Thymus dysfunction in the 22q11 deletion syndrome

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Löpningens essens, dess kärna, är att man anstränger sig till det yttersta inom sina begränsningar. Det är en metafor för själva livet – och för mig även för skrivandet. Troligen kan de flesta löpare instämma i det.

Haruki Murakami

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ABSTRACT

Introduction The 22q11.2 deletion syndrome (22q11DS) is associated with heterogeneous clinical findings, including T-cell immunodeficiency resulting from thymus hypoplasia. Newborn screening programs based on the quantification of T-cell receptor excision circles (TRECs) identify infants with severe combined immunodeficiency, as well as a number of infants with 22q11DS.

Aim To study the outcome of TRECs at birth in infants with 22q11DS, and to investigate if low numbers of TRECs are predictive of persistent thymus dysfunction in individuals with 22q11DS.

Method TRECs were retrospectively quantified by PCR using the original newborn screening cards from 48 infants with 22q11DS (Paper I). A follow-up of individuals with low numbers of TRECs (22q11Low, N=10), normal numbers of TRECs (22q11Normal, N=10) and matched healthy controls (N=10), was performed, including quantification of TRECs, flow cytometry for characterization of lymphocyte subsets, deep sequencing of T-cell receptor repertoires, and PCR for assessment of telomere lengths (Paper II). High-density arrays were used for autoantibody profiling (Paper III).

Results A considerable proportion of infants with 22q11DS had abnormal numbers of TRECs at birth (Paper I). At follow-up (median age 16 years), the 22q11Low group had lower TRECs, lower proportions of naïve T cells, aberrant T-cell receptor repertoires (Paper II) and more autoantibodies (Paper III), as compared to the 22q11Normal group and to healthy controls. Many autoantibody specificities were shared between the two 22q11DS groups.

Conclusion Newborn screening with TRECs identifies a subpopulation of infants with 22q11DS, in whom low numbers of TRECs at birth are associated with long-term immune aberrations, necessitating follow-up.

Keywords 22q11.2 deletion syndrome, TREC, thymus

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SAMMANFATTNING PÅ SVENSKA

Brässen (thymus) är ett livsnödvändigt organ beläget högt upp i brösthålan. I thymus utvecklas T-lymfocyter som är en typ av vita blodkroppar. Dessa ger oss ett brett skydd mot olika smittämnen. I thymus sker även en utsortering av de T-lymfocyter som skulle kunna reagera mot kroppens egna vävnader. Detta för att begränsa risken för utveckling av autoimmunitet.

22q11-deletionssyndromet (22q11DS) förekommer hos ca 1:2000 nyfödda och uppstår då en liten bit av arvsmassan på kromosompar 22 saknas. Knappt 1% av nyfödda med syndromet saknar thymus och har inga T-lymfocyter alls. Utan tidig behandling drabbas de av svåra infektioner och dör inom första levnadsåret. Övriga barn med syndromet har en thymus, men oftast är den liten och bildningen av T-lymfocyter nedsatt. Nyligen introducerades en ny analys i den allmänna screeningen av nyfödda (”PKU-provet”). Analysen påvisar en restprodukt som bildas när T-lymfocyter mognar i thymus (T-cell receptor excision circles, TRECs). Metoden utvecklades för att hitta barn med svår kombinerad immundefekt, som beror på medfödd avsaknad av T-lymfocyter. Även andra svåra T-cellsbrister hittas och enligt rapporter från USA, identifieras oväntat många barn med 22q11DS.

Studierna i denna avhandling syftar till att ta reda på hur stor andel av barn med 22q11DS som kan identifieras i nyföddhetsscreening med TREC, samt att undersöka om låga TRECs i nyföddhetsperioden är kopplat till mer uttalad immunologisk påverkan på lång sikt. Studierna utgick från en grupp barn med 22q11DS. Analys av TREC på sparade PKU-prov visade låga värden hos en betydande andel, vilket kunde ha lett till identifiering i nyföddhetsperioden. Bland dem fanns avlidna, vars chanser till botande behandling hade ökat vid tidig upptäckt. Långtidsuppföljning med blodprov visade mer uttalade immunologiska förändringar och en större mängd antikroppar riktade mot kroppsegna strukturer (autoantikroppar) hos barnen som hade låga TREC vid födelsen, jämfört med barn med syndromet som hade normala TREC. De påvisade förändringarna talar för bestående nedsatt thymusfunktion och liknar de som ses vid åldrande.

Baserat på dessa fynd anser vi att barn med 22q11DS som hittas i nyföddhetsscreening och har nedsatt thymusfunktion bör erbjudas uppföljning, för att förebygga infektioner och för att tidigt uppmärksamma utveckling av autoimmun sjukdom.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Lingman Framme J**, Borte S, von Döbeln U, Hammarström L, Óskarsdóttir S. Retrospective analysis of TREC based newborn screening results and clinical phenotypes in infants with the 22q11 deletion syndrome. J Clin Immunol. 2014 May; 34(4): 514-9. doi:10.1007/s10875-014-0002-y.

II. **Framme JL**, Lundqvist C, Lundell AC, van Schouwenburg PA, Lemarquis AL, Thörn K, Lindgren S, Gudmundsdottir J, Lundberg V, Degerman S, Zetterström RH, Borte S, Hammarström L, Telemo E, Hultdin M, van der Burg M, Fasth A, Óskarsdóttir S, Ekwall O. Long-Term Follow-Up of Newborns with 22q11 Deletion Syndrome and Low TRECs. J Clin Immunol. 2022 Apr; 42(3): 618-633. doi: 10.1007/s10875-021-01201-5.

III. **Lingman Framme J**, Hennings V, Lundell A-C, Thörn K, Lundqvist C, Lindgren S, Lundberg V, Telemo E, Fasth A, Óskarsdóttir S, Ekwall O. Proteome wide autoantibody profiling in the 22q11.2 deletion syndrome. In manuscript.

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Gudmundsdottir J, Thorgeirsdottir S, Lundbäck V, Göngrich C, **Lingman Framme J**, Kindgren E, Rydenman K, Ludviksson B, Bjarnadottir H, Runarsdottir S, Nilsson S, Zetterström RH, Ekwall O, Lindgren S. Normal neonatal TREC and KREC levels in early onset juvenile idiopathic arthritis. Clin Immunol. 2023 Mar 4:109277. doi: 10.1016/j.clim.2023.109277.

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CONTENT

ABBREVIATIONS

22q11DS	22q11.2 deletion syndrome
AIHA	autoimmune hemolytic anemia
AIRE	autoimmune regulator
ANA	anti-nuclear antibody
APECED	autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS1	autoimmune polyendocrine syndrome type 1
CHARGE	coloboma, heart defects, choanal atresia, growth retardation, genital abnormalities, and ear abnormalities syndrome
CDR3	complementary-determining region 3
CVID	common variable immunodeficiency
DBS	dried blood spot
DN	double negative thymocyte
DNA	deoxyribonucleic acid
DP	double positive thymocyte
EBV	Epstein-Barr virus
FISH	fluorescence in situ hybridization
GLILD	granulomatous lymphocytic interstitial lung disease
GvHD	graft-versus–host disease
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IFN	interferon

IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
ITP	immune thrombocytopenic purpura
JIA	juvenile idiopathic arthritis
KREC	kappa-deleting recombination excision circle
LCR	low copy repeat
MHC	major histocompatability complex
MLPA	multiplex ligation-dependent probe amplification
NGS	next generation sequencing
OPLS-DA	orthogonal projection to latent structures by means of partial least-squares discriminant analysis
OTFCS2	otofaciocervical syndrome type 2
PKU	phenylketonuria
RAG	recombination activating gene
RF	rheumatoid factor
RT-qPCR	Real-Time quantitative polymerase chain reaction
SCID	severe combined immunodeficiency
SP	single positive thymocyte
TCR	T-cell receptor
cTEC	cortical thymic epithelial cell
mTEC	medullary thymic epithelial cell

Th2	T-helper type 2 cell
Th17	T-helper type 17 cell
TRA	tissue-restricted antigen
TREC	T-cell receptor excision circle
TSLP	thymic stromal lymphopoietin
WES	whole-exome sequencing
WGS	whole-genome sequencing
WHO	World Health Organization

1 INTRODUCTION

The basis for this PhD project was my clinical interest in inborn errors of immunity (IEI). It all started with a child who came to the emergency department during my residency in pediatrics. It turned out that the child had an IEI, and as I was reading up on the differentials, I learned that there are hundreds of diseases classified as IEI. They are typically associated with an increased susceptibility for infections, although many are also linked to increased risks to develop autoimmunity, allergy and cancer. Consequently, affected individuals may present in almost any subspecialty in medicine. I reasoned that these patients were an underrecognized group, and felt I wanted to learn more. With all the respect for the complex immune system, this has been part of my life plan ever since that day.

A PhD project seemed like a good way to proceed, and it would not have been possible without my three supervisors. Professor Anders Fasth has been a clinical mentor to me, and he taught me about scientific methodology, as we performed an evidence-based review together. Sólveig Óskarsdóttir shared with me her expertise in the 22q11.2 deletion syndrome (22q11DS), and invited me to collaborate in the first study of this PhD project. Professor Olov Ekwall accepted me as PhD student in his research group, *Team thymus,* and has been the main supervisor for the projects leading to this thesis.

The focus of this thesis is 22q11DS, which is associated with a T-cell immunodeficiency resulting from thymus hypoplasia. When newborn screening programs based on quantification of T-cell receptor excision circles (TRECs) were introduced in some US states, in order to detect infants with severe combined immunodeficiency (SCID), it turned out that a number of infants with 22q11DS were also identified in the screening. While SCID is a fatal disease, which can be cured with timely hematopoietic stem cell transplantation, the prognostic value of low numbers of TRECs at birth in 22q11DS was less clear.

This thesis studies the prognostic value of TRECs at birth in individuals with 22q11DS, and investigates if low numbers of TRECs at birth are predictive of persistent thymus dysfunction in individuals with 22q11DS. The introduction in Sections 1.1-1.4 of this thesis includes reviews on the 22q11DS, newborn screening with TREC, the thymus and thymus defects, respectively. This is followed by the presentation and discussion of **Papers I–III** that form the basis for this thesis in Sections 2-7.

1.1 THE 22Q11.2 DELETION SYNDROME

The 22q11DS is the most common microdeletion syndrome, with an estimated incidence of 1/2,000–1/3,000 live births (1, 2).

Affected individuals present a spectrum of symptoms and signs of varying severity. The most-common clinical findings in infants include heart defects, hypoparathyroidism, palatal abnormalities, and immunodeficiency resulting from hypoplasia of the thymus (2). In older children and adults, developmental delays and learning disabilities, as well as behavioral and psychiatric disorders contribute to the morbidity (2). Both children and adults with 22q11DS display typical facial features, which may however be subtle (3).

Before the genetic etiology of 22q11DS was known, authors reported on several case series with partially overlapping phenotypes. During the 1960s, the pediatric endocrinologist Angelo DiGeorge reported on children with hypoparathyroidism and thymus hypoplasia (4). Heart defects were later added to the phenotype, and individuals with that triad of clinical findings were referred to as having *DiGeorge syndrome (5)*. During the 1970s, there were reports of children with palatal anomalies, anomalous facies, heart defects and learning disabilities collectively termed *the velocardiofacial syndrome,* as well as children with cardiac defects affecting the outflow tract and typical facies, designated as *the conotruncal anomaly face syndrome* (6, 7).
With advances in genomics and access to a new diagnostic technique based on fluorescence in situ hybridization (FISH) in the 1990s, it became clear that most of the individuals with these syndromes shared a common etiology for their condition: a submicroscopic deletion on chromosome 22 (8, 9). The current convention is to refer to the condition as *22q11.2 deletion syndrome* when the underlying molecular diagnosis has been confirmed (10).

The underlying cause of the malformations in 22q11DS is defective embryogenesis, which will be reviewed in more detail for the thymus gland in Section 1.3.

Genetics

In the q11.2 region of chromosome 22, there are several gene segments with similar nucleotide sequences. These segments are often referred to as low copy repeats (LCRs). Due to the high sequence homology of these segments,

this region is prone to errors during recombination in meiosis, which can result in duplications or deletions (11). An overview of the most common deletions in the 22q11.2 chromosomal region are depicted in Figure 1.

Approximately 85% of individuals with 22q11DS have hemizygous deletions that encompass 3 Mb, corresponding to LCR22A–D, which are referred to as typical deletions (2, 11). Overall, 5%–10% of individuals with 22q11DS have smaller deletions, a 1.5-Mb deletion affecting LCR22A–B or a 2-Mb deletion affecting LCR22A–C. These deletions give rise to phenotypes similar to those associated with the LCR22A–D deletions (2, 11).

Deletions encompassing 1.5 Mb of LCR22B–D or 0.7 Mb of LCR22C–D are referred to as atypical nested deletions and they confer milder phenotypes, reflecting the fact that they do not include the critical genes in the proximal region. The frequencies of these deletions in 22q11DS cohorts are not as well-described, mainly because they were not detected by FISH, which was the diagnostic method that was previously used (2, 11).

The typical deletions are de novo in 90% of cases, whereas the atypical nested deletions are more often inherited (2).

The 3-Mb region that is typically deleted in the majority of the patients contains 46 protein-coding genes and 7 genes coding for microRNAs, along with genes coding for non-coding RNAs and pseudogenes (2). It is beyond the scope of this thesis to review comprehensively all of these genes. A few of the genes for which haploinsufficiency is known to contribute to the phenotype in persons with 22q11DS will be described briefly. One of the most intensely studied genes is *TBX1,* which maps to the LCR22A–B region and codes for the T-box transcription factor 1. Mouse models have helped to reveal that this transcription factor is important for the formation of the heart, the thymus, and the parathyroid glands and it also plays a role in the development of the blood vessels in the brain (12-14). Haploinsufficiency of the Crk-like Adaptor Protein L (*CRKL)* gene, which is encoded in the LCR22B–D region, is known to contribute to the heart defects seen in individuals with atypical nested deletions, and in the mouse, haploinsufficiency of this gene contributes to thymus hypoplasia (15, 16). Furthermore, the DiGeorge Syndrome Critical Region 8 (*DGCR8)* gene, which is encoded in the proximal region, plays a role in the formation of micro RNAs (17), which in turn regulate the expression of other genes during embryogenesis (18).

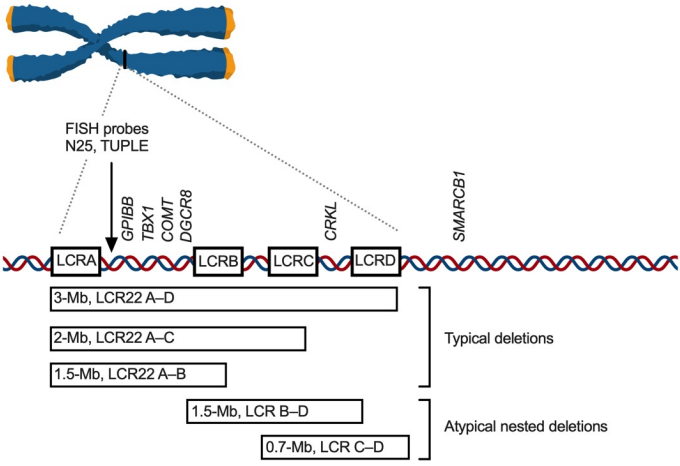

Figure 1. Schematic overview of the most common deletions in the 22q11.2 chromosomal region. The approximate positions of the probes used for fluorescence in situ hybridization (FISH) and of the genes mentioned in the text are marked. Adapted from Rozas MF, Benavides F, Leon L, Repetto GM. Orphanet J of Rare Dis. 2019; 14: 195. With permission: http://creativecommons.org/licenses/by/4.0/

Despite the advances made in genomics and the characterization of genes within the deleted region, the extensive phenotypic variability of the syndrome remains an enigma (2). Individuals with the same genetic defect display different phenotypes, which is true also for homozygous twins (19). Plausible explanations for the variable expression of phenotypes include variants in genes located in the 22q11.2 region on the intact chromosome, modifier genes outside the region, epigenetic modifications and environmental triggers (20).

Diagnostics

Clinical heterogeneity may explain why the 22q11DS is under-recognized by physicians in various sub-specialties (21, 22). According to a previous study, the median delay in making a diagnosis is approximately 4.5 years, and many individuals with 22q11DS see numerous sub-specialists and go through multiple medical investigations during this time (23). An early confirmation of the diagnosis confers the possibility of receiving targeted interventions and management according to recommended guidelines (24, 25). This can improve the long-term outcome, reduce the burden of disease for the individual and lower the healthcare costs (23, 26). Furthermore, a molecular diagnosis facilitates appropriate genetic counseling (27).

