. Atopic diseases were diagnosed in 411 thymectomized individuals (IR, 60.1; 95% CI, 54.3-65.9) and 218

surgery controls (IR, 77.0; 95% CI, 66.8-87.2) (aHR, 0.73; 95% CI, 0.62-0.86). Asthma was diagnosed in 326 thymectomized (IR, 47.1; 95% CI, 42.0-52.2) and 181 surgery controls (IR, 63.1; 95% CI, 53.9-72.3) (aHR, 0.69; 95% CI, 0.58-0.83).

Hazard ratios are presented as forest plots in Figure 21.

	N events (%)		Adjusted Hazard Ratio (HR) (95%CI)		
	Thymectomy	Surgery controls		HR (95%CI)	P-value
Autoimmune	141 (2.5%)	42 (1.9%)		1.57 (1.11-2.22)	**0.01**
DM1	27 (0.48%)	4 (0.18%)		3.16 (1.08-9.21)	**0.04**
Hypothyroidism	32 (0.57%)	5 (0.22%)		3.03 (1.17-7.83)	**0.02**
Hyperthyroidism	5 (0.09%)	1 (0.04%)		3.05 (0.35-26.35)	0.31
JIA	17 (0.30%)	8 (0.35%)		0.96 (0.41-2.25)	0.93
Rheumatic diseases	11 (0.19%)	5 (0.22%)		1.08 (0.37-3.14)	0.89
Inflammatory Bowel Disease	7 (0.12%)	5 (0.22%)		0.68 (0.21-2.18)	0.51
Psoriasis	12 (0.21%)	4 (0.18%)		1.59 (0.51-4.98)	0.43
Celiac Disease	42 (0.75%)	16 (0.71%)		1.17 (0.66-2.10)	0.59
Cancer†	26 (0.46%)	11 (0.48%)		1.07 (0.52-2.19)	0.85
Hematologic	10 (0.18%)	1 (0.04%)		3.74 (0.48-29.44)	0.21
Solid	17 (0.30%)	10 (0.44%)		0.84 (0.38-1.86)	0.66
Infections**	3573 (63.1%)	1042 (45.8%)		1.42 (1.33-1.52)	**<0.001**
Pneumonia	291 (5.1%)	107 (4.7%)		1.10 (0.88-1.37)	0.41
According to cause					
Viral	3370 (59.5%)	990 (43.5%)		1.40 (1.30-1.50)	**<0.001**
Bacterial	1024 (18.1%)	337 (14.8%)		1.26 (1.11-1.43)	**<0.001**
Fungal/protozoan	169 (3.0%)	60 (2.6%)		1.25 (0.93-1.69)	0.14
Atopic	411 (7.6%)	218 (10.1%)		0.73 (0.62-0.86)	**<0.001**
Asthma	326 (5.9%)	181 (8.3%)		0.69 (0.58-0.83)	**<0.001**
Rhinitis	57 (1.0%)	28 (1.2%)		0.79 (0.50-1.26)	0.33
Eczema	77 (1.4%)	33 (1.5%)		0.96 (0.64-1.44)	0.84

*Figure 21. Hazard ratios - thymectomized compared to surgery controls. Sex- and age-adjusted hazard ratios with 95% confidence intervals. †Only one health care visit listing cancer required, **Infections were analyzed as recurrent events using 1 month's grace period; JIA = juvenile idiopathic arthritis; DM1 = type 1 diabetes mellitus.*

In summary, increased risks were detected for overall autoimmune diseases, type 1 diabetes and hypothyroidism. The risk of cancer was not significantly different. The risks of infections were increased, both for viral and bacterial infections. The risk of asthma was reduced in the thymectomy group.

4.3.4 Thymectomy compared to general population

Incidence rates and hazard ratios

Incidence rates are presented in Paper III, table 1; and hazard ratios as supplementary material, as for the surgery control group.