Based on ultrasonographic findings for the fetus, 22q11DS may be suspected already in the prenatal phase (27). Conotruncal heart defects are the most-specific findings in affected infants (27). Less-specific findings that may indicate 22q11DS include thymic abnormalities, increased nuchal translucency, renal abnormalities, cleft palate, skeletal defects and polyhydramniosis (27). When such features are present, second-tier screening test is generally offered, often with invasive sampling of the amniotic fluid or chorion villus (27). Non-invasive analysis of fetal DNA in maternal plasma is another option, although those test results need to be confirmed by chromosomal array from an invasive test or a blood sample when the infant is born (27).

In the post-natal phase, genetic testing is recommended at an early stage once there is a clinical suspicion of the syndrome. Chromosomal microarrays are recommended as a first and untargeted approach, as they detect copy number variations of the whole genome and determine the sizes of any detected variations (10). The traditional FISH technique is based on probes (TUPLE and N25) that bind to the proximal LCR22A–B region, which explains why individuals with atypical nested deletions have been missed by this technique (2). Multiplex ligation-dependent probe amplification (MLPA) is a rapid, PCR-based technique, which has the advantages over FISH that it includes the whole 22q11.2 region and it determines the size of the deletion (10). Modern techniques such as whole exome sequencing (WES) and whole genome sequencing (WGS) can be used to detect variants that are confined to single genes (such as *TBX1*) or to estimate the dosage of single genes, although they are not used to determine the sizes of deletions (10).

IMMUNE DEFICIENCY IN PERSONS WITH 22q11DS

Immune deficiency was part of the original description of the syndrome by Angelo DiGeorge, and has been investigated in many studies since then (4). Since the early descriptions of the syndrome, there have been considerable advances in the laboratory methods, and the historical studies have the natural limitation of using the methods that were available at that time. As an example, in the historical studies naïve and memory T-cell subsets were often not assessed separately. Furthermore, in previous studies from the pre-genomic era referred to herein, the cohorts were defined on the basis of clinical findings rather than on the basis of a molecular diagnosis of 22q11DS.

T lymphopenia

T-cell lymphopenia is seen in approximately 70%–85% of children affected by 22q11DS (28, 29). It was postulated already in the 1960s that the severity of the immune deficiency was dependent upon the extent of the thymus hypoplasia (30). A minority of infants with 22q11DS have a profound T-cell deficiency related to severe thymus hypoplasia or athymia (31), which will be discussed in more detail in Section 1.4.

The dynamics of the T-cell compartment of infants with 22q11DS differs from that of healthy infants, in whom there is an intense export of naïve T cells from the thymus (thymopoeisis) before birth and during the first year of life, resulting in transient lymphocytosis and the attainment of diverse T-cell repertoires (32, 33). After the first year of life, the T-lymphocyte counts in healthy children gradually decline, reflecting the start of the thymic involution (33).

Most infants with 22q11DS have a mild T-cell deficiency. Their T-lymphocyte counts do increase during the first year of life, but they lack the intense thymopoeisis seen in healthy infants (34). As the T-lymphocyte counts of healthy infants decline, the differences compared to 22q11DS children are blunted (34-36). After the first year of life, the T-lymphocyte counts in 22q11DS children decrease, albeit at a slower pace compared to healthy children (34, 35).

The increase in T-lymphocyte numbers during the first year of life in infants with 22q11DS might partly be explained by a slow, albeit steady, generation of naïve T cells from the hypoplastic thymus (37). In addition, homeostatic proliferation of T-lymphocytes in the periphery is known to compensate for a low thymic output under lymphopenic conditions. Upon binding of their

peptide-MHC complex, naïve T cells are converted into memory T cells, which is followed by their rapid clonal expansion (38). Finally, there is some evidence of slow peripheral proliferation of naïve T lymphocytes in 22q11DS and in other T-lymphopenic conditions (29, 38).

The T-cells of individuals with 22q11DS are generally considered to have a normal functionality (36, 39-42). There are reports of diminished proliferative responses in individuals with 22q11DS with increased age (35), and in individuals with more-severe T-cell lymphopenia, as well as in adults with 22q11DS that have developed antibody deficiency similar to common variable immunodeficiency (CVID) (43). One previous study showed reduced telomere lengths in naïve T-helper lymphocytes in adults with 22q11DS (29), and one recent study reported increased expression of cell-surface markers associated with exhaustion on T cells of children with 22q11DS (44).

The homeostatic events that occur in the T-cell compartment of infants with 22q11DS result in qualitative aberrations, such as inverse ratios of naïve cells to memory cells and restricted T-cell receptor repertoires. Furthermore, the increased replicative history of T cells might eventually impair their capacities to proliferate and function (29, 44). In summary, these aberrations are similar to those seen in the ageing immune system, where they are thought to contribute to the increased risks of infections, autoimmune disease and malignancies seen in the elderly population (45). The long-term consequences of early senescence of the immune system in individuals with 22q11DS remain to be elucidated.

T-cell receptor repertoires

A few previous studies have attempted to assess the T-cell receptor repertoires in 22q11DS, and abnormalities have been reported by all of the research groups (29, 46-48). Those studies were based on either of the two techniques that were available at that time. Flow cytometry enables the quantification of common variants of the T-cell receptor (Vβ families) (49). Spectratyping is a PCR-based method that assesses qualitative alterations of the T-cell receptors by counting the number of nucleotides that make up the variable part of the receptors, referred to as complementary-determining regions (CDR3) (49). Cancrini et al. assessed longitudinally the T-cell receptor repertoires of children aged 2-19 months and reported qualitative alterations. The cytotoxic T lymphocytes were particularly affected, although there was improvement over time (47). McLean-Tooke et al. studied the T-cell receptor repertoires of children with 22q11DS of different ages and found abnormalities in a majority of the subjects. Both T-helper cells and

cytotoxic T cells were affected, and T-cell receptor abnormalities were linked to low numbers of recent thymic emigrants (48).

Regulatory T cells

Regulatory T cells in 22q11DS have been investigated in a few previous studies, and the absolute numbers of regulatory T cells have been found to be reduced at all ages, as compared to controls (39, 50-53). One study showed unaffected proportions of regulatory T lymphocytes in young children with 22q11DS, as compared to healthy controls, whereas the proportions were decreased in older children (50).

B cells

The 22q11DS is generally considered to be associated with a T-cell deficiency, and the numbers of B lymphocytes in affected individuals are typically normal (35, 41, 50, 54, 55). As flow cytometry panels evolved to include markers of naïve and memory B lymphocytes, the skewing of subpopulations within the B-lymphocyte compartments became evident. Several studies have reported increased proportions of naïve B-lymphocytes and decreased proportions of memory B lymphocytes in patients with 22q11DS (39, 50, 55, 56). In addition, the study conducted by Derfalvi et al. showed a decreased level of somatic hypermutations in the B cells of individuals with 22q11DS (56). B-lymphocytes need stimuli from T-lymphocytes in order to mature into antibody-producing B-lymphocytes (56). As evidence for a B-cell intrinsic defect in 22q11DS is currently lacking, these findings are interpreted to reflect a consequence of insufficient T-cell help (56).

Immunoglobulins

Previous studies have shown an increased prevalence of hypogammaglobulinemia in individuals with 22q11DS. This includes immunoglobulin A (IgA) deficiency, which has been reported for up to 13% of cases (41, 57-59). In individuals with 22q11DS, the levels of immunoglobulin M (IgM) are decreased, as compared to age adjusted cutoffs in up to 29% of cases, and cases of selective IgM deficiency have also been reported (54, 59-61). Hypogammaglobulinemia with low levels of immunoglobulin G (IgG) or low levels of IgG1 or IgG2 subclasses along with increased frequency of infections have been reported in a subset of children and in adults with 22q11DS (43, 55, 58, 59). Some of the adults with 22q11DS had a combination of low IgA and IgG levels, similar to what is seen in patients with CVID (43). In a registry-based study, 2%–3% of patients with 22q11DS were found to be in receipt of immunoglobulin substitution therapy (59). According to a few studies of 22q11DS, the

low levels of IgG and/or IgA seen in infancy increase to normal or even supra-normal levels in many individuals in late childhood and early adulthood (35, 55, 62). This a bit contradictory, considering the CVID-like phenotype which some individuals develop.

In further support of a dysfunctional humoral immune system, a reduced response to polysaccharide antigens has been observed in a proportion of 22q11DS individuals (41, 54, 58, 63).

Infections in individuals with 22q11DS

Most children with 22q11DS suffer from an increased frequency of infections, which persists in adulthood in approximately one-third of the subjects (21, 35, 58, 64-67). The majority of the infections involve the upper respiratory tract, such as bronchitis, otitis and sinusitis (35, 64), although pneumonia is also increased (21, 65). The opportunistic infections that characterize severe T-cell defects are uncommon, except in the rare cases of congenital athymia (68). Severe infections such as septicemia and intra-abdominal infections have also been reported in association with 22q11DS, and constitute the second most common cause of death (58, 67, 69). Although the infectious disease problems were associated with decreased numbers of T-helper cells in patients with 22q11DS in one study (61), most studies show no such association (35, 54, 64). However, unsurprisingly, infections seem to increase in cases of immunoglobulin deficiency or lack of protective antibody titers to *Streptococcus pneumoniae* (41, 58). Recent publications have identified anatomic anomalies involving the palate and throat as risk factors for recurrent respiratory infections in 22q11DS (64).

Malignancies in individuals with 22q11DS

Although not comprehensively studied, an increased risk of malignancy has been reported in 22q11DS (70, 71). In a retrospective multicenter study that included 687 individuals (mostly children) with 22q11DS, there were six cases of malignancy, corresponding to a frequency of 0.9% (70). Another retrospective study from a single center reported two cases of malignancy in 467 individuals with 22q11DS, corresponding to 0.4% (64). If the reported frequencies of malignancy in 22q11DS are compared with the reported cumulative risk of 0.2% for children 0–14 years of age in the general population (72), it may not be as much increased as previously suggested (70). If there is an increased risk of malignancy in 22q11DS, the underlying mechanism may be that genes in the deleted region have a direct effect on malignancy risk, or that immune surveillance is impaired due to the immunodeficiency in 22q11DS (70). Bi-allelic inactivation of the tumor suppressor gene *SMARCB1* located in the distal regions of 22q11.2

is associated with rhabdoid tumors, and subsequent haploinsufficiency, as seen in 22q11DS infers a risk, which is illustrated by case reports (73-75). The gene for catechol-*O*-methyltransferase (*COMT),* which is located in the proximal region of 22q11.2, plays a role in detoxification of environmental carcinogens. Haploinsufficiency of this gene has been proposed to have a role in hepatoblastoma, which has been reported in children with 22q11DS (70, 76). Epstein-Barr virus (EBV) is known to drive lymphoproliferative complications in patients with inborn errors of immunity (77). In line with this, there exists a number of case reports of EBV-positive lymphomas in persons with 22q11DS (78-84).

Allergies in individuals with 22q11DS

Previous studies have obtained conflicting results regarding the prevalence of allergies in persons with 22q11DS. One retrospective study reported on the presence of atopy in two-thirds of children with 22q11DS, and an increased risk of atopy with low T-lymphocyte counts (85). Those authors suggested that T-lymphopenia and subsequent homeostatic proliferation leads to skewing of T-lymphocyte subpopulations towards the T-helper type 2 lymphocyte (Th2), which predisposes for atopy (85). Th2 skewing has been documented in animal studies, and for the severe T-lymphopenic state seen in patients with Omenn syndrome (86). One previous study documented Th2 skewing in adults with 22q11DS (42). For comparison, an Italian registry-based study of 415 individuals with 22q11DS documented a normal prevalence of asthma in persons with 22q11DS (6.7%), although the risk was higher for those with low numbers of T lymphocytes (54). A British retrospective report of 467 individuals with 22q11DS (mostly children) from a single center reported of allergic manifestations in 24%, as well as an association with low levels of IgM (64). Finally, a French retrospective cohort reported allergy in 27% and asthma in 19% of children with 22q11DS, and an increased risk of allergy in association with low cytotoxic T lymphocytes (61).

AUTOIMMUNITY IN 22Q11DS

Many studies have reported an increased risk of autoimmunity in individuals with 22q11DS, as compared to the general population. The reported prevalence of 22q11DS is in the range of 4%–30%, as compared to 5%–10% in the general population (87). This wide range is explained by the different methodologies used in the published studies, the differences in age between the included study participants, and inconsistencies related to how autoimmunity was defined. The outcomes of previous studies that have investigated autoimmunity in patients with 22q11DS are shown in Table 1.

Several factors are known to predispose to autoimmunity in the general population, including old age and female sex (45, 88). Environmental factors such as infections may also contribute to autoimmunity. During infections, self-antigens of injured tissues are exposed to antigen-presenting cells that are already primed to provide the co-stimulatory signals needed to activate lymphocytes (89). Furthermore, antigens from pathogens may resemble self-antigens, paving the way for cross-reactions (molecular mimicry) (89). The genetic background plays a role in the development of autoimmunity, and particularly the major histocompatability (MHC) genes, since the encoded MHC complexes determine which specific peptides from a given antigen will be presented for the immune system on the cell surface. In addition, autoimmunity can result from a loss of central tolerance if self-antigens are not properly presented to maturing T cells in the thymus, during the positive and negative selection processes (reviewed in Section 1.3). Failure to generate functioning regulatory T cells leads to impaired peripheral tolerance (90). T-cell lymphopenia also predisposes to autoimmunity, as briefly mentioned previously. The homeostatic processes that serve to reduce T lymphopenia favor the expansion of T cells with high affinity receptors for self-antigen-MHC (38).

Many studies have set out to investigate the cause for autoimmunity in 22q11DS. Recently, studies have shown an association between low numbers of naïve T cells and autoimmunity in 22q11DS (54, 64, 91-94). A large retrospective study has shown that low numbers of B cells are linked to autoimmunity in cases of 22q11DS (54). Another study has reported that low numbers of switched memory B cells are linked to a clear increase in the risk to develop ITP (92). In comparison, some studies have failed to show an association with low numbers of B cells and increased risk for autoimmunity in cases of 22q11DS (64, 91). Hypogammaglobulinemia and/or selective antibody deficiency have, however, been reported to occur concomitantly with immune thrombocytopenic purpura (ITP) (35, 43), or more generally together with autoimmunity in 22q11DS (58). Two studies have shown an increased risk for autoimmunity in individuals with 22q11DS, who have increased frequency of infections (95) or severe infections (61), respectively, although these results have not been confirmed by others (50, 58). Based on the thymus hypoplasia observed in persons with 22q11DS, it is reasonable to suspect that autoimmunity in 22q11DS results from a failure of central tolerance induction. Thymus tissues from individuals with 22q11DS are, however, scarce in terms of availability for studies, so no firm conclusions can be drawn on this subject. Furthermore, it is possible that the defective embryogenesis in 22q11DS leads to aberrant exposure of self-antigens to immune cells, paving the way for autoimmune processes.

Autoimmune hematological diseases

Autoimmune hematological diseases such as ITP and autoimmune hemolytic anemia (AIHA) are often described as the most-common autoimmune manifestations in young children with 22q11DS, although these conditions can also appear in adulthood (43, 91, 92). Autoimmune neutropenia has been described less frequently in individuals with 22q11DS (61). The prevalence of ITP in children with 22q11DS may be more than 100-times higher than in the general population, and recurrent ITP or the combination of ITP and AIHA (Evans syndrome) also seems to occur frequently (94). Moreover, the response to standard treatment of autoimmune hematological manifestations seems to be less efficient in 22q11DS, as compared to controls (67). Recent studies have shown an increased risk of ITP with decreased counts of naïve T-helper lymphocytes (91, 92) and with reduced switched memory B lymphocytes (92). One previous study reported on the co-occurrence of ITP and hypogammaglobulinemia in adults with 22q11DS (43).

Defining ITP in 22q11DS poses a challenge owing to the baseline characteristic of low platelet counts, associated with the syndrome. This mild thrombocytopenia is thought to be caused by haploinsufficiency of the *GPIBB* gene, which is located within the deleted gene segment on chromosome 22 in >90% of individuals with 22q11DS . This gene codes for the glycoprotein IBb (GPIBb), an essential component of the receptor that binds von Willebrand factor, which is necessary for the platelets to initiate hemostasis by adhesion to injured endothelium (96). An increased platelet volume has also been proposed as consequence of haploinsufficiency of *GPIBB* in 22q11DS, although this finding does not appear to be consistent in adults (97). Bi-allelic mutations of the same gene give rise to the more-severe bleeding disorder of Bernard-Soullier syndrome with large platelets (megatrombocytopenia) (96). Bernard-Soullier syndrome has been described in individuals with 22q11DS who have a dysfunctional *GPIBB* allele. This warrants a clinical work-up for this disease in individuals with 22q11DS who present with a bleeding disorder at an early age (96).

Table 1. Studies reporting on autoimmunity in 22q11 deletion syndrome.

Study	Subjects N	Age (range) in years	Gender	AI (%)	ATD (%)	AH (%)	Other AI conditions
Basset **(65)**[a]	78	31.5[m] (21–41)	36 M/42 F	25 (32%)	20 (26%)	5 (6%)	n.a.
Björk **(43)**	26	25[Md] (18–44)	10M/16 F	11 (42%)	6 (23%)	4 (15%)	1 JIA
Cancrini **(67)**	228	<18	112M/116F	24 (11%)	4 (2%)	11 (5%)	n.a.
Crowley **(91)**	721	All ages	346 M/375F	30 (4%)	18 (2%)	7 (1%)	5 JIA
Deshphande **(54)**[b]	415	4.6[Md] (0–46)	220M/195F	24 (6%)	8 (2%)	6 (1%)	1 JIA, 1 RA 1 DM, 1 alo
DiCesare **(95)**	50	6[m] (0–17)	28M/22F	9 (18%)	n.a.	n.a.	n.a.
Gennery **(58)**	32	1–22	19M/13F	6 (19%)	-	2 (6%)	1 JIA,1 vasc 2 RP
Giardino **(64)**	467	11[m] (0–30)	284M/183F	30 (6%)	7 (1%)	9 (2%)	6 JIA, 3 IBD 3 pso, 2 vit 1 DM, 4 alo 1 SLE
Jawad **(35)**	195	1.5[Md] (0–9)	n.a.	15 (8%)	-	8 (4%)	4 JIA, 1 vit 1 pso, 1 RA
Levy-Shraga **(98)**	48	11[m] (4–18)	28M/20F	9 (19%)	2 (4%)	4 (8%)	3 CD
Lima **(99)**	59	9[Md] (1–54)	28M/31F	6 (10%)	3 (5%)	1 (2%)	1 JIA, 2 CD
Mahe **(61)**	86	<18	45M/41F	11 (13%)	4 (5%)	3 (3%)	2 JIA, 1 DM 1 IBD
Montin **(92)**[bb]	294	<18	n.a.	72 (24%)	n.a.	23 (8%)	n.a.
Óskarsdóttir **(21)**[c]	100	7[Md] (0–19)	46M/54F	6 (6%)	3 (3%)	2 (2%)	1 JIA, 1 CD
Ricci **(93)**	44	13[m] (7–19)	23M/21F	10 (23%)	6 (14%)	1 (25%)	1 alo, 1 vit 1 CD
Ricci **(100)**[d]	73	<18	n.a.	n.a.	16 (22%)	n.a.	n.a.
Shugar **(101)**[d]	169	10[m] (1–18)	99M/77F	n.a.	16 (9%)	n.a.	n.a.
Stagi **(102)**[d]	30	10[Md] (1.5–44)	10M/20F	n.a.	8 (27%)	n.a.	n.a.
Sullivan **(103)**	60	<18	n.a.	7 (23%)	1 (2%)	2 (3%)	2 JIA, 1 vit
Tison **(94)**	130	8[m] (0.5–23)	67M/63F	11 (8%)	3 (2%)	6 (5%)	2 JIA, 1 pso
Wahrmann **(104)**	98	9[Md] (0–15)	53M/45F	29 (30%)	n.a.	n.a.	n.a.
Yu **(105)**	87	7[Md] (2–15)[e]	50M/47F	11 (13%)	n.a.	n.a.	n.a.

AI, autoimmunity; ATD, autoimmune thyroid disease; AH, autoimmune hematologic disease; M, males; F, females; m, mean; Md, median; a, this study only reported features present in > 5% of participants; b, registry study; bb, registry study focusing on AH; c, this study reported presenting findings; d, these studies focused on ATD; JIA, juvenile idiopathic arthritis; n.a., information not available; RA, rheumatoid arthritis or other rheumatologic condition; DM, diabetes mellitus; alo, alopecia; vas, vasculitis; RP, Raynaud´s phenomenon; IBD, inflammatory bowel disease; pso, psoriasis; vit, vitiligo; CD, celiac disease; e, interquartile range.

Autoimmune thyroiditis

Shugar et al. have reported the prevalence of manifest thyroid disease in a cohort of children with 22q11DS as being 9.5%, with a mean age at presentation of 8 years (101). That study did not report on the presence of thyroid autoantibodies or any ultrasonographic findings, so no conclusions can be drawn on the cause of the thyroid disease. In a recent prospective, longitudinal study, Ricci et al. reported that 21.9% of patients with 22q11DS developed autoimmune thyroid disease before the age of 18 years, with a mean age at onset of 10 years (100). They defined a diagnosis of autoimmune thyroid disease as being based on positive thyroid autoantibodies, typical ultrasonographic findings (inhomogeneous echotexture, hypo-echogenicity, vascular overflow), and typical findings in blood samples (100). It is known that anatomic anomalies, such as thyroid hypoplasia, are present in 50% of individuals with 22q11DS (102, 106). Ricci et al. confirmed these findings and stated that such anomalies were present at equal frequencies in individuals with 22q11DS and autoimmune thyroid disease as in individuals without autoimmune thyroid disease (100). Several studies report on a female predisposition for thyroid disease in 22q11DS, although the female to male ratio seems blunted, as compared to the clear female to male ratio of 19:1 of autoimmune thyroid disease in the general population (100-102). Despite the difficulties associated with comparing studies that include participants of different ages, the prevalence values mentioned above are discrepant with the reported prevalence rates of hypothyroidism: 0.7% by Ryan et al. and 1.5% by Giardino et al. (31, 64). Therefore, I find it resonable to conclude that thyroid disease warrants active surveillance in 22q11DS, throughout childhood and into adulthood.

Juvenile idiopathic arthritis (JIA)

There are several reports of arthritis in patients with 22q11DS, with a prevalence of 4% being proposed in an earlier study (107). Clinically, the arthritis is described as resembling extended oligoarticular JIA or polyarticular JIA, with a moderate or severe course, and in a few cases, it is described as destructive. One report stated that 2 out of 13 patients with JIA and 22q11DS were positive for rheumatoid factor (RF) and 6 out of 13 were positive for antinuclear antibodies (ANA) (108, 109). Interestingly, 4 out of 13 patients with JIA and 22q11DS had IgA deficiency (108). An earlier report stated that patients with 22q11DS and arthritis had permissive HLA-types, although this was not confirmed in all cases (107). Our experience, from approximately 200 individuals with 22q11DS seen at our center, does not suggest such a clear overrepresentation of JIA, as only two of our patients presented with arthritis (21). In accordance with our expericence,

a nationwide study from Norway revealed no cases of JIA and only 1/59 patients with psoriasis arthritis (99).

Other autoimmune diseases

Celiac disease and inflammatory bowel disease (IBD) are less well-studied in relation to 22q11DS, although a few studies have failed to show increased prevalence rates of these diseases in patients with 22q11DS (110, 111).

Autoantibodies in 22q11DS

A few studies have investigated the occurrence of selected autoantibodies in patients with 22q11DS. Gennery et al. assessed autoimmune symptoms and the presence of antibodies to five well known autoantigens in 30 children with 22q11DS. Nine of those children had autoantibodies, whereof five children had concurrent autoimmune symptoms (58). Lima et al. performed a nationwide study of 59 individuals of different ages with 22q11DS in Norway and found that 58% had autoantibodies to any of the 20 solid organ autoantigens that were studied (99). Ricci et al. reported that 8 out of 44 children were positive to ANA or thyroid antibodies, without having autoimmune symptoms, which was associated with lower recent thymic emigrant T-helper cells, as compared to the children with 22q11DS who had no autoantibodies or autoimmune symptoms (93). In a study of autoimmune thyroid disease in 22q11DS, a large proportion of study participants were ANA positive, which was linked to an increased risk for thyroid disease (100).

1.2 NEWBORN SCREENING WITH TRECS

The era of newborn screening

The fundamental purpose of newborn screening is to identify infants who have severe congenital diseases in the asymptomatic phase, in order to enable preventive measures and treatment, before any damage has occurred (112). Large-scale screening using dried blood samples was first used for the detection of phenylketonuria (PKU) in the USA in the 1960s (113). In PKU, the metabolism of the amino acid phenylalanine is impaired, which leads to its accumulation in the blood and eventual harm to the central nervous system. Affected individuals will develop cognitive impairment, if they are not fed a diet that is restricted for phenylalanine from birth (114). The PKU screening system was based on a swift and economical method developed by Robert Guthrie and Ada Susie, in which blood drawn from a heel prick was applied to absorbent paper (the Guthrie card), which was then sent by mail to the screening laboratory. Standard-size discs were punched from the dried blood spots (DBS), followed by incubation of the discs on agar plates that contained bacterial cultures and inhibitory media. The presence of phenylalanine in the sample prevented the inhibition of bacterial growth and the levels of phenylalanine in positive tests were estimated by the size of bacterial growth around the disc (113).

Key principles for newborn screening systems

As more diseases were considered for screening, concerns were raised regarding ethical, legal and economic issues, and there was a need for a frame-work that would guide decision-making as to which diseases should be screened. In 1968, James Wilson and Gunnar Jungner published a report on behalf of the World Health Organization (WHO) titled: *Principles and practice on screening for disease (115).* In summary, the principles for screening listed by Wilson and Jungner are that: the disease should be a significant health problem, for which the natural history is known and includes an asymptomatic stage; appropriate treatment should be available; and there should be consensus regarding which persons to treat. They further stated that there must be a suitable screening test that is acceptable to the population, and that testing and treatment for the disease should be economically justifiable (115). Whereas the original Wilson and Jungner criteria mainly address issues regarding the *disease*, there have been subsequent addendums that more-specifically address the issues concerning *newborn screening programs* (116). Furthermore, local amendments are often made to adjust the principles to the infrastructure, society and

healthcare systems in screening countries or regions, as exemplified by the 15 criteria that are applied in Sweden (117). Despite the addendums and amendments, the original principles proposed by Wilson and Jungner still to this day form the basis for decision making in relation to screening (115).

The quest for a screening test for SCID

SCID is an inborn error of adaptive immunity, which is fatal within the first years of life if left untreated (118). SCID can result from pathogenic variants in a number of genes that regulate the differentiation of T lymphocytes (118). However, regardless of the genetic cause, SCID is typically characterized by the complete absence of, or very low numbers of non-functioning autologous T cells (119). Although B cells may be present in cases of SCID (referred to as T-B+ SCID), they are non-functioning due to the lack of T-cell help (118). In congenital athymia, the maturation of T cells is also impaired, which may result in a severe T-cell deficit that is similar to SCID (68, 120). The term SCID is, however, traditionally reserved for conditions with an intrinsic hematopoietic defect (119).

Clinical diagnosis of SCID is challenging, since affected infants often appear healthy at birth, and the symptoms from infections are unspecific and present gradually (118). If treatment with hematopoietic stem cell transplantation (HSCT), gene therapy or enzyme replacement is initiated before infections have occurred, the prognosis is excellent, with a survival rate of 95% (121). Based on the nature of the disease, SCID was considered as a candidate for newborn screening, although for a long period of time there was no suitable screening test (122). In 2005, Chan and Puck reported that an assay based on the quantification of T-cell receptor excision circles (TRECs) applying Real-Time quantitative polymerase chain reaction (RT-qPCR) to blood that was eluated from the Guthrie card could be used, as TRECs function as biomarkers for naïve T cells (123). TRECs are small, circular pieces of DNA that remain in the cell after T-cell receptor gene rearrangement (124). The process by which TRECs are formed will be reviewed in more detail in Section 1.3. Population-based newborn screening with the TREC assay was initiated in Wisconsin in 2008, and a decade later all states of the US had implemented TREC screening programs (125, 126). Subsequently, several European countries, as well as Israel, Taiwan and New Zeeland have added TREC quantification to their screening programs, and several regions around the world are doing pilot studies. In Sweden the TREC screening program was launched in August 2019 (127).

Identification of 22Q11DS in TREC screening programs

As the first TREC screening programs from the US reported their outcomes, it was clear that the sensitivity for the detection of SCID was close to 100% (125, 128, 129). It was also clear that the TREC screening assays identified cases of T lymphopenia with origins other than typical SCID. Preterm infants may present with low numbers of TRECs that self-resolve, and may therefore be considered "false positives" (127, 130). Second to prematurity, 22q11DS was the dominant cause of non-SCID T lymphopenia identified in the screening programs, and in some TREC screening programs, the identified 22q11DS cases have even out-numbered the identified SCID cases (129, 131). Based on the prevailing knowledge that complete athymia and subsequent lack of T cells is present in less than 1% of infants with 22q11DS (31), the numbers of infants with 22q11DS identified in TREC-screening programs are perhaps surprising. Although the screening programs are all very sensitive in terms of picking up cases of classical SCID, the proportion of identified infants with 22q11DS is not uniform. Table 2 shows the number of infants with 22q11DS identified in TREC screening pilot studies and implemented population based screening programs.

TREC screening methodologies

It is beyond the scope of this thesis to review the different TREC screening methods in detail. In summary, different methodologies uniformly pick up cases of typical SCID with very low numbers of TRECs. However, further comparisons regarding cutoffs or actual TREC counts are hindered by the different methodologies used (132, 133).

The TREC screening programs for newborns operating in different countries vary in several aspects, for example regarding the methodology used for the TREC assay, the applied cutoffs, and the follow-up algorithms. Some screening programs use a commercially available method, while others use methods that were developed in-house. Some programs apply a single-plex RT-qPCR for TREC as the first tier and add a second tier RT-qPCR of for a house keeping gene, such as *ACTB* or *RNaseP* to verify the successful extraction of DNA from the Guthrie card, but only if there is a failure to quantify the TRECs (134). Others run a duplex RT-qPCR for TREC and a house keeping gene as the first tier (129). In a few programs or pilot studies, a triplex RT-qPCR was performed as the first tier, as they also include enumeration of kappa-deleting recombination excision circles (KREC) as a biomarker for the generation of naïve B cells (127, 132, 135, 136). The simultaneous enumeration of TRECs and KRECs offers the advantage of differentiating between T-B+ SCID and T-B- SCID, which gives an indication of the underlying genetic diagnosis, which in turn can be important

Table 2. Outcome of TREC newborn screening programs or pilot studies

Study	Region	Cutoff TRECs (copies/μL)	Method	Period	Infants N	SCID N	22q11DS N
Amatuni (137)	California	<40 <22–18[a]	In house, PE	2010–2017	3,252,156	50 1:65,043	47 1:69,194
Argudo-Ramirez (138)	Catalonia	<34–24[a]	PE	2017–2018	129,614	1 1:129,614	5 1:25,923
Audrain (139)	France	<21	PE	2015–2017	190,517	3 1:63,505	4 1:47,629
Chan (123)	Singapore	<18	PE	2019–2020	35,888	0	1 1:35,888
Chien (140)	Taiwan	<40	In house	2010–2011	106,391	2 1:53,196	5 1:21,278
Gans (141)	New York	<200	In house	2010–2017	199[b]	3	6
Gizewska (135)	Poland/ Germany	<6	SPOT-it™	2017–2020	44,287	1 (1)[e] 1:44,287	0
Gröngrich (127)	Sweden	<15–6[a,d]	SPOT-it™	2019–2020	115,786	2 1:57,893	4 1:28,946
Hale (142)	Massachu-setts	<252	In house	2009–2019	720,038	9 (5)[e] 1:80,004	40 1:18,000
Kwan (131)[c]	11 US States	various	various		3,030,083	50 1:60,601	78 1:38,847
Lev (143)	Israel	<36–17[a]	PE	2015–2020	937,953	32 1:29,311	10[f] 1:93,795
Liao (144)	Taiwan	<30	In house	2012–2015	253,999	2 1:126,999	6 1:42,333
Strand (145)	Norway	<25[g]	In house	2018–2019	88,000	3 1:29,333	0
Verbsky (146)	Wisconsin	<40–25[h]	In house	2008–2011	207,696	5 1:41,539	5 1:41,539
Vogel (129)	New York	<200–125[a]	In house	2010–2012	485,912	10 1:48,591	18 1:26,995
Wakamatsu (132)	Japan	<31	PE	2017–2021	137,484	2 68,742	5 1:27,497

PE, PerkinElmer Enlite™ neonatal TREC kit; a, cutoffs were lowered; b, cohort with T-cell lymphopenia identified in TREC newborn screening; c, this study reported the outcome of TREC newborn screening in 11 US States; d, TRECs/3.2 mm punch from the newborn screening card; e, combined immunodeficiency; f, reported as DiGeorge syndrome without information on molecular genetic diagnosis; g, TRECs 25–5/μL reported only if gene panel performed on newborn screening card confirmed molecular genetic diagnosis; h, cutoff was increased.

for treatment decisions (132, 147). Quantification of KRECs also enables the identification of inborn errors of B lymphocytes, although the drawback of the combined TREC-KREC assay is its higher cost and the relatively high frequencies of false positives, which are largely explained by the secondary effect on the infant of the immunosuppressive treatment given to some mothers during pregnancy (132, 147). In addition, the identification of B-cell deficiencies with the KREC assay might be less coherent with the Wilson and Jungner criteria (115). Upon implementation, the programs generally adjust the cutoffs so as to reduce the frequency of false positives, while maintaining adequate sensitivity to detect cases of SCID (127, 137, 143).