During follow-up 141 thymectomized individuals (IR, 19.7; 95% CI, 16.5-23.0) and 1045 controls (IR, 12.3; 95% CI, 11.6-13.1) were diagnosed with any autoimmune disease (aHR, 1.64; 95% CI 1.38-1.96). Significant increase in aHR for specific diagnoses was found for hypothyroidism (aHR, 4.94; 95% CI, 3.27-7.46), juvenile idiopathic arthritis (aHR, 1.85; 95% CI, 1.11-3.09), rheumatic diseases (aHR, 1.89; 95% CI, 1.00-3.57), and Celiac disease (aHR, 1.96; 95% CI, 1.42-2.72). In the thymectomy group 26 individuals were diagnosed with any kind of cancer (IR, 3.6; 95% CI, 2.2-4.9) compared to 199 in the general population (IR, 2.3; 95% CI 2.0-2.7) giving an increased aHR of 1.61 with 95% CI 1.07-2.43. When analyzed separately the aHR was not significantly increased; 1.88 (95% CI, 0.96-3.66) and 1.48 (95% CI, 0.90-2.45) for hematologic and solid cancers, respectively. Infections were diagnosed at 3573 separate occasions in the thymectomized (IR, 490; 95% CI, 474-506) and at 13,094 occasions for the general population controls (IR, 153; 95% CI, 151-156) (aHR, 3.18; 95% CI, 3.07-3.30) with similarly increased aHR 3.45 and 3.63 for viral and bacterial infections respectively. Atopic diseases were diagnosed in 411 thymectomized individuals (IR, 60.1; 95% CI, 54.3-65.9) and in 3063 individuals of the general population controls (IR, 36.9; 95% CI, 35.6-38.3) (aHR, 1.62; 95% CI, 1.46-1.79). Significant results were found for Asthma with an aHR of 1.84 with 95% CI 1.64-2.07.

Hazard ratios are presented as forest plots in Figure 22.

	N events (%)		Adjusted Hazard Ratio (HR) (95%CI)		
	Thymectomy	General population controls		HR (95%CI)	P-value
Autoimmune	141 (2.5%)	1045 (1.9%)		1.64 (1.38-1.96)	**<0.001**
DM1	27 (0.48%)	281 (0.50%)		1.13 (0.76-1.67)	0.55
Hypothyroidism	32 (0.57%)	78 (0.14%)		4.94 (3.27-7.46)	**<0.001**
Hyperthyroidism	5 (0.09%)	31 (0.05%)		2.04 (0.79-5.26)	0.14
JIA	17 (0.30%)	106 (0.19%)		1.85 (1.11-3.09)	**0.02**
Rheumatic diseases	11 (0.19%)	72 (0.13%)		1.89 (1.00-3.57)	**0.05**
Inflammatory Bowel Disease	7 (0.12%)	166 (0.29%)		0.52 (0.25-1.12)	0.09
Psoriasis	12 (0.21%)	101 (0.18%)		1.51 (0.83-2.75)	0.18
Celiac Disease	42 (0.75%)	250 (0.44%)		1.96 (1.42-2.72)	**<0.001**
Cancer†	26 (0.46%)	199 (0.35%)		1.61 (1.07-2.43)	**0.02**
Hematologic	10 (0.18%)	64 (0.11%)		1.88 (0.96-3.66)	0.06
Solid	17 (0.30%)	143 (0.25%)		1.48 (0.90-2.45)	0.13
Infections**	3573 (63.1%)	13 094 (23.1%)		3.18 (3.07-3.30)	**<0.001**
Pneumonia	291 (5.1%)	568 (1.0%)		5.88 (5.10-6.77)	**<0.001**
According to cause					
Viral	3370 (59.5%)	11 287 (19.9%)		3.45 (3.32-3.59)	**<0.001**
Bacterial	1024 (18.1%)	3217 (5.7%)		3.63 (3.38-3.90)	**<0.001**
Fungal/protozoan	169 (3.0%)	1009 (1.8%)		2.06 (1.75-2.42)	**<0.001**
Atopic	411 (7.6%)	3063 (5.5%)		1.62 (1.46-1.79)	**<0.001**
Asthma	326 (5.9%)	2116 (3.8%)		1.84 (1.64-2.07)	**<0.001**
Rhinitis	57 (1.0%)	771 (1.4%)		0.88 (0.67-1.15)	0.34
Eczema	77 (1.4%)	782 (1.4%)		1.15 (0.91-1.45)	0.24

*Figure 22. Hazard ratios - thymectomized compared to general population controls. Sex- and age-adjusted hazard ratios with 95% confidence intervals. †Only one health care visit listing cancer required, **Infections were analyzed as recurrent events using 1 month's grace period; JIA = juvenile idiopathic arthritis; DM1 = type 1 diabetes mellitus.*

In summary, increased risks were detected for overall autoimmune diseases, hypothyroidism, juvenile idiopathic arthritis, rheumatic diseases and celiac disease. The risk of any kind of cancer was increased, but when analyzed separately for hematologic and solid forms of cancer the aHR was not significantly increased. The risks of all types of infections were increased. The risk of asthma was increased.

5 DISCUSSION

5.1 Paper I.

Naive and memory T cells

This study on immunologic effects of early childhood thymectomy showed a quantitative defect in the T cell compartment at both 18 months and 18-year follow-up, with both a relatively early pronounced difference but also a long-lasting effect as observed at 18-year follow-up. The analyzed subsets showed a prominent increase in proportions of memory versus naive T cells consistent with previously published results (39, 40, 45, 46). The results also showed a substantial decrease in the absolute numbers of naive T cells, which indicates a defective production and/or maintenance of this subset in thymectomized individuals. In contrast, the memory T cells maintained normal numbers, which is most likely due to peripheral proliferation of the seeded clones.

Lymphopenia and congenital heart defect

The preoperative lymphocyte numbers were lower than previously published reference values for healthy infants (97-100). Increased incidence of lymphopenia has been described in infants with congenital heart defects, and in a study by Cabrera et al. 90 out of a total of 280 infants had preoperative lymphocyte counts below $3.0x10^9$/L (103) which is in concordance with our results.

Recent thymic emigrants

In Paper I, recent thymic emigrants were defined as $CD45RA^+CD31^+$ cells as first described by Kimmig et al. in 2002 (25). The data from our control group showed that approximately half of the T helper cells are $CD45RA^+$, and of these approximately 80% co-expressed CD31 which is in line with results from previous publications (25, 60, 97). Lower proportions of CD31 co-expression has been reported in older persons but generally with a large individual variation; 10-80% (104). The co-expression of CD31 in our healthy control group showed only a small individual variation, with a mean of 81%, ranging from 76 to 90% whereas in the thymectomy group the mean was much lower, 55% and the variation much larger, or 25 to 89%, similar to the results found in older individuals (104). The implication of these results is unclear, but since all but one thymectomized individuals also had no detectable TRECs this indicates a severely affected production of recently generated T cells. A possible residual thymic function therefore seems an unlikely explanation for the observation that some thymectomized

individuals have a normal amount of $CD31^+$ cells. Also, there is some evidence indicating that $CD45RA^+$ cells can retain their CD31 expression in spite of peripheral proliferation (60).

T cell receptor excision circles

Most previous publications have shown a decreased number of TRECs in thymectomized individuals (40, 42, 45, 49, 58) which was also seen in Paper I. Even subtotal thymectomy seems to result in lower TRECs for at least one year after surgery (105). On the other hand, van Gent et al. (50) noticed an initial decrease in TRECs, lasting at least 5 years after surgery, but later in life a normalization of TREC numbers was observed. This effect was sometimes seen decades after the thymectomy and a regrowth of thymic tissue was postulated as an explanation to this phenomenon, which was supported by MRI findings in most patients. In our study, no data was collected on possible remaining thymic tissue but all patients underwent an early (<6m of age) near-total (>90% assessed by surgeon) thymectomy. An undetectable TREC level in 10 out of 11 indicates a severely affected thymic function even 18 years after surgery. Both Halnon et al. (40) and Ogle et al. (58) have shown a substantial decrease in TREC numbers, and in 6/11 and 10/20 subjects, respectively, the TREC amount was below the PCR analysis detection limit. Residual thymic tissue was confirmed visually during re-operation in one study (40), and in those cases TREC numbers were not as low as in the group without any residual thymus. Taken together, these results indicate that sparing a part of thymic tissue during surgery could be important and presumably could lessen the immunological impact of early childhood thymectomy.