TREC screening follow-up algorithms

Absent or very low numbers of TRECs on the newborn screening card in a term infant, in a situation with normal amplification of the house-keeping gene, is highly indicative for SCID (126). In most screening programs this leads to an urgent referral for a fresh blood sample, for the enumeration of lymphocyte subsets by flow-cytometry, and consultation by a pediatric immunologist (126, 134). The flow-cytometry panels typically include markers for T lymphocytes (CD3+), T-helper cells (CD4+), naïve T-helper cells (CD45RA+), cytotoxic T cells (CD8+), natural killer cells (CD56+) and B-cells (CD19+). An overview of the TREC screening algorithm in Sweden is provided in Figure 2.

As an alternative to an urgent referral, some programs obtain a new screening card prior to referral (137, 143). This approach is also often applied for preterm infants, and for infants who are in intensive care, as this is associated with high rate of false positives (137). A second screening card may also be obtained in cases with intermediate numbers of TRECs (134).

If T-cell lymphopenia is verified with flow-cytometry, molecular genetic studies of genes known to cause SCID are usually performed as the next step, although the approach for this is often not described in detail in published reports. Furthermore, the threshold of T-cell lymphopenia for when molecular genetic studies is performed also varies between programs (126, 137). Genetic studies need to include known causes of congenital athymia, which is not always the case (141), and will be discussed further in Section 1.4.

The TREC newborn screening program in Norway is unique in the sense that it has integrated next generation sequencing (NGS) of a gene panel on the newborn screening card as a second-tier test. The panel includes genes that are known, to cause SCID, but also other genes known to cause T-cell defects (145). In the Norwegian screening program, TREC values <5/μL are reported as urgently positive, raising a high suspicion of SCID, whereas TREC values 5-25/μL are reported only if the gene panel is positive (145). This approach

has the advantages of less false positives in the intermediate interval of TREC 5-25/uL, as well as faster turnaround time for the true positives (145). Depending on the program, and on the clinical presentation of the infant, additional blood tests may be performed. For example, *in vitro* tests of T-cell proliferative response are often performed (129) and T-cell receptor repertoire analyses may be performed, especially if an infant presents with an Omenn syndrome phenotype (148).

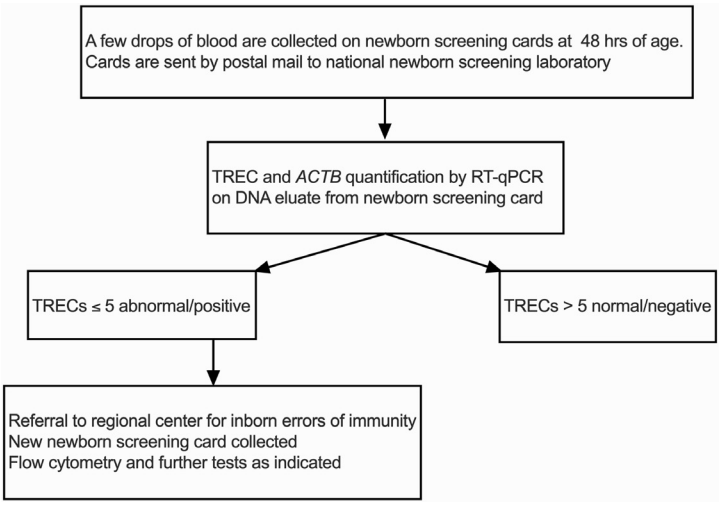

Figure 2. Overview of the present TREC screening algorithm in Sweden.
The applied cutoff is ≤ 5 TREC copies/ 3.2 mm punch from the screening card.

1.3 THE THYMUS

Historically the thymus was considered to be a redundant organ of no great importance.

The ground-breaking discoveries made by Jacques Miller in 1961 proved that the thymus is a vital organ that is essential for immune function. Miller showed that thymectomy in newborn mice led to their wasting and eventually to their death (149). He also noted that thymectomized mice did not reject skin grafts from other strains or species, and suggested that the lymphocytes leaving the thymus were selected cells (149).

Further insights into the importance of the thymus came from observations of rare human conditions. In 1965 Angelo DiGeorge described the autopsy findings of children who lacked the parathyroid glands and the thymus, and he stated these deficiencies were due to defective embryogenesis of the pharyngeal apparatus (4). We now know that this condition is often caused by 22q11.2 deletion (8). DiGeorge considered the condition to be a human counterpart to thymectomy in newborn animals, and since the thymus had been absent during gestation, he claimed it was an even better model to study than thymectomized mice (150). T-lymphocyte development starts in the first trimester of pregnancy, and the newborn term infant is already equipped with a repertoire of long-lived T lymphocytes, which explains why congenital thymus aplasia is much more detrimental than postnatal thymectomy (151). In comparison, newborn mice are not as developed, and the lifespan of their T lymphocytes is shorter; consequently, neonatal thymectomy of mice is fatal (151). Despite the differences between humans and mice in terms of thymus development and the generation of T lymphocytes, much of our knowledge originates from murine models.

THYMUS ORGANOGENESIS

The pharyngeal arches are five paired structures situated on either side of the pharyngeal foregut in the human embryo. They give rise to bone, cartilage, muscles, nerves, glands and connective tissues in the head and neck region. The thymus is formed together with the inferior parathyroid glands from the third pharyngeal pouch, starting at 5 weeks of gestation (152). The pharyngeal pouches are the inner folds of the pharyngeal arches, each of which is composed of three layers that give rise to different cells and organs of the midline region: an outer layer of ectoderm, an inner layer of endoderm, and a core of mesenchyme originating from the mesoderm and neural crest cells from the neural plate. A schematic illustration of a human embryo and the pharyngeal apparatus is shown in Figure 3.

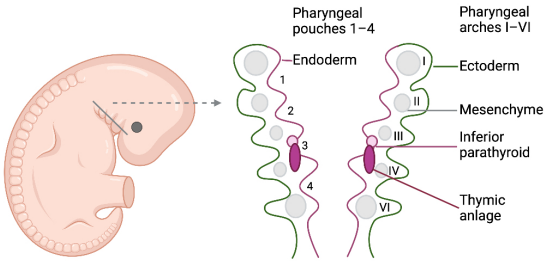

Figure 3. Schematic illustration of a human embryo to the left, with a cross-section of the pharyngeal region to the right. The endodermal epithelium is depicted in purple and the ectodermal epithelium in green, whereas the mesenchymal core is depicted in gray. Pharyngeal arches are numbered I–XI, whereas pharyngeal pouches are numbered 1–4. The inferior parathyroid (light pink) and the thymic anlage (purple) form togheter from the third pharyngeal pouch. Adapted from Gordon, J. Hox genes in the pharyngeal region: how Hoxa3 controls early embryonic development of the pharyngeal organs. Int. J. Dev. Biol. 2018; 62: 775 - 783. Doi: 10.1387/ijdb.180284jg - With the permission of UPV/EHU Press. Created with Biorender.com

Studies carried out in mice support the notion that the formation of the third pharyngeal pouch and the thymus anlage is the result of a complex network of activities of transcription factors, such as Pax1, Pax9, Hoxa3 (152, 153), Eya, Six1 and Six4 (154). Moreover, the T-box transcription factor 1 (Tbx1) and chromodomain helicase DNA-binding domain 7 (Chd7) are important for the segmentation and vasculature of the arches, and thus for the formation of the entire midline region (155, 156).

In the second stage of the thymus organogenesis the transcription factor Forkhead box N1 (Foxn1) dictates the patterning of the thymic epithelium. Foxn1 is also crucial for the subsequent growth of the organ, the establishment of the three-dimensional architecture of the tissue, and the maintenance of the organ throughout adulthood (157, 158).

In line with the findings obtained from animal models, genetic variants involving some of these transcription factors may lead to thymus aplasia or hypoplasia also in humans (reviewed in Section 1.4).

THYMIC ORGANIZATION

Histologically, the thymic tissue is organized into compartments with separate functions. The cortex is densely packed with immature lymphocytes (thymocytes) and cortical thymic epithelial cells (cTECs), while the medulla is composed of medullary thymic epithelial cells (mTECs), and thymocytes, and other cells of hematopoietic origin, such as dendritic cells, B cells, macrophages, eosinophils and neutrophils (159).

Thymus stroma and thymic epithelial cells

The thymic stroma is composed of an extracellular matrix, as well as several cell types, including TECs that originate from the endoderm of the pharyngeal pouch, together with vascular endothelial cells, vascular mural cells and fibroblasts, all of which originate from the mesenchyme and neural crest (16, 154, 159). Growth factors produced by mesenchymal cells and by lymphoid progenitors activate the transcription of Foxn1 in TEC progenitors, thereby promoting their proliferation and differentiation into cTECs and mTECs (159). The thymic stroma forms a three-dimensional scaffold, which facilitates the bi-directional interactions of stromal cells with progenitor lymphocytes that are migrating from the bone marrow to the thymus. The crosstalk that occurs between the cells is essential for the development of both thymocytes and TECs (159).

THYMUS FUNCTION

T-cell development

Lymphoid progenitors populate the thymus from the 8th week of gestation (160). In the postnatal period, thymocyte progenitors enter the thymus through fenestrated capillaries in the corticomedullary junction and adhere to selectins on the TECs. The entering lymphoid progenitors receive stimuli from the cTECs and acquire T-cell characteristics, and this is followed by their proliferation. These stimuli include delta-like 4 (DL4), which signals via the Notch1 receptor on thymocytes and IL-7 (161). At this stage, the thymocytes do not express CD4 or CD8 and are thus labeled double negative (DN). DN thymocytes are guided by chemokine receptors to migrate to the cortex (159), as depicted in Figure 4.

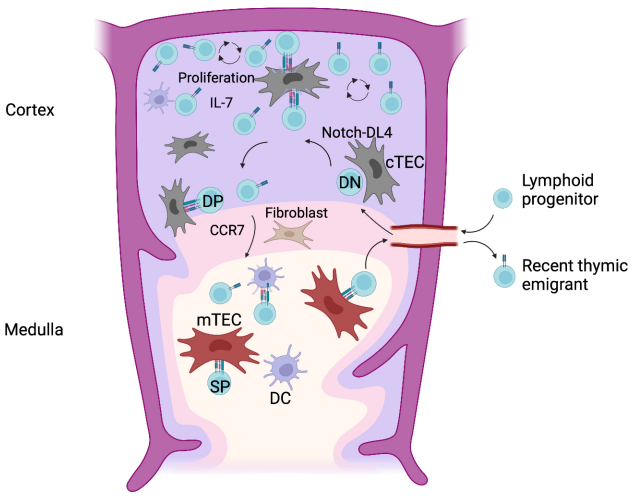

Figure 4. Schematic illustration of T-cell development in a thymic lobule. Lymphoid progenitors enter at the cortico-medullary junction and receive delta-like 4 signals via the Notch1 receptor, followed by generation of T-cell receptors. Double negative (DN) thymocytes interact with cTEC via the T-cell receptor, which mediates proliferation. Double positive (DP) thymocytes that bind peptide-MHC on cTEC are positively selected and migrate to the medulla where self-reactive single positive (SP) thymocytes are deleted by negative selection upon interaction with DC and mTEC. Thymocytes are depicted in turquoise, cTECs in gray, mTECs in red, dendritic cells (DC) in purple and fibroblasts in brown. Created with Biorender.com

Generation of a diverse T-cell receptor repertoire

In the cortex, the DN thymocytes start to express T-cell receptors (TCR) that can specifically bind the self-antigens presented on MHCs on cTECs.

The TCRs are heterodimers, and in αβ T cells they are composed of an α-chain and a β-chain, both of which have a constant part for anchoring and a variable part for antigen binding (162). The diversity of the receptors results from the largely random recombination of the variable (V), joining (J) and (for the β-gene) diversity (D) gene segments that encode the variable part of the receptors. The β-chain of the receptor is rearranged first, with one of the 2 possible D-gene segments pairing with any of the 12 J-gene segments, followed by pairing of any of the 42 V-gene segments to the DJ. Once the β-chain is successfully rearranged, it pairs with an invariant pre-TCR α-chain to form a pre-TCR. Signaling through the pre-TCR, plus the DL4-Notch interaction provides the necessary signals for the genomic rearrangement of the 43 V-gene and 58 J-gene segments of the α-gene locus and the concurrent expression of co-receptors CD4 and CD8, thereby making the thymocytes double-positive (DP) (162).

The combinatorial diversity is further reinforced by the junctional diversity that results from nucleotide deletions or insertions during the recombination events, resulting in 10^{15} hypothetical specificities of the αβ-TCR (163).

Formation of TRECs

The rearrangement of gene segments described above is initiated and tightly regulated by recombination activating gene (RAG) proteins, while repair mechanisms involve non-homologous end-joining. RAG proteins mediate double-strand breaks in the DNA at the *recombination signal sequences* that define the coding gene sequences at both ends, followed by ligation of the coding ends (164). The DNA that is located between the recombined V-D-J gene segments is excised, and the blunt ends of the signaling sequence ligate to form circles, which are termed signal joint T-cell receptor excision circles (sjTRECs). The TRECs are stable, remain in the progenitor cells and do not replicate during mitosis (124). The sjTRECs generated during recombination of the α-chain (the δRec-ψJα TRECs) are particularly well suited as biomarkers for the generation of naïve T cells used in newborn screening (165). This is because the α-chain is rearranged late in the process of T-cell maturation, after the major proliferative steps occur in thymopoiesis, and 70%–80% of newly formed αβ-T cells will carry this TREC (164) (Figure 5).

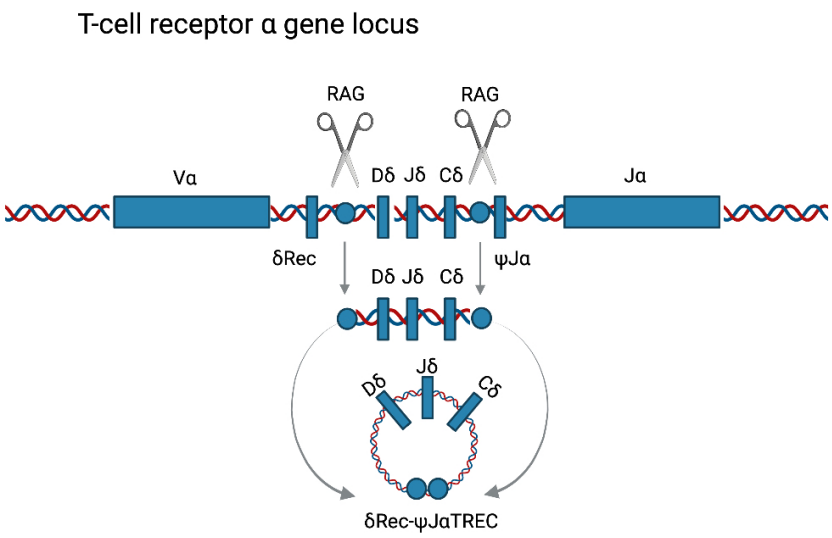

Figure 5. Schematic illustration of the formation of a δRec-ψJα TREC. Recombination activation gene (RAG) proteins mediate double-strand breaks in DNA and the removed DNA strand forms a circle, the δRec-ψJα TREC.

Positive selection to make the TCR repertoire individualized

The DP thymocytes that express a functional TCR that recognizes self-antigens presented on the MHCs of cTECs are subject to positive selection. In this way, a TCR repertoire is generated that is both diverse and individualized. Compared to other antigen-presenting cells, cTECs have unique proteolytic activities, enabling them to present broad repertoires of self-antigen peptides on MHCs (166). Even so, only 1%–5% of DP thymocytes bind self-antigens/MHC with the appropriate affinity, providing them with signals to survive and proliferate (166). In contrast, the majority of DP thymocytes bind insufficiently and undergo death by neglect (166). Positively selected DP thymocytes mature into single-positive (SP) CD4 or CD8 thymocytes, depending on whether they received survival signals via MHC II or MHC I.

Negative selection to purge self-reactive T cells from the repertoire

Negative selection is the process whereby thymocytes that bind self-peptide-MHC complexes with high affinity suffer apoptotic death, resulting in a largely self-tolerant repertoire of mature T cells (167). Although some negative selection occurs in the cortex, the bulk of negative selection takes place in the medulla (167). The SP thymocytes are attracted to the medulla as they start to express CCR7 (159). Through the expression of RANKL and CD40L, they promote the maturation and proliferation of mTECs (168). The medulla is populated by an array of cell types that collectively contribute to presenting almost complete coverage of self-antigens to thymocytes (Figure 4).

Medullary TECs have unique abilities to present thousands of antigens that are normally expressed in peripheral tissues (tissue-restricted antigens, TRAs), largely under the transcriptional control of the autoimmune regulator (AIRE) (167, 169). A classic example of such promiscuous gene expression by mTECs is that of insulin, which is otherwise expressed only by islet cells of the pancreas. The presentation of insulin by mTECs promotes the deletion of insulin-specific T lymphocytes, which otherwise have the potential to induce diabetes (170). Each TRA is presented by a minor fraction of mTECs at any given time-point, so thymocytes need to scan many antigen-presenting cells to be comprehensively exposed to self-antigens. In order to expand the area and timespan for a specific TRA to be presented, TRAs can be transferred from mTECs to be presented by medullary dendritic cells.