Regulatory T cells

In mice, thymectomy before day 3 after birth has long been known to have severe consequences such as reduced body weight and wasting, organ specific autoimmune diseases, fewer recirculating T cells and impaired T cell immunity whereas later thymectomy, after day 7, does not have such severe consequences (106, 107). Although the precise mechanism of this effect is unclear, the severe autoimmune manifestations of early childhood thymectomy are presumably a consequence of impaired Treg development (108, 109). A few studies have addressed the impact of early childhood thymectomy on Tregs in humans. Eysteinsdottir et al. (44) showed a preserved normal proportion of $CD4^+CD25^+$ cells in thymectomized individuals, and no difference in their expression of CD127 and Foxp3 transcription factor. Halnon et al. (49) reported similar results with retained cell numbers and proportions of Tregs defined as $CD4^+CD25^{hi}FOXP3^+$, but not surprisingly with a decrease in the naive Treg subset, i.e. those co-

expressing CD45RA. A report by Schadenberg et al. (52) shows, on the other hand, lower absolute numbers but an increased proportion of $CD4^+FOXP3^+$ after thymectomy. They also saw a significantly lower percentage of $FOXP3^+$ cells co-expressing CD31, a marker of RTEs. Our results are in agreement with the latest report, with a decreased number of total Tregs but an unaffected proportion. We did not analyze CD31 on Tregs, but on naive $CD4^+CD45RA^+$ T cells, CD31 expression was decreased in thymectomized individuals. Thymectomy thus seems to affect Tregs leading to lower Treg numbers, but the unaffected Treg proportion of $CD4^+$ cells may indicate that they are relatively spared after thymectomy.

Telomere length

Leukocyte telomere length is dependent upon a number of factors such as initial inherited length, replicative history, telomerase activity and other internal cellular factors. It can be used as a marker of replicative potential, and a shorter leukocyte telomere length has been coupled to a number of aging-related diseases (74). The results from Paper I showed a shorter telomere length in $CD8^+$ cells in thymectomized individuals. Telomere lengths of $CD4^+$ cells were shorter in the thymectomized compared with the controls, but the difference was not statistically significant, $p=0.067$. We find this lack of significance most likely due to our small sample size, as telomere lengths of $CD4^+$ and $CD8^+$ cells from the same individual have been shown to correlate with each other(74). No difference between groups was detected in B cells, which is not surprising since thymectomy should primarily affect the T cell population. Shorter telomere length in $CD8^+$ cells implies a decreased replicative potential.

T cell receptor variable β chain

The results indicated perturbations in the TCR Vβ usage with signs of oligoclonality. This is in line with published results from other studies(42, 44, 46, 54, 57). Interestingly, the perturbations were more pronounced in the $CD8^+$ T cell subset, a finding shared with Sauce et al.(46). Although the majority of the previous publications used spectratyping, a PCR based method analyzing the frequency of defined homologous TCR families rather than flow cytometry, the results are comparable. The analysis of the TCR Vβ utilization with flow cytometry allows differentiation between so-called families of different β-chains (sharing at least 75% nucleotide homology with each other) where perturbations become evident in case of a clonal expansion, or possibly due to clonal deletion (110). The method is crude, and primarily detects clonal expansions. If gaps in the repertoire are evenly distributed among the different TCR Vβ families the method will fail to detect them, as this would not change the proportions between families in any given individual. This limitation leads to the conclusion that a detection of

relatively modest variations using TCR Vβ analysis does not exclude a severely affected immune repertoire.

Clinical data

Knowledge about possible long-term clinical consequences of thymectomy is sparse. Many studies have included clinical data but the studies are few, often the follow-up time is short, or very variable, and the patient cohorts are small. In our study the thymectomy group reported more frequent otitis media infections, pneumonia, upper respiratory infections and more severe infections necessitating hospital admission. The interpretation of these findings is complicated by their underlying cardiac problem. Only one individual in each group reported an autoimmune disease but allergies were reported more frequently in thymectomized individuals. Due to the small number of participants no conclusions can be drawn from these results other than stating that the lack of information about possible clinical consequences of early childhood thymectomy needs to be addressed in an adequately powered study.

Summary

In summary, early childhood thymectomy leads to distinct immunological aberrances. There was a decrease in the production of new T cells, evident as low naive T cell numbers and a near absence of TRECs. A peripheral T cell expansion seemed to occur concomitantly with a relative increase of memory T cells with diminished replicative potential and signs of T cell receptor oligoclonality. The immunological changes seen in these young adults at 18-years follow-up are similar to characteristics of the immune system of aged individuals (32) and could increase their risk of prematurely acquiring diseases related to altered immune function seen in the elderly, such as infections, autoimmune diseases and malignancies.

5.2 Paper II.

The results of the study revealed an impact of early childhood thymectomy on the diversity of the T cell immune repertoire with an estimated clonality index 1.8 times higher in $CD4^+$ T cells and 2.7 times higher in $CD8^+$ T cells. These results were in line with the results from Paper I, and earlier studies analyzing diversity with other methods(42, 44, 46, 53, 54). Interestingly, the effect seems to be more profound in the cytotoxic T cell subset, a finding shared by Sauce et al.(46). We agree that this could be due to different proliferation responses after antigen stimulation, where stimulated $CD8^+$ T cells accumulate in the periphery in higher numbers than $CD4^+$ cells, thereby

skewing the immune repertoire. In fact, even in healthy individuals, clonal expansion seems to introduce larger repertoire biases in $CD8^+$ T cells than $CD4^+$ T cells(111). Thymectomized individuals showed a larger variation in the clonality index compared to the healthy controls. The effects of thymectomy on the TCR repertoire, the amount of TRECs and naive T cell production have been shown to be augmented by chronic cytomegalovirus (CMV) infection(46, 54). It would have been interesting to determine the CMV status of the study participants, as it could be an important modifying factor, where a chronic CMV infection would probably increase the clonality index of the $CD8^+$ T cell subset.

Qualitative differences between thymectomized individuals and controls in the *TCRB* were minor, with only a slight but statistically significant increase in the number of junctional deletions in productive rearrangements in $CD8^+$ T cells. This finding can perhaps be explained by the observed increased oligoclonality, if by chance the expanded clones carried a higher number of junctional deletions.

The negative correlation observed between the number of both $CD4^+$ and $CD8^+$ T cells and the clonality index is explained only by naive T cell numbers; no correlation exists between number of memory T cells and clonality index. Thus, individuals with a decreased thymic output, and fewer naive T cells, have less repertoire diversity than those with many naive T cells, independent of a possible compensatory peripheral proliferation. The loss of diversity can indeed also occur with abundant peripheral proliferation, but according to these results the naive T cell number seems to be a stronger determinant of diversity.

The clinical impact of the decreased repertoire observed after thymectomy remains unknown. However, a decreased overall capability to recognize foreign or malignantly transformed epitopes can presumably affect certain disease risks, such as infections and cancer.

5.3 Paper III.

The extent of thymectomy

Neither verification of thymectomy, nor information on the extent of thymectomy, can be gathered through the clinical registers used in this study. To estimate the frequency and degree of thymectomy, additional surgical information from Gothenburg was analyzed. The validation analysis indicated that nearly half of the study group has experienced a total or near-

total thymectomy and that 50% or more of the thymus has been removed in nearly all cases. The obvious conclusion is that the thymectomy group includes individuals that have only had a part of their thymus removed. These individuals are actually quite interesting. First, they can be regarded as an ideal control group, should complete information be available on an individual basis. Second, the remaining thymic tissue probably lessens the impact of thymectomy(51). If so, the inclusion of these individuals leads to an underestimation of the effect of thymectomy in this study.