Recent studies based on single-cell RNA sequencing have revealed a previously unknown heterogeneity of mTECs (171, 172). Some mTECs have the unique ability to mimic extra-thymic epithelial cells by adopting the activation of transcription factors and gene expression patterns of those peripheral cells (172). Subsequently, thymic mimetic cells acquire phenotypes that resemble the corresponding cells in the periphery, and provide peripheral antigens to reinforce negative selection and central tolerance induction. Examples of mimetic cells are thymic tuft mTECs, myoid mTECs, neuroendocrine mTECs, microfold mTECs and ionocyte mTECs (172).

Other antigen-presenting cells, such as B cells, also reside in the thymic medulla and have been proposed to have a role in central tolerance induction (173). Furthermore, medullary fibroblasts present fibroblast-specific antigens under the control of lymphotoxin, which is produced by maturing thymocytes (174).

Regulatory T cells

Negative selection results in the deletion of most of the thymocytes that have high affinity TCRs against self-peptide-MHC complexes, while only a low number of T lymphocytes egress from the thymus as naïve effector T cells. The elimination of self-reactive CD4 SP T cells is, however, incomplete, as some self-reactive CD4 SP T cells are diverted into FoxP3-expressing regulatory T cells. Regulatory T cells act to suppress activated self-reactive T cells in the periphery (175).

1.4 THYMUS DEFECTS

Congenital thymus defects can result from defective organogenesis, defective TEC development or malfunctioning of TECs.
Failure of thymus development can also be secondary to congenital hematopoietic defects, and in the postnatal situation the thymus can acquire damage from iatrogenic insults. Physiological processes may also clinically mimic primary thymic defects.

DEFECTIVE ORGANOGENESIS

Congential athymia in 22q11DS

The 22q11DS is the most common cause of defective organogenesis leading to congenital athymia (68, 120). Even so, congenital athymia affects only a small minority of all infants with 22q11DS. According to the multicenter study by Ryan and colleagues, 2 out of 263 patients (0.8%) had severe immunodeficiency consistent with athymia (176). This corresponds reasonably well with recent reports from TREC-based newborn screening programs. For example, when 3,252,156 newborns were screened in California, 4 had athymia requiring thymus transplant, corresponding to 0.3% –0.5% of the infants with 22q11DS, based on an assumed birth prevalence of 1/2,148 to 1/4,000 (1, 137, 177).

The absence of a thymus shadow on x-radiograms or the absence of a visible thymus at heart surgery is a rather common finding in patients with 22q11DS, although this does not always correspond to athymia. In most cases, there are T lymphocytes present in the peripheral blood, reflecting a maldescent of the thymus and the presence of ectopic thymus tissue (21).

True athymia results in a severe immunodeficiency similar to SCID, which is fatal if left untreated. Affected infants present with infections caused by bacteria, viruses and fungi, including opportunistic pathogens. Failure to clear these infections may result in organ damage. Vaccinations with live strains of bacteria or viruses entail serious risks for these patients (24).

The immunophenotyping of infants with athymia is usually consistent with T-B+ SCID, with T-lymphocyte counts less than 50 x 10^6/L and a lack of lymphocyte responses to mitogen stimulation (178). However, up to 30%

of affected infants have oligoclonal T cells that give rise to skin rash, gastrointestinal symptoms, lymphadenopathy, eosinophilia and increased levels of IgE, resembling Omenn syndrome (68).

Thymus hypoplasia in 22q11DS

Although complete athymia is a rare manifestation of 22q11DS, milder forms of thymus hypoplasia are present in the majority of patients with 22q11DS (2, 21, 29). Hypoplasia of the thymus in 22q11DS is often proposed as an explanation for the various degrees of T lymphopenia seen in most patients with the syndrome (reviewed in Section 1.1). The architecture and function of the thymus have only scarcely been studied in individuals with 22q11DS (53).

One recent report used a murine model of 22q11DS to study the composition of the thymic stroma. Mice with compound haploinsufficiency for Tbx1 and Crkl displayed the typical 22q11DS phenotype, including hypoplastic thymuses with reduced cellularity (16). The numbers of cells of mesenchymal origin, and specifically fibroblasts were particularly reduced. The authors have argued that defective TGF-β signaling from mesenchymal cells onto TECs affects TEC function and that this explains why compound heterozygotes express significantly less of Foxn1-target genes (16).

In a previous study of human 22q11DS thymuses, the authors described alterations to early thymocyte development and disturbed corticomedullary distribution (53).

The main etiology leading to athymia or thymus hypoplasia in 22q11DS is thought to be haploinsufficiency of the *TBX1* gene, which maps within the deleted region. TBX1 regulates the expression of thousands of genes by histone modification/epigenetic modifications and ensures that chromatin is available as a target to other regulatory factors (179). *TBX1* is expressed in the pharyngeal epithelia and the pharyngeal mesoderm, but not in the neural crest (180).

TBX1 haploinsufficiency

The importance of *TBX1* for thymus development is underlined by the fact that individuals with the 22q11DS phenotype who lack the common 3-Mb deletion of chromosome 22q11.2 but have small deletions encompassing *TBX1* or heterozygous variants in the coding sequence of *TBX1* display the typical phenotype, with heart defects, thymus hypoplasia, hypocalcemia and cleft palate (181).

Furthermore, individuals with 22q11DS with distal deletions not including *TBX1* have better thymopoiesis, as compared to those with proximal deletions (182).

TBX2 haploinsufficiency

Heterozygous missense variants of *TBX2* were found in a low number of individuals affected by a multisystem malformation disorder, that resembles 22q11DS. Three related individuals had heart defects, thymus aplasia or signs of hypoplasia, dysmorphic facies, skeletal abnormalities and cleft palate. Tbx2 knock-out animal models show striking phenotypic similarities, supporting *TBX2* as a candidate gene for this disorder (183). However, more patients need to be identified in order to confirm this hypothesis.

FOXI3 haploinsufficiency

The gene *FOXI3* gene located on chromosome 2p11.2 codes for a transcription factor of the Forkhead box family and is expressed in the ectoderm and endoderm of the pharyngeal apparatus. Recent reports have described seven infants with microdeletions encompassing *FOXI3,* who were identified as having low numbers of TRECs in newborn screening programs. These infants exhibited various degrees of T lymphopenia and some had concurrent hypocalcemia (184, 185).

Defective organogenesis, toxic agents

Retinoic acid

Retinoic acid interacts with TBX1 and plays a role in the formation of the pharyngeal arch arteries and the third pharyngeal pouch (154). High levels of retinoic acid are teratogenic, which became evident when pregnant women who were treated with isoretinoin for acne gave birth to children with multiple malformations resembling the 22q11DS phenotype (186), including thymus aplasia (68).

Ethanol

Maternal alcoholism is known to lead to fetal alcohol syndrome, which resembles the 22q11DS phenotype with regard to facial abnormalities, heart defects and neurobehavioral problems (187). Fetal ethanol exposure has also been associated with chronic impairment of T-cell immunity in affected individuals (187, 188). Furthermore, studies in mice have underlined the detrimental effect of ethanol exposure on thymus development (189, 190), although the mechanisms behind this remain to be elucidated.

Defective organogenesis, gestational diabetes

Although 22q11DS and CHARGE are the most-common causes of congenital athymia, infants born to mothers who have diabetes constitute the third-largest group (68). A possible mechanistic explanation for the athymia in this group is that elevated glucose induces retinoic acid in the pharyngeal region, consequently lowering the level of TBX1.

DEFECTIVE TEC DEVELOPMENT

CHARGE

Most cases of coloboma, heart defects, choanal atresia, growth retardation, genital abnormalities and ear abnormalities (CHARGE) syndrome are caused by haploinsufficiency of chromodomain helicase DNA-binding gene 7 (*CHD7*), which is located on chromosome 8q12 (191). CHD7 is important for formation of the pharyngeal arches, where it is expressed in the mesoderm and endoderm. CHD7 is also considered important for the development of cTECs and mTECs via BMP4 regulated FOXN1 expression (191). The CHARGE phenotype shows overlap with some aspects of the 22q11DS phenotype, including the presence of heart defects, cleft palate, and T-cell lymphopenia of various severity caused by thymus hypoplasia or aplasia (192). Cases with oligoclonal T cells and the Omenn syndrome phenotype have been reported (68, 193). CHARGE constitutes the second-most common cause of congenital athymia (68).

Down syndrome, Trisomy 21

Down syndrome is associated with severe and recurrent infections and a predisposition for autoimmune disease. Lymphopenia is a common finding, although only a small minority of infants with Down syndrome present with abnormal TRECs on newborn screening (137, 194). While the mechanism underlying these phenomena is probably multifactorial, there is evidence that thymus dysfunction contributes to the immune phenotype. Affected individuals have hypoplastic thymuses with reduced cellularity, affecting both the lymphoid and TEC compartments. The medullary compartment is characterized by enlarged Hassall´s corpuscles, indicating early senescence of mTECs (53, 195, 196). Furthermore, increased expression of AIRE is evident, consistent with the fact that affected individuals have three copies of the *AIRE* gene (197). These factors might affect the thymocyte selection processes and contribute to the predisposition to develop an autoimmune disease.

Nude-SCID syndrome, *FOXN1* deficiency

The gene for the transcription factor FOXN1 is encoded on chromosome 17p11.2 and is selectively expressed in thymic epithelia and in the skin. Homozygous mutations in the gene give rise to a similar phenotype in mice and humans, characterized by congenital alopecia, nail dystrophy, tight skin and a rudimentary thymus where the epithelium has failed to differentiate, and eventually giving rise to a severe immunodeficiency (nude-SCID) (198-200). With the introduction of TREC-based newborn screening programs, infants with a slightly different phenotype and heterozygous (n=25) or compound heterozygous (n=2) mutations in *FOXN1* have recently been identified. These infants had low numbers of TRECs and pronounced T-cell lymphopenia, although there was some improvement in these aspects with time, especially for the T-helper lineage. The infants suffered from recurrent infections, which were severe in some cases and mostly of viral etiology. Some individuals had nail dystrophies and sparse hair, although they did not have alopecia (201-204).

PAX1 deficiency

A SCID-like phenotype caused by congenital athymia has recently been described in eight individuals with otofaciocervical syndrome type 2 (OTFCS2), which is caused by bi-allelic loss-of function variants in the *PAX1* gene located on chromosome 20p11.2 (205, 206). The OTFCS2 phenotype includes short stature, dysmorphic facies, abnormal ears, impaired hearing, branchial cleft cysts, anomalous vertebrae, mild intellectual disability, and various degrees of T-cell deficiency due to thymus hypoplasia or aplasia. The described phenotype is consistent with the fact that *PAX1* is expressed in the mesenchymal cells forming the intervertebral discs and cochlea, but also in the endoderm and, subsequently, in TECs (205, 206).

MALFUNCTIONS OF TECS

APS1/APECED

The AIRE protein encoded on chromosome 21q22.3 is indispensable for the promiscuous gene expression and negative selection that is mediated by mTECs (207, 208). The importance of AIRE is illustrated by the autosomal recessive disorder autoimmune polyglandular syndrome type 1 (APS1), also referred to as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), which is characterized by multiorgan autoimmunity, including hypoparathyroidism, adrenal insufficiency and mucocutaneous

Candida infections (209). APS1 cases with later onset and milder phenotypes have been ascribed to autosomal dominant mutations in *AIRE* (210).

More recently, studies conducted in mice have shown that the transcription factor forebrain-expressed zinc finger 2 (Fezf2) acts in cooperation with chromodomain helicase binding protein 4 (Chd4) to control the expression levels of hundreds of tissue-restricted antigens in mTECs, independently of Aire (211). Evidence of associated diseases in humans is currently lacking.

Gene variants affecting cTECs

cTECs are equipped with specialized proteasomes that degrade proteins and produce peptides for presentation on MHC.
The thymoproteasome contains the catalytic subunit β5t, which is encoded by *PSMB11* and is responsible for the production of self-peptides bound to MHC I. In mice, homozygous variants of *PSMB11* impair the positive selection of CD8+ thymocytes, resulting in a restricted TCR repertoire of CD8+ T cells. In humans, homozygosity for the corresponding gene variants is associated with an increased risk for Sjögren´s syndrome (212).

TTC7A deficiency

Bi-allelic mutations in the gene for tetratricopeptide repeat domain-7A (*TTC7A*) lead to intestinal atresia and a combined immunodeficiency (213). TTC7A is expressed in the epithelia of the gut, and in the thymus and to a lesser extent in thymocytes, indicating that the immunodeficiency linked to this condition could be secondary to thymus dysfunction (213).

NFKB2 deficiency

Haploinsufficiency of *NFKB2* (encoding nuclear factor-κB subunit 2) typically gives rise to a deficiency of adrenocorticotropic hormone (ACTH) with variable immunodeficiency (DAVID) syndrome. Affected individuals present with early onset hypogammaglobulinemia and/or various forms of T cell-mediated autoimmunity, most often in the form of alopecia (214). Studies in mice have shown that *NFKB2* deficiency results in a reduced number of mTECs, as well as reduced transcription of Aire in residual mTECs (215). Although expression of *NFKB2* is not confined to the thymus stroma, it is possible that impaired central tolerance mechanisms account for the autoimmunity seen in humans affected by *NFKB2* deficiency.

SECONDARY THYMUS DEFECTS

The development and maintenance of a functioning thymus stroma are dependent upon cross talk with thymocytes. Subsequently, inborn errors of immunity that affect lymphoid progenitor cells will have a negative impact on thymus development. In the classical forms of SCID, T cells are typically absent, and the thymus does not develop. In comparison, hypomorphic mutations in SCID genes allow for development of some T cells with residual function, giving rise to an oligoclonal repertoire of T cells that fails to support proper maturation of the stroma, resulting in impaired negative selection and aberrant formation of regulatory T cells (216, 217). The resulting phenotypes range from SCID with severe immune dysregulation, as in Omenn syndrome, to milder forms with granulomas or autoimmunity as the only manifestation (218). Although much of the current knowledge of secondary thymic defects derive from detailed study of humans with *RAG1* or *RAG2* deficiency and mice with mutations in orthologue genes, other conditions affecting V(D)J recombination (e.g., DCLRE1C deficiency, DNA ligase IV deficiency and Cernunnos) and other forms of T-cell deficiency with residual T-cell function may present with immune dysregulation, presumably involving the same pathophysiological mechanisms (216-218).

The *PRKDC* (protein kinase, DNA-activated, catalytic subunit) gene encodes a protein kinase that is implicated in non-homologous end-joining which is crucial for V(D)J recombination. In addition, PRKDC is required for the AIRE-dependent expression of TRAs in mTECs, adding a mechanism that might contribute to the autoimmunity and the presence of autoantibodies observed in patients with *PRKDC* mutations (219).

THYMIC INVOLUTION

Thymic output of naïve T cells peaks during fetal life and in the first year of life, and this peak is followed by a gradual decline of thymopoesis with age (220). The decline of thymic output is paralleled by a diminished thymus mass and a disrupted organization of the cortical and medullary compartments (221). A shift in cell composition is also evident with ageing, with reductions in the numbers of thymocytes and TECs in favor of increases in the numbers of fibroblasts and amounts of adipose tissue (221). In mice, age-dependent declines in the expression levels of Aire and MHC II have been demonstrated, which result in decreased presentation of TRA and impaired induction of central tolerance induction (222). In addition to the

effects of age on the thymic stroma, the ageing thymus is provided with a decreasing number of lymphoid progenitors, such that with age the progenitors are more prone to develop into the myeloid linage rather that lymphoid cells (222).

The mechanism underlying the age-related involution of the thymus is likely multifactorial, involving a combination of thymus-intrinsic and -extrinsic factors. Throughout life, the fine-tuned network of interacting cells is vulnerable to the repeated insults posed by extrinsic factors, such as stress, infections and hormonal alterations.

IATROGENIC INSULTS TO THE THYMUS

Thymectomy

The most-obvious iatrogenic insult to the thymus is thymectomy, especially if it is done in the neonatal period. Thymectomy is performed in children who are undergoing open surgery for heart defects, to facilitate access for the surgeon. Long-term follow-up studies after neonatal thymectomy show immunological alterations consistent with early immunosenescence, such as low numbers of TRECs, low numbers of naïve T cells and disrupted TCR repertoires (223-225). Although most thymectomized individuals are asymptomatic during childhood, epidemiological studies show a marked increase in the risk of autoimmunity and a slight increase in the risk of cancer after childhood thymectomy (226). The authors of that study stated that due to the young age of the participants (mean age, 14 years), the full effect of thymectomy might not yet be evident (226). The follow-up studies of thymectomized children demonstrate the importance of the thymus for maintaining self-tolerance into adulthood. Considering the high prevalence of heart defects in individuals with 22q11DS, some will be subjected to thymectomy (227). Based on their underlying immune defect, these individuals might be more susceptible to the effects of thymectomy, as compared to children without genetic defects, although definitive evidence is still lacking.

Radiation, chemotherapy, graft-versus-host-disease

The thymus is essential for the reconstitution of T-cell immunity following HSCT, and preserved thymic function is beneficial in terms of the long-term prognosis. After HSCT, children tend to regenerate their T-cell compartments faster than adults, reflecting the fact that children generally have superior thymic capacity (228). The thymus is sensitive to radiation, chemotherapy

and glucocorticoids, which are often part of the HSCT treatment regiments (229). Although rapidly proliferating thymocytes might the be most sensitive to such treatments, studies in mice have also demonstrated a substantial loss of TECs (230). Furthermore, the thymus is a target of graft-versus–host disease (GvHD), which results in reduced cellularity of the organ and reduced thymopoiesis, sometimes leading to persistent impairment of immunity (228). Current research is aimed at establishing a therapy to prevent thymus injury and to support thymus regeneration post-HSCT (229).

DIAGNOSIS OF CONGENITAL ATHYMIA

Congenital athymia is a rare condition that results in an inability to develop T cells. Affected infants may appear healthy at birth, and the onset of symptoms from opportunistic infections are often insidious and unspecific, rendering the diagnosis of the condition challenging (231). Cases of congenital athymia are identified through newborn screening using TREC, although screening is not available worldwide. Importantly, TREC screening followed by the enumeration of T lymphocytes does not differentiate T-B+ SCID of hematopoietic origin from congenital athymia. It is therefore vital that screening algorithms include second-tier tests that can differentiate congenital athymia from T-cell lymphopenia of hematopoietic origin, as these conditions require different treatment strategies to ensure long-lasting reconstitution of T-cell immunity. As a first measure, the molecular genetic studies need to assess not only SCID, but also all known causes of congenital athymia. While gene panels based on NGS identify variants in specific genes, they are not as sensitive to detect copy number variations such as the microdeletion in 22q11DS (10). Even so, 22q11DS can be detected with this approach, by application of software that detects variants in dosage of specific genes in the typically deleted region, e.g., *TBX1* (145). In order to determine the size of the deletion, other methods such as chromosomal microarrays need to be performed (10). As an example, a chromosomal array is performed when SCID is ruled out in all identified infants with T-cell lymphopenia in the Catalonian TREC screening program (138). If no genetic cause is identified in an infant with T-B+ SCID immunophenotype, *in vitro* models can be used to differentiate congenital athymia from T-B+ SCID of hematopoietic origin. Two recent reports have demonstrated that hematopoietic stem cells from athymic donors were able to differentiate into double positive T lymphocytes upon co-culture with growth factors and murine stroma cells providing delta like signaling (232, 233).

Treatment with thymus transplantation

The therapy of choice for congenital athymia involves the transplantation of cultured, allogeneic thymus tissue. A tissue graft is acquired from unrelated children undergoing cardiac surgery. HLA matching is not performed, although there is some evidence that incompatability with regard to AB0-blood group gives slower reconstitution of the T-cell compartment (231). In order to prevent GvHD, the tissue is cultured to remove the thymocytes and infants with symptoms consistent with Omenn syndrome receive conditioning therapy (68). Tissue slices are then introduced into the musculature of the thigh of the recipient. The reported long-term survival of thymus transplantation is approximately 75%, with infections being the main cause of death.

2 AIMS

The overall aim of this thesis was to study the outcome of TRECs at birth in infants with 22q11DS, and to investigate if low TRECs are predictive of persistent thymus dysfunction in individuals with 22q11DS.

Specific Aims

Paper I

To study the frequency of low numbers of TRECs at birth in infants with 22q11DS, and to examine possible correlations between low numbers of TRECs and diminished T-lymphocyte numbers and a more-severe clinical course during the first year of life. An additional aim was to evaluate if a second-tier test could be applied on the newborn screening card to verify the haploinsufficiency of *TBX1*.

Paper II

To investigate if low numbers of TRECs at birth in individuals with 22q11DS are associated with long-term restrictions in thymopoiesis and signs of premature immunological senescence, manifested as low numbers of naïve T cells, low numbers of TRECs, restricted TCR repertoires, and short telomere lengths. A second aim was to study the effects of low numbers of TRECs on additional available laboratory and clinical variables.

Paper III

To assess if low TRECs at birth in individuals with 22q11DS are associated with long-term deficits in central tolerance, monitored as increased prevalence of autoantibodies. An additional aim was to identify novel syndrome-specific autoantibodies.

3 PATIENTS AND METHODS

An overview of **Papers I-II** is provided in Figure 6.

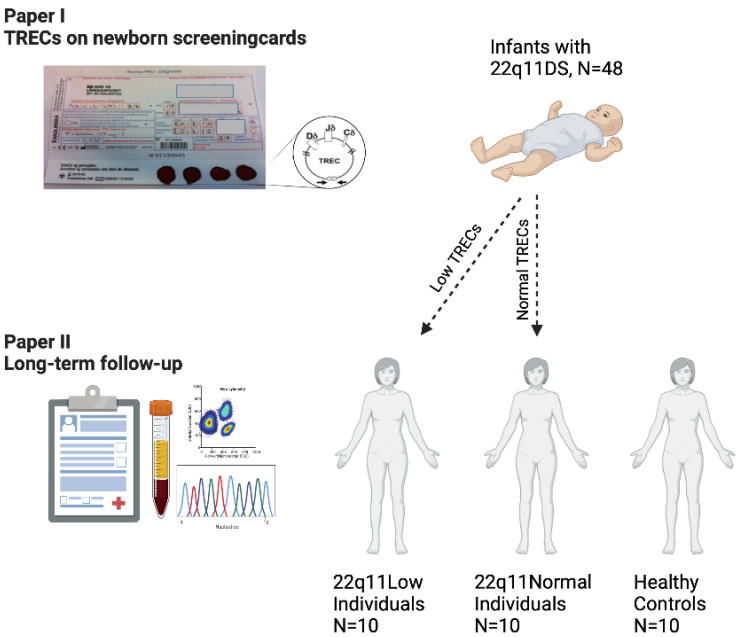

*Figure 6. Schematic overview of methods and participants in **Paper I** and **Paper II**.*

*For **Paper I**, a retrospective analysis of TRECs was performed by RT-qPCR on original newborn screening cards from 48 individuals with 22q11DS.*

*For **Paper II**, a long-term follow-up was performed for: 10 individuals with 22q11DS and low numbers of TRECs at birth (22q11Low); 10 individuals with 22q11DS and normal numbers of TRECs at birth (22q11Normal); and 10 healthy controls, with the groups matched for age and gender. The follow-up included quantification of TRECs in fresh, peripheral blood, flow cytometry for the characterization of lymphocyte subsets, deep sequencing of T-cell receptor repertoires, and PCR for the assessment of telomere lengths, as well as a health questionnaire and review of the available health records. Created with Biorender.com.*

Subjects

For **Papers I–II** the participants were recruited from the Queen Silvia Children´s Hospital in Gothenburg, which is a national reference center for pediatric immunology and pediatric cardiac surgery. Since 1997, there is a multidisciplinary collaboration regarding 22q11DS in the region, including specialists from pediatric immunology, cardiology, endocrinology, a cleft-lip and palate team, neurology, neuropsychology and neuropsychiatry, physiotherapy, an orofacial team, clinical genetics, and adult infectious diseases at separate departments of the Sahlgrenska University Hospital. Children under the age of 18 years with suspected or confirmed diagnosis of 22q11DS are generally referred to the pediatric immunology clinic from the subspecialists in the collaboration or from out-patient clinics in the region. Previous studies carried out by Sólveig Óskarsdóttir et al. have resulted in a well characterized cohort and improved awareness of the syndrome within the region (3, 21, 24, 43, 177). **For Paper III**, an extended cohort was recruited, mainly from the Queen Silvia Children´s Hospital in Gothenburg, although individuals with 22q11DS seen at Halland Hospital, Halmstad were also invited to participate. Participants in **Paper I–II** were recruited in 2012 and in 2016–2017, respectively, whereas the extended cohort in **Paper III** were recruited in 2022.

All subjects had their 22q11DS diagnosis confirmed by FISH, MLPA or chromosomal micro array.

3.1 PAPER I

Subjects

To investigate the frequency of low numbers of TRECs at birth we invited all children with 22q11DS, who were aged <18 years and had a previous or present referral to the Queen Silvia Children´s Hospital to participate. Deceased patients who would have been less than 18 years of age at the point of inclusion were also included.

Retrieval of newborn screening cards

In Sweden, all newborn screening cards are sent for analysis at the centralized laboratory at Karolinska University Hospital in Solna. Dating back to 1975, the used screening cards have been catalogued and kept in cold storage, for the purposes of quality control, refinement of methods, and research (234). We retrieved the stored screening cards, that were originally obtained at birth from our study participants with 22q11DS. For comparison, we also retrieved the screening cards from selected infants with other known inborn errors of immunity, such as T-B+ SCID (*IL2RG* deficiency), T-B- SCID (*RAG* deficiency) and a classical B-cell deficiency (X-linked agammaglobulinemia, *BTK* deficiency). In addition, 750 prospectively collected screening cards from anonymous newborns were used as controls. Previous studies showed that Guthrie cards that were properly stored could be used for retrospective analyses (235, 236).

TREC-KREC assay

A triplex RT-qPCR assay was used for the quantification of TRECs, KRECs, and the house-keeping gene *ACTB* from the original newborn screening cards. The method was developed in-house at the Karolinska Institute, Huddinge, and the same method was used in the trials preceding implementation of population based TREC screening in Sweden (147). The method is described in detail in **Paper I**.

TBX1 assay

An additional RT-qPCR was performed on the same DNA eluate from the dried blood spots, as described in **Paper I**, to quantify the expression levels of *TBX1* and the reference gene *PTBP1*. *TBX1* maps to the deleted region on chromosome 22, while *PTBP1* maps to chromosome 19.

Immunophenotyping

The absolute counts of T lymphocytes (CD3+), T-helper lymphocytes (CD4+), cytotoxic T lymphocytes (CD8+) and B lymphocytes (CD19+) were determined by standard methods of flow cytometry at the time of diagnosis, and the results were retrospectively obtained from the medical charts. The analyses were performed at the Department of Clinical Immunology, Sahlgrenska University Hospital. Lymphopenia was defined as an absolute count below the 10^{th} percentile, as compared to age-matched reference values (33).

Clinical outcome

A chart review was performed to obtain the following information from the first year of life:

- The presence of a heart defect identified at echocardiography
- The presence of hypocalcemia (as defined by the treating physician)
- The occurrence of severe bacterial infection (i.e., any episode of pneumonia, septicemia, meningitis or osteomyelitis); and
- The occurrence of severe or recurrent viral infections (defined as one episode requiring hospital admission or more than six episodes in total)

Group comparisons and statistical analysis

The study participants with 22q11DS were divided into two groups, based on the cut-off value of 8 TRECs /μL. This cut-off was used in the pilot study, preceding population based newborn screening in Sweden (147). The resulting groups were compared with regards to lymphocyte counts and the presence of heart defects, hypocalcemia and severe and recurrent infections during the first year of life. The Mann-Whitney U-test was used for comparison of continuous variables, while Fisher´s exact test was used for comparison of categorical variables.

3.2 PAPER II

Paper II describes the results of a long-term follow-up study of infants with 22q11DS and low numbers of TRECs at birth.

Subjects

Based on the outcomes of the TREC assay on the newborn screening cards in **Paper I**, we included the 10 individuals with lowest (22q11Low) and highest (22q11Normal) TREC respectively, and 10 healthy controls who were matched for age and gender (Figure 6).

Chart review and health questionnaire

A review of the health records was performed for the 22q11DS participants. In addition to the information already retrieved for **Paper I**, we also noted the presence of malformations, neurodevelopmental disorders, autoimmunity, allergies and asthma (as defined by the treating physician). All study participants filled out a questionnaire that contained questions regarding their health, history of vaccinations, infections, allergies and autoimmunity. The presence of significant and prolonged infections was defined as described in **Paper II**.

TRECs at follow-up

TRECs and the reference gene *GAPDH* were quantified in DNA samples obtained from fresh, whole blood using RT-qPCR, as previously described by van Zelm et al. (164), and detailed in **Paper II**. TRECs are reported as number of TRECs per million cells.

Immunophenotyping

Fresh, whole blood was used for the enumeration of lymphocyte subsets by flow cytometry. The analyses were performed at the Department of Clinical Immunology, Sahlgrenska University Hospital in accordance with well-established and standardized methods (237, 238). Details regarding the definition of cell types, staining, and gating strategies can be found in **Paper II**.

Sequencing of T-cell receptor repertoires

DNA was prepared from sorted lymphocyte subsets, followed by amplification of six replicates of 50 ng of DNA from each sample of naïve T-helper lymphocytes and naïve cytotoxic T lymphocytes, and one replicate of

100 ng of DNA from each memory subset. Subsequently, each replicate was subjected to Illumina sequencing of the rearranged *TRB* genes, encoding the β chain of the T-cell receptor. The sequencing of six replicates from the naïve T-helper lymphocytes and naïve cytotoxic T lymphocytes enables calculation of a clonality score (239). Briefly, the occurrence of the same clone (with identical V gene and amino acid sequence of the CDR3 region) in replicate samples indicates higher clonality. Details of the procedure, including definition of the cell types, staining, and gating strategy applied for the flow cytometry-based sorting of cells are provided in **Paper II**.

Telomere length analysis

Telomere lengths were assessed by PCR using the DNA from sorted lymphocyte subsets, as described previously (240, 241) and detailed in **Paper II**.

Immunoglobulins and specific antibodies

Standardized methods were used to the samples of serum, to assess the total levels of immunoglobulins, as well as the abilities of IgG antibodies to recall antigens to *Haemophilus influenzae* type b, *Streptococcus pneumoniae,* tetanus toxoid, cytomegalovirus and Epstein-Barr virus. The analyses were performed at the Departments of Clinical Immunology and Microbiology at Sahlgrenska University Hospital.

FASCIA and ELISPOT

The *in vitro* immune responses of T lymphocytes were assessed by flow cytometric assaying for specific cell-mediated immune responses in activated whole blood (FASCIA) (242). An enzyme-linked immunospot assay (ELISPOT) was used to assess the *in vitro* immunoglobulin production capabilities of B lymphocytes (243). Both analyses, which were described in **Paper II,** were performed at the Department of Clinical Immunology, Sahlgrenska University Hospital.

Cytokines

The commercially available V-plex assays were used to measure the levels of cytokines and C-reactive protein (CRP) in plasma (MSD, Rockville, MD). The levels of the following cytokines were measured: IFN-γ, IL-1β, IL-10, IL-13, IL-17A, IL-21, and thymic stromal lymphopoietin (TSLP).

Multivariate discriminant analysis

A multivariate discriminant analysis (orthogonal projection to latent structures by means of partial least-squares discriminant analysis, OPLS-DA) based on the entire dataset was used to screen for differences between the

groups (SIMCA software; Sartorius Stedim Data Analytics AB, Umeå, Sweden).

Group comparisons and statistical analysis

The Kruskal-Wallis test, followed by Dunn´s correction, was used for univariate comparisons between groups, except for comparisons of telomere lengths, for which a linear mixed model was applied.

3.3 PAPER III

In **Paper III**, a proteome wide profiling of autoantibodies was performed in the 22q11Low and 22q11Normal individuals, who also participated in Paper II. The study was performed in three steps, as outlined in Figure 7. The methods are briefly described below and a detailed description is provided in **Paper III.**

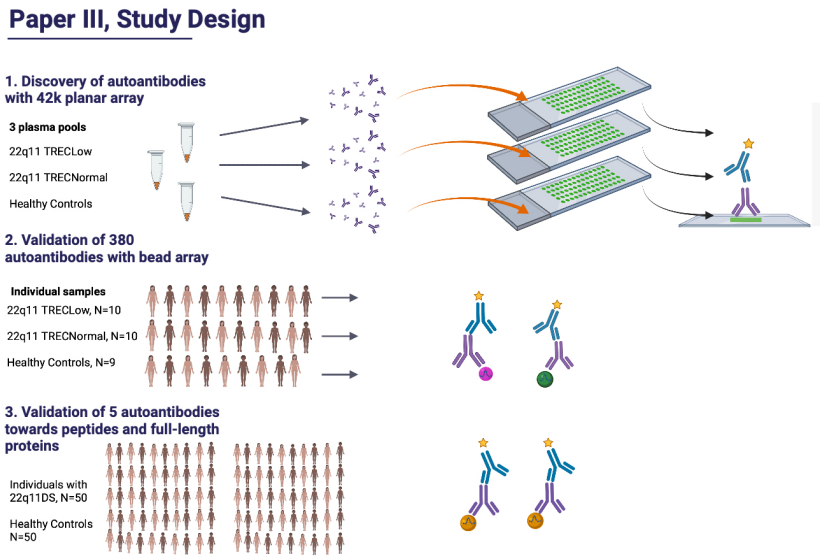

*Figure 7. Schematic overview of study design in **Paper III**. In phase I (discovery phase) a planar array with 42,100 peptides was used to screen for autoantibodies in pools of plasma from the three study groups: individuals with 22q11DS and low TRECs at birth (22q11Low), individuals with 22q11DS and normal TRECs at birth (22q11Normal) and healthy controls. In phase 2 (first validation phase) a bead array was used to screen for autoantibodies in individual samples from: 10 22q11Low individuals, 10 22q11Normal individuals, and 9 healthy controls. The array covered 380 antigens that were reactive in both plasmapools from 22q11DS individuals in phase I. In phase 3 a bead array was performed for validation of autoantibodies to 5 peptide antigens and the corresponding full-length proteins in individual samples from 50 individuals with 22q11DS and 50 healthy controls. Created with Biorender.com.*

Subjects

Subjects from the study in **Paper II** were included in Phase 1 and Phase 2. For Phase 3, the cohort was expanded to include 50 individuals with 22q11DS and 50 healthy controls, matched for age and gender.

Phase 1, Discovery phase

A planar array with 42,100 human peptide antigens printed on glass slides was used to screen for IgG autoantibodies in pools of plasma from the three groups. The planar array, which was developed as part of the Human Protein Atlas project, was previously validated for high-throughput detection of potential autoantibodies (244, 245). The peptide antigens consist of sequences of 16-202 amino acids (mean, 81 amino acids), which were selected based on their unique representations of their corresponding full-length proteins and their low levels of homology with other proteins (244). The binding of IgG in the sample to the antigens printed on the array was detected with a secondary anti-human antibody.

Phase 2, Validation phase 1

Based on the results from Phase 1, antigens for which there was reactivity in both the 22q11Low and 22q11Normal plasma were selected for further analysis with a bead based array, as previously described (246). In total, 380 antigens were coupled to magnetic beads. Each individual sample was incubated with a suspension of the coupled beads. Autoantibodies directed against coupled antigens were detected through the binding of a fluorescent secondary anti-human antibody. The median fluorescence intensity (MFI) was calculated based on the detection of at least 50 beads of each identity by the Luminex-based technology.

Phase 3, Validation phase 2

Five antigens were selected for further validation, based on their high frequencies of reactivity in the 22q11DS samples and the high intensity levels of the reactive signals. The biological properties of these antigens and their potentials as autoantigens were also considered during the selection process. The peptide antigens and their full-length protein counterparts were coupled to magnetic beads, and the reactivities to those antigens in he samples from an extended cohort were assessed.

Data analysis

The planar array (Phase 1) and the bead arrays (Phases 2 and 3) that were used to detect autoantibodies generated different signal variations and different backgrounds. This warranted separate strategies to define reactive signals for the two methods, respectively, and similar approaches have been

applied in previous studies (246, 247). For the planar array, the data were log2-transformed and the mean MFI generated from the binding to all 42,100 antigens was calculated. A reactive signal was defined at three levels of stringency: the lower cutoff, a signal greater than the sample mean + 2SD; the intermediate cutoff, a signal greater than the mean + 4SD; and the higher cut-off, a signal greater than the mean +8 SD. The Chi^2-test was applied to test for differences in the proportions of reactive antigens between the pooled samples.
For the bead array, antigen specific cutoffs were calculated. The data were log2-transformed and a reactive signal was defined as a signal greater than the mean MFI in the control group + 3 SD. Fisher´s exact test was used to test for differences in the proportions of reactivity to an antigen between the groups.

Methodologic limitations

The main limitation of **Paper I** was the retrospective design of the study. Although original newborn screening cards have been used in other retrospective studies (235, 236), we cannot exclude the possibility that the process of storage affects the outcome of the TREC assay. On the other hand, the use of stored newborn screening cards enabled us to perform the study, which would have been postponed at least a decade if a prospective inclusion was to be applied. The inclusion of individuals with known 22q11DS entails a risk of inclusion bias, towards more-severely affected infants. The cohort was, however, comparable to other 22q11DS cohorts in terms of the presence of heart defects, hypocalcemia and T-cell lymphopenia, and the subjects were referred from various subspecialties for a multidisciplinary evaluation (2). Although the genetic defects in our study participants were not described in detail, all the participants had their diagnosis confirmed by standard genetic methods and, in addition, *TBX1* haploinsufficiency was confirmed by analysis of the newborn screening cards.

The main limitation of **Paper II** was the low number of participants, which yielded inadequate power in some of the univariate statistical analyses. The number of individuals with 22q11DS available for inclusion was limited, not least in the 22q11DSLow group, as three of them died in infancy. We could have increased the power of the statistical analyses by including more 22q11Normal participants, and more healthy control subjects, or performed a multicenter study. Despite the low numbers, most of the variables pointed in the same direction, towards persistent impairment of thymopoiesis and premature immunosenescence in the individuals with 22q11DS and low numbers of TRECs at birth. These findings were reinforced by the clear separation of the groups in the multivariate analysis. Another limitation of

Paper II was the follow-up time (median, 16 years). This could be considered a long period of time in many aspects, and it was sufficient to detect differences between the groups in terms of laboratory variables. Even so, the individuals were still young at follow-up, and the follow-up time was insufficient for evaluation of how low numbers of TRECs at birth affect the clinical outcome.

The main concern of the explorative method used in **Paper III** was the use of peptide fragments instead of full-length proteins. The peptide fragments on the planar array are linear and lack the secondary structures and post-translational modifications of full-length proteins (244, 248). The bead array used in Phases 2 and 3 may allow some secondary structures to form. The main strength of the planar array is its high density, covering approximately 94% of the human proteome (244, 248). The lack of pre-defined cutoffs for positive outcomes was another challenge in **Paper III**, as positive outcomes had to be defined separately for Phase 1 and Phases 2 and 3, respectively.

4 RESULTS

4.1 PAPER I

Subjects

Forty-eight individuals with 22q11DS, born in 1993–2010, were included (33 females, 15 males). Most of the subjects had a heart defect (81%), while 40% had hypocalcemia during the first year of life. Bacterial and viral infections were noted in 21% and 44%, respectively.

Overall, 37 of the 88 eligible individuals with 22q11DS did not reply to the invitation, which was sent once by postal mail. Three individuals declined to participate.

TREC-KREC assay

The infants with 22q11DS had significantly lower numbers of TRECs, as compared to the prospectively screened anonymous newborns. Nine of 48 infants with 22q11DS (19%) had TREC numbers below the cut-off of 8 copies/μL (defined as abnormal). The KREC numbers did not differ between the infants with 22q11DS and anonymous newborns (Figure 8).

Lymphocyte subsets

When the entire group of 48 infants with 22q11DS was considered, T-cell lymphopenia was noted in a majority (67%), and was more frequent for T-helper cells (63%), than for cytotoxic T cells (48%). B-cell lymphopenia was noted in 21% of the individuals (Figure 2 in **Paper I**).

TBX1

The normalized level of expression of *TBX1* was consistently lower in 22q11DS individuals, being approximately half that of the control group of anonymous newborns (Figure 3 in **Paper I**).

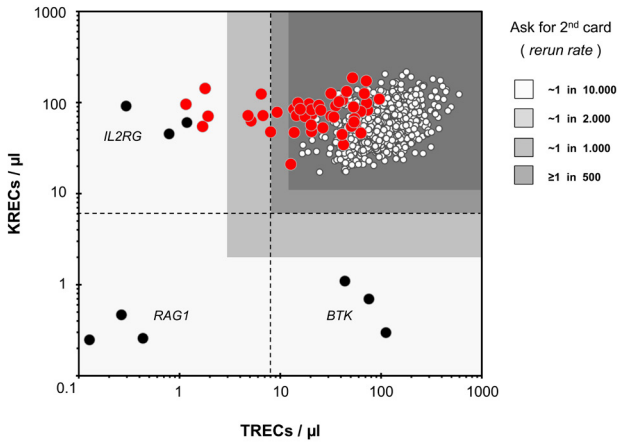

Figure 8. Numbers of TRECs and KRECs per µL of blood from the newborn screening card, in infants with 22q11DS (red dots), infants with inborn errors of immunity (black dots), and in anonymous newborns (white dots). The dotted lines represent the cutoffs that were used in the pilot study, preceding population-based newborn screening in Sweden (147). The areas shaded in gray depict the how different cutoffs affect the frequency with which an infant from the general population would be called back for new samples. IL2RG, i.e., T-B+ SCID; RAG1, i.e., T-B- SCID; BTK, X-linked agammaglobulinemia i.e., B cell-deficiency. Reproduced with permission from Springer Nature (249).

Impact of abnormal TRECs on lymphocyte counts

When infants with 22q11DS and abnormal numbers of TRECs were compared to the 22q11DS group with normal numbers of TRECs, the absolute counts of cytotoxic T lymphocytes at diagnosis were significantly lower ($400x10^6$/L vs. $580x10^6$/L). There were no differences regarding the number of T-helper lymphocytes or B lymphocytes (Table I in **Paper I**).

Impact of abnormal numbers of TRECs on the clinical course

A larger proportion of the infants with 22q11DS and abnormal numbers of TRECs had significant viral infections during the first year of life (67%), as compared to infants with 22q11DS and normal numbers of TRECs (38%). There were no differences between the two groups regarding bacterial infections during the first year of life, the presence of heart defects or hypocalcemia (Table I in **Paper I**).

4.2 PAPER II

Subjects

The three groups in this study were comparable regarding age and gender:
22q11Low: 6 females, 4 males, median age of 15.8 years, range 8.1–22.5 years
22q11Normal: 7 females, 3 males, median age of 15.8 years, range 7.7–23.1 years; and
Healthy controls: 6 females, 4 males, median age of 15.5 years, range 9.2–22.2 years.

From the original cohort of 48 individuals with 22q11DS, three infants with abnormal numbers of TRECs had succumbed. The median TREC count for the included 22q11Low individuals was 8 copies/μL (range 1–16), as compared to the median TREC count in the 22q11Normal group, which was 62 copies/μL (range 43–96)

A detailed description of the subjects is provided in **Paper II**.

Clinical outcomes

The frequencies of reported significant infections did not differ between the two groups of 22q11DS participants, although both groups reported more infections than the healthy controls. Autoimmunity was noted in 3 out of 10 individuals in the 22q11Low group, as compared to 1 out of 10 in the 22q11Normal group and in the healthy controls. The groups did not differ concerning diagnosed allergies or asthma.

TRECs and naïve T lymphocytes

When TRECs were quantified in whole blood at follow-up, the TREC numbers were significantly lower in the 22q11Low individuals, compared to both the healthy controls and the 22q11Normal group (Figure 9 a).

At follow-up, the absolute counts and proportions of T-helper cells were significantly lower in the 22q11Low group, as compared to the healthy controls and these differences were most evident for the naïve T-helper cells (Figure 9 b, c).

The absolute counts and proportions of naïve cytotoxic T cells were lower in the 22q11Low group, as compared to the healthy controls (Figure 9 d, e).

When comparing the 22q11Low group and the 22q11Normal group, there were no differences in the absolute counts of naïve T-helper lymphocytes or naïve cytotoxic T cells, although the 22q11Low group showed decreased proportions of naïve T-helper cells as compared to the 22q11Normal group (Figure 9 b-e).

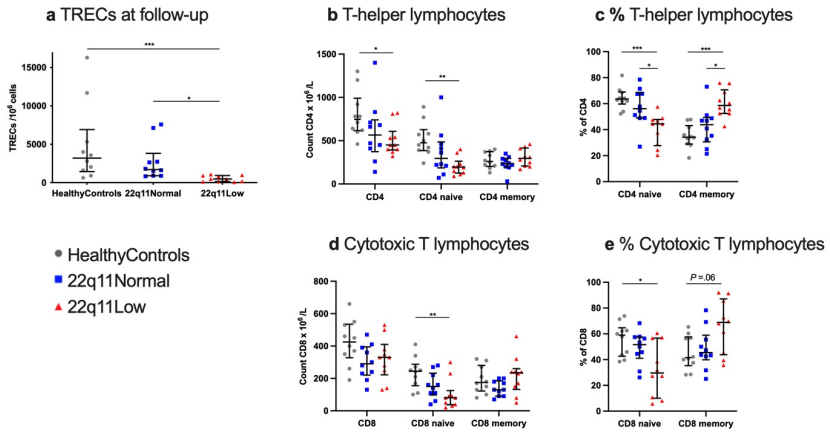

*Figure 9. Outcomes for the TRECs and T-lymphocyte subtypes at follow-up. Numbers of TRECs per million cells in whole blood **(a)**. T-helper lymphocyte counts **(b)** and proportions **(c)**. Cytotoxic T-lymphocyte counts **(d)** and proportions **(e)**. The 22q11Low individuals are depicted with red triangles, the 22q11Normal individuals with blue squares and healthy controls with gray dots. Reproduced with permission from Springer Nature (250).*

T-cell receptor Vβ repertoires

When comparing the 22q11Low group to the healthy controls and to the 22q11Normal individuals, skewed usage towards the dominant V genes 19, 12-3 and 29-1 was noted (Figure 10). In addition, two J-gene segments were used less-frequently in the naïve T-helper lymphocytes of 22q11Low individuals, as compared to the healthy controls and 22q11Normal individuals (data not shown).

Telomere lengths and clonality scores

Comparing the 22q11Low group to the healthy controls, the relative telomere lengths of the naïve cytotoxic T cells were shorter and there was a trend to higher clonality scores. The relative telomere lengths and clonality scores of the naïve T-helper cells did not differ between the three groups, and there were no differences in relative telomere lengths for memory T-cell subsets or B cells (Figure 2, **Paper II**).

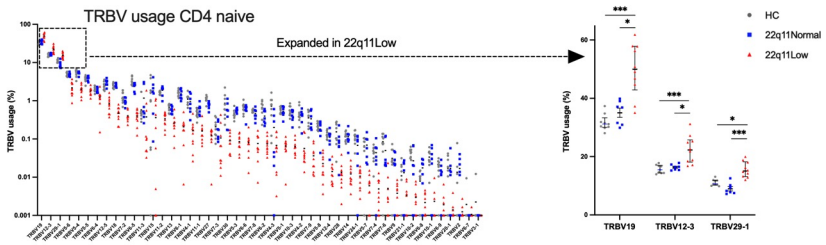

Figure 10. The usage levels of TRVβ genes in 22q11Low individuals (red triangles) 22q11Normal individuals (blue squares) and healthy controls (gray dots). The genes are ordered according to usage rate in 22q11DS individuals, with the most-frequently used genes on the left and the least-frequently used genes on the right. The inset to the far right shows a magnification of the most-frequently used genes Reproduced with permission from Springer Nature (250).

Multivariate analysis

When all available data from the evaluation at follow-up were included in a multivariate discriminante analysis, a clear separation between the three groups was observed (Figure 11 a). The distinction by the model of the 22q11Low group from the healthy controls was robust, as indicated by R2Y=0.89 and Q2=0.86, where R2Y estimates the accuracy of the model, and Q2 the predictive ability of the model (251).

As expected, the differences between the groups regarding the following variables contributed to this separation: TREC counts and proportions of naïve and memory T-helper lymphocytes and cytotoxic T lymphocytes.
The multivariate analysis revealed that large proportions of T-helper type 17 cells (Th17) were associated with the 22q11Low group (Figure 11 b). Univariate comparisons confirmed a significantly larger proportion of Th17 cells in 22q11Low individuals (17%) compared to healthy controls (6%).

The multivariate analysis showed that large proportions of naïve B lymphocytes were associated with both groups of 22q11DS individuals, while there was a negative association with memory B lymphocytes for both groups. Univariate comparisons verified that both the 22q11Low and 22q11Normal groups had larger proportions of naïve B lymphocytes than the healthy controls (70% and 74% vs. 60%), whereas the proportions of class-

switched memory B cells were smaller compared to those of the healthy controls (3.4% and 3.9% vs. 7.2%), (Figure 3, **Paper II**).

The groups did not differ with regard to T-lymphocyte proliferative responses *in vitro*, B-lymphocyte immunoglobulin producing capacity *in vitro* or antibodies to recall antigens (data not shown).

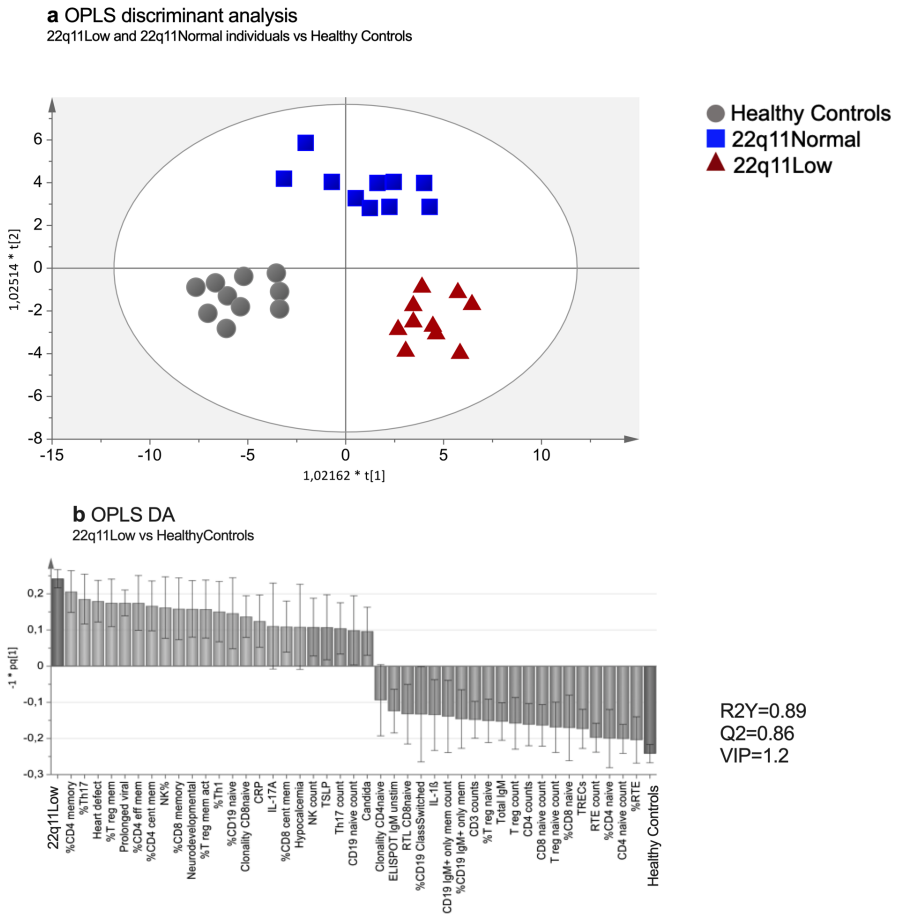

Figure 11. Multivariate discriminant analysis based on the entire dataset. OPLS-DA score plot of 22q11Low individuals (red triangles), 22q11Normal individuals (blue squares) and healthy controls (gray dots) (a). OPLS-DA column-loading plot showing how the variables associate with the 22q11Low group and healthy controls, respectively (b). Reproduced with permission from Springer Nature (250).

4.3 PAPER III

Subjects

The subjects in Phases 1 and 2 were previously described in **Paper II**. For Phase 3, the cohorts were expanded to include 50 individuals with 22q11DS and 50 healthy controls. The characteristics of these study participants are described in Table 1 in **Paper III**.

Phase 1, planar array

A schematic three-dimensional overview of the reactivity pattern in one pooled sample is shown in Figure 12.

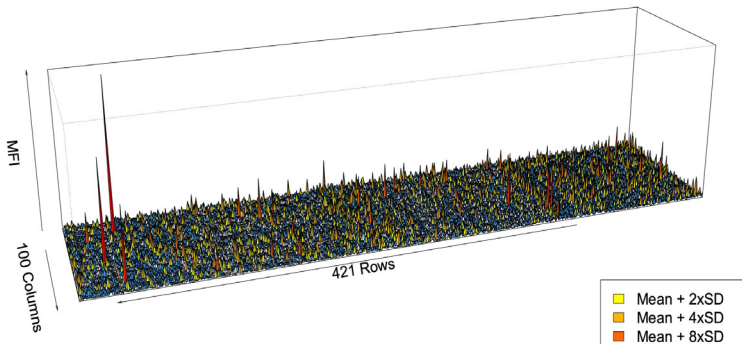

Figure 12. Schematic 3D overview of the reactivities to 42,100 antigens in the planar array for one of the pooled samples. Reactive signals with intensity > mean + 2SD are illustrated by yellow peaks, reactive signals with intensity > mean + 4 SD are illustrated by orange peaks, whereas reactive signals with intensity > mean + 8SD are illustrated by red peaks. With permission from Ronald Sjöberg, Affinity Proteomics, SciLife lab.

The 22q11Low group showed autoantibodies to more of the 42,100 antigens on the array, as compared to the healthy controls when applying all three cutoff levels (lower cutoff: 1399 vs. 998 antigens; intermediate cutoff: 309 vs. 140 antigens; and higher cutoff: 41 vs. 23 antigens), (Figure 2 in **Paper III)**.

The 22q11Low group did not differ from the 22q11Normal group with respect to the proportion of the 42,100 antigens that were reactive at the lower cutoff (1399 vs. 1472 antigens), while it had more reactive antigens than the 22q11Normal group, both at the intermediate cutoff (309 vs. 227 antigens) and at the higher cutoff (41 vs. 20 antigens), respectively, (Figure 2 in **Paper III)**.

The 22q11Normal group had autoantibodies to a greater proportion of the 42,100 antigens than the healthy controls at the lower cutoff (1472 vs. 998 antigens), and at the intermediate cutoff (227 vs. 140 antigens), while the proportion of reactive antigens did not differ between the two groups at the higher cutoff (20 vs. 23 antigens), (Figure 2 in **Paper III)**.

A larger proportion of reactive antigens was shared between the 22q11Low and the 22q11Normal group at the lower cutoff and at the intermediate cutoff, as compared to the proportion shared by any of these groups with the healthy controls, (Figure 2 in **Paper III)**.

Phase 2, bead array

The bead array was based on 380 antigens that were reactive in the pooled samples from both groups of individuals with 22q11DS in the planar array. In total, 146 of these antigens were verified as reactive in the individuals with 22q11DS, whereof 89 antigens were reactive in one individual, 32 reactive antigens were shared by 2 individuals, 16 reactive antigens were shared by 3 individuals, 5 antigens were shared by 4 individuals, 3 antigens were shared by 5 individuals and 1 antigen was shared by 8 out of 20 individuals with 22q11DS, (Figure 3 in **Paper III)**.

Based on the high frequencies of reactivity in the 22q11DS samples, and the strong intensities of the signals, the following five antigens were selected for further study: OTUD5, TNFRSF11B, MTFR1L, LDLRAD1 and RAB35.
Five samples were positive for antibodies directed against the antigen ovarian tumor deubiquitinase 5 (OTUD5; deubiquitinating enzyme, DUBA), (Figure 3 in **Paper III)**. The OTUD5 protein suppresses type I interferon immune responses (252). Germline variants in the *OTUD5* gene are associated with a

syndrome with multiple congenital anomalies and neurodevelopmental problems (253).
Four samples were positive for antibodies directed against the antigen TNF superfamily member 11b (TNFRSF11B; osteoprotegerin, OPG; osteoclastogenesis inhibitory factor OCIF), (Figure 3 in **Paper III)**. The TNFRSF11B protein is a cytokine receptor for TNF11B/RANKL. This protein has a role in the development of osteoporosis and rare forms of osteopetrosis, and in the development of the immune system (254, 255).
Four samples were positive for antibodies towards the antigen fission Regulator 1 Like (MTFR1L), (Figure 3 in **Paper III)**.
Three samples were positive for antibodies towards the antigen mitochondrial Low Density Lipoprotein Receptor Class A Domain Containing 1 (LDLRAD1), (Figure 3 in **Paper III)**.
Two samples were positive for antibodies towards the antigen Ras-related 35 (RAB35). This protein is involved in membrane trafficking and has been linked to Parkinson´s disease (256, 257), (Figure 3 in **Paper III)**.

Phase 3

Bead arrays analysis were performed with the five selected antigens and their full-length protein counterparts. The reactivities that were demonstrated towards the peptide antigens OTUD5, MTFR1L, LDLRAD1 and RAB35 in Phase 2 were largely confirmed in Phase 3. With the expansion of the 22q11DS cohort, only a few new individuals with autoantibodies towards the peptide antigens were identified, which was also noted in the expanded group of healthy controls, (Figure 4 in **Paper III)**. The antigen TNFRSF11B did not pass the technical criteria and was excluded from further analysis. The reactivities that were demonstrated towards peptide antigens were not confirmed in the analysis of the corresponding full-length proteins.

Patterns of reactivities

Two individuals with 22q11DS shared reactivity to 6 antigens: CLDN6, MTFR1L, NAA35, NAGK, PKD1L3 and TCTE, while another pair of 22q11DS individuals shared reactivity to the three antigens BRF1, CA2 and SARNP. In addition, three pairs of participants shared reactivity to two antigens, respectively, (Figure 5 in **Paper III)**.

When the Gene Ontology Resource and the Panther tool were used to assess the molecular functions of the 146 antigens that were verified as reactive in the bead array, many of the antigens shared molecular functions related to receptor binding, as displayed in Table 2 in **Paper III**.

5 DISCUSSION

Identification of infants with 22q11DS from newborn screening

In **Paper I**, we show that a considerable proportion of infants with 22q11DS can be identified through TREC newborn screening. The TREC numbers in infants with 22q11DS ranged from zero-, to values overlapping those from newborn controls, which presumably reflects the various degrees of thymus impairment in 22q11DS infants. The broad distribution of TRECs in our 22q11DS cohort illustrates that the cutoff applied in the screening program will have an impact on the fraction of all infants with 22q11DS that are identified. This is reflected in the variation in numbers of identified infants with 22q11DS reported by different screening programs (126, 129, 131, 144). The TREC screening programs were primarily implemented to identify infants with SCID or complete athymia and some newborn screening programs have since the start lowered their cutoffs to increase the specificity to detect SCID (127, 137, 143, 258). This will reduce the number of identified cases with non-SCID T-cell lymphopenia such as 22q11DS.

While the early identification of infants with SCID or congenital athymia facilitates timely immune restoring therapy, there is no generally accepted treatment for the immunodeficiency in infants with 22q11DS and thymus hypoplasia and the benefits of early recognition are less obvious. Our study shows that all infants with 22q11DS with abnormal numbers of TRECs at birth have T-cell lymphopenia at the time of diagnosis and lower numbers of cytotoxic T cells and more viral infections during the first year of life, as compared to infants with 22q11DS with normal numbers of TRECs. In a similar retrospective study of infants with 22q11DS carried out in Norway, Gul et al. showed an association between low numbers of TRECs and higher prevalence of T-helper cell lymphopenia at diagnosis (259). While there is no immune-restoring treatment for these infants, early diagnosis and immune work-up facilitate measures to prevent infections and make safe decisions regarding the administration of live vaccines and blood transfusions (24). An additional retrospective study conducted by Barry et al. reported on the outcomes for 11 infants with 22q11DS who presented with low numbers of TRECs in newborn screening (260). Six of the 11 infants had no other evident symptoms of the syndrome at the time of screening and received a diagnosis of the syndrome only after identification in the screening (260). The authors emphasized that the identified infants had comorbid conditions that were recognized during the syndrome-specific work-up that followed (260).

Recognition of 22q11DS through newborn screening raises ethical concerns that need to be discussed. The TREC screening programs were implemented to identify SCID, whereas the 22q11DS is less coherent with the Wilson and Jungner criteria (115). The 22q11DS is undeniably associated with considerable morbidity in many individuals, although the clinical spectrum is wide with some individuals having mild symptoms or delayed presentation as late as in adulthood, for example with psychiatric symptoms (2). But , there is no treatment available to cure the genetic defect.

There are PCR-based assays for the quantification of *TBX1* or other genes located in the deleted region on the newborn screening card, as shown in our study and by others (1, 144). In Norway, a next-generation sequencing panel based on DNA eluted from the newborn screening card and targeting SCID-associated genes has been integrated into the TREC-screening program as a second-tier test (145). The panel also includes the testing of *TBX1* dosage (145). Targeted screening for 22q11DS is often offered based on typical clinical findings, such as observed heart defects in fetuses and infants, although in the absence of typical anatomical anomalies the diagnosis is often delayed (21-23). According to previous studies, the mean age at diagnosis of 22q11DS is 4–7 years years in pediatric cohorts, and the presence of cardiac defects and palatal anomalies was associated with younger age at diagnosis (21, 22, 67). Furthermore, a study by Palmer et al. showed that patients with 22q11DS were seen by physicians from a median of seven sub-specialty areas prior to diagnosis (23). Based on these findings, it is understandable that most parents of affected children, as well as most adults with 22q11DS are in favor of screening for 22q11DS (261, 262). They argue that a correct and timely diagnosis prevents unnecessary trials and facilitates access to appropriate care and support (262). Furthermore, several authors from the 22q11DS research community advocate for general screening for 22q11DS, using the same basis for their arguments (1, 2). The advantages of an early diagnosis need to be balanced against the possible risk of imposing unnecessary anxiety and stress on parents of mildly affected children, with the subsequent risks for negative impacts on bonding (263). If second-tier screening for 22q11DS is not part of TREC screening, some affected infants without clear clinical signs may initially be considered to have idiopathic, self-resolving T-cell lymphopenia. For example, 22 out of 55 infants with idiopathic T-lymphopenia identified in the TREC screening program in California eventually turned out to have a syndromic disorder (137). This emphasizes that one cannot rely on the detection of syndromic stigmata to diagnose 22q11DS, as these might be subtle, even for the experienced physician (3). While there is no formal evidence yet to support general

screening for 22q11DS, it is reasonable to conclude that genetic testing for the syndrome should be offered to infants who present with low numbers of TRECs in newborn screening and typical SCID ruled out.

In **Paper I**, one infant had zero TRECs in the retrospective analysis of the newborn screening card, indicating athymia with complete absence of T cells. This infant died from a CMV infection at 5 months of age, while awaiting a thymus transplant. The infant had no obvious stigmata of the underlying disorder, emphasizing the importance of TREC screening to identify infants with congenital athymia in a timely manner. The follow-up algorithms for TREC newborn screening programs need to include measures to differentiate infants with congenital athymia from infants with typical T-B+ SCID. This is important, as thymus transplantation is the therapy of choice in athymia, rather than HSCT, which is the common treatment for SCID (68, 120, 264, 265). Thymus transplantation allows for the reconstitution of an autologous T-cell compartment (68, 120), whereas stem cells provided by HSCT fail to develop into mature T cells in the absence of a thymus, and only the engraftment of post-thymic T cells is possible. This is reflected by the poor long-term outcome for HSCT in athymic patients (264, 265). Follow-up algorithms for infants with T-B+ SCID phenotypes need to include genetic studies that detect the known causes of hematogenic defects causing SCID, as well as all the known causes of congenital athymia described herein. Since the implementation of TREC newborn screening programs, novel genetic etiologies for congenital athymia have been described, and it is likely that the number of known genetic defects leading to athymia will increase even further in the future (184, 185, 201, 202, 205, 206). In addition, congenital athymia can have non-genetic causes, as exemplified by infants born to diabetic mothers (68). In such cases, *in vitro* models that support T-cell differentiation, i.e., artificial thymic organoids, can help differentiate T-cell lymphopenia due to congenital athymia from SCID caused by primary hematopoietic defects (232, 233). The availability of such models has been limited to a few research centers, whereas they need to be accessible in all regions where TREC screening is implemented.

The long-term effects of low numbers of TRECs at birth in individuals with 22q11DS

The long-term effects of low numbers of TRECs at birth in individuals with 22q11DS were assessed in **Paper II**. At follow-up, the 22q11Low group could be distinguished from the 22q11Normal group and from the healthy controls based on lower numbers of TRECs and smaller proportions of naïve T-helper cells, indicating that abnormal numbers of TRECs at birth is predictive of a persistent limitation of thymus output in this group.

Consequently, the effects of homeostatic events on the T-cell compartment were also more-evident in the 22q11Low group, as exemplified by qualitative aberrations in the receptor repertoires of the T-helper cells, and shorter telomere lengths, as well as a trend towards increased clonality of the naïve cytotoxic T lymphocytes. A previous study of adults with 22q11DS reported similar findings (29). These aberrations are often referred to as signs of immunologic senescence, which typically develop as the thymus becomes progressively involuted with increasing age (45). A similar immunologic profile has been reported following childhood thymectomy (223, 266). Immunologic senescence contributes to the impaired immune competence seen in elderly individuals, and might partly explain the increased frequency of infections, autoimmunity and cancer observed with increasing age (45). The 22q11Low individuals in the present study did not report more-frequent or more-severe infections compared with the 22q11Normal group. An increased frequency of infections was instead reported in both groups of 22q11DS individuals, as compared to the healthy controls. This is in line with previous studies, which have mainly associated infectious problems in 22q11DS with anatomic anomalies of the palate and throat, rather than with T-cell lymphopenia (64). Recent studies based on large 22q11DS cohorts have, however, linked T-cell lymphopenia in persons with 22q11DS to an increased risk for autoimmunity and immune dysregulation in the form of atopy (54, 64, 91-94). Furthermore, a large epidemiologic study reported on increased risks for autoimmunity and cancer following childhood thymectomy (226). The individuals in our study were still young at follow-up, which means that there is a risk that the immunologic aberrations might progress with age, leading to increased risks for autoimmune diseases and other immune-dysregulatory complications.

In **Paper III**, we used a high-density array to demonstrate an increased frequency of autoantibodies with stronger reactivities in the 22q11Low group, as compared with the 22q11Normal group and healthy controls. Our findings of a broader array of autoantibodies in the 22q11Low group are in line with those of previous studies associating T-cell lymphopenia in 22q11DS, and particularly low numbers of naïve T-helper cells, with autoimmunity (54, 64, 91-94). The T-cell