The timing of thymectomy

In the study children thymectomized before the age of five were included. This age range is rather wide. A narrower age criteria would perhaps have been more appropriate as age at thymectomy has been shown to affect the immune system differentially. Halnon et al. showed that thymectomized individuals who underwent surgery before the age of two years both had a lower number of TRECs and less naive regulatory T cells than those exposed to surgery after two years of age, indicating that earlier surgery had a greater effect on thymic output(49). To the contrary more recent reports indicate somewhat diminished effect of thymectomy very early in life. Zlamy et al. showed a more prominent reduction of $CD8^{+}CD31^{+}$ naive T cells in those thymectomized at 1-2 years of age compared to those before one year of age(54). Also, in a study by Silva et al., eight thymectomized individuals with the lowest sjTREC levels were all thymectomized after 12 months of age(57). There is evidence of a regenerative capability of the neonatal thymus; and van Gent et al. have speculated that very early thymectomy may ultimately have less immunological impact as opposed to thymectomy at an older age(50). A modifying effect of the timing of thymectomy is plausible, but due to considerations on the power of the study, this relatively wide age range was determined.

The selection of a control group

The definition of a representative control group was challenging. The risks attributed to thymectomy can be regarded as superimposed on risks related to an underlying cardiac defect. The comparison to the surgery control group is perhaps less likely to be confounded than comparison to the general population control group. The use of the surgery control group was an attempt to increase comparability with the thymectomy group, through the selection of individuals who share the basic feature of a congenital cardiac defect. However, important differences remain. The types of the underlying cardiac defects differ and with it inherent differences, known and unknown, between the two groups. Furthermore, the extent of the surgical interventions differs, and with it for example the rate of complications, such as infections.

Matching in case-control studies is difficult and sometimes unintentionally creates biases(112). Although such matching generally should be avoided some exceptions exist, such as in our case, where it is impossible to control for the presence of a congenital cardiac defect in addition to thymectomy without the selection of a control group. The final decision was to analyze the data using both the surgery control group, as well as a general population group.

Comparing with the surgery controls

Increased risks were observed for autoimmune diseases (hypothyroidism, diabetes) and infections. Immunologic effects of early childhood thymectomy could hypothetically constitute one of many components in the disease mechanisms, but of course other possibilities still remain, related to other differences between the thymectomy groups, and the surgery control group.

The only adjustments in risk calculations were for age, and sex. The thymectomy group had fewer females, whereas the surgery control group had more. The cause is probably the known gender differences in the prevalence of the various congenital heart defects(113). For example, surgeries due to patent ductus arteriosus used to define individuals in the surgery control group, is twice as common in girls.

Decreased risks were found for asthma in the thymectomy group. Again, thymectomy is a possible causal component, but we also speculate that this could be due to differences in the underlying cardiac disease. Cardiac asthma is presumed to be caused by a pulmonary congestion rather than by an atopic immunologic mechanism(114). Congenital heart defects certainly differ in their tendency to induce cardiac asthma, thereby differentially affecting asthma incidence.

The aHR for hematologic cancer was increased to 3.74 but the 95% CI was wide, 0.48-29.4, and therefore not statistically significant. Nonetheless this increase is alarming and needs further investigation. Thymectomy might affect the risk of hematologic malignancy through at least two different mechanisms. First, an increase in peripheral T cell proliferation induced by the reduced thymic output, could increase the overall chance of a malignant transformation(115, 116). Second, as the immune system is important during carcinogenesis(117), the reduced TCR diversity might decrease the ability of the immune system to effectively recognize, and eliminate, malignantly transformed cells.

Comparing with the general population

Compared with the general population the thymectomy group had an increased risk for hypothyroidism, a finding also seen for the surgery control group. In addition, increased risks were observed for juvenile idiopathic arthritis, rheumatic diseases and celiac disease. The increased risk for type 1 diabetes in the surgery control group was not repeated, but could be due to the limited number of outcome events, where only four patients in the surgery control group developed type 1 diabetes, compared to 27 in the thymectomy group. In general, the thymectomy group has a significant increased risk of developing autoimmune disease compared with the general population.

The overall risk of cancer was significantly increased, but when hematologic and solid forms of cancer were analyzed separately the difference was no longer significant, presumably due to the small number of events in the thymectomy group. As discussed previously this finding is both interesting and alarming, and merits further investigation.

As expected, the risks of infections were increased even more compared with the general population than compared with the surgery control group. Presumably, this is in part due to the lack of underlying cardiac condition in the general population control group. The risk of asthma was increased, opposite to the surgery control group comparison. This could be due to the underlying cardiac condition, an immunologic effect, or a combination of these.

The follow-up time period

The oldest individuals included in this study were born in 1973, and were therefore only 37 years of age at the end of follow-up. In addition, the older individuals were fewer due to a lower number of pediatric cardiac surgeries during the earlier study period. The youngest individuals had only a very short follow-up time, only one year. A stratification of the data by chronological age, or age at thymectomy, would have been informative, but unfortunately impossible due to loss of power with too few individuals in each category.

While the importance of a functional thymus during fetal development is unquestioned, the role of the human thymus in postnatal life is more debated. As thymic output in healthy individuals continues to decrease over the decades of human life-span(118), the effects of early childhood thymectomy perhaps do not become overt until later in life when peripheral T cell proliferation of the already seeded clones has become exhausted and the increasingly limited T cell receptor repertoire passes the threshold of causing clinical consequences. Therefore, a longer follow-up time would be valuable to detect any long-term effects of thymectomy.

6 CONCLUSION

For the past 40 years, major advancements in pediatric cardiac surgery have taken place, allowing long-term survival of children with serious congenital heart malformations that have been exposed to thymectomy during early life-saving surgery. Our research confirms that thymectomy early in life has a long-lasting immunologic impact. As presented in Paper I, early childhood thymectomy led to T cell lymphopenia, especially in the naive T cell compartment where a peripheral T cell expansion seemed to occur concomitantly with a relative increase of memory T cells. The T cells showed signs of a decreased replicative potential, a restricted T cell repertoire with signs of oligoclonality, and evidence of a severely affected thymic output. The effect on the T cell repertoire diversity was further confirmed in Paper II, where the diversity was clearly decreased for both helper, and especially prominent in cytotoxic T cells. In general, the results from Papers I and II are indicative of a persistent and severe immunologic dysfunction even 18 years after thymectomy with changes reminiscent of those seen in aged individuals(32). Theoretically, this could increase their risk of prematurely acquiring diseases related to altered immune function seen in the elderly, such as infections, autoimmune diseases and malignancies. The clinical importance is evident from the results in Paper III where we detect elevated risks for certain autoimmune diseases, cancer and infections following early childhood thymectomy. Although a causal effect has not been proven, taken together our results are supportive of a conservative approach in pediatric cardiac surgery, avoiding total thymectomy, if possible.

7 FUTURE PERSPECTIVES

Individuals that have been thymectomized early in life continue to grow in numbers, as well as in age. The results presented in this thesis reveal immunologic aberrations and increased disease risks are observed. However, the results do not allow a causal conclusion to be drawn regarding clinical consequences. To be able to provide good and evidence based clinical care from the neonatal period, including the early life-saving cardiac surgery, until adult, and even old, age the possible clinical consequences of thymectomy need to be determined.

There seems to be considerable difference between countries in the clinical practice regarding the removal of the thymus, and perhaps also between individual surgeons. Further clinical studies, with a follow-up time of decades, and a detailed individual clinical information such as for instance age at thymectomy and degree of thymectomy, are needed. For this, thymectomy needs to be noted in surgery reports, and should be assigned a specific surgical code, preferably including information regarding whether a total, or a partial, thymectomy has been performed. Such detailed information enables the definition of a more suitable control group, for example by comparing disease risks of those that have been partially thymectomized to those that have been totally thymectomized. This would minimize confounding issues due to the underlying cardiac defect and severe clinical illness related to the primary disease and it´s treatment. The results of such a study could support evidence based clinical guidelines improving patient care, not only during cardiac surgery, but also providing an opportunity of individualized targeted clinical follow-up. For the time being, based on the results of the current study, we find caution advisable during early cardiac surgery, and recommend sparing at least a part of the thymus, if possible.

Understanding the effects of early childhood thymectomy on the complex immune system and ultimately on our health is, and will be, a continuous challenge for interested researchers and clinicians. We need to know that what we do is safe. If we do not know, the decent thing to do is to find out.

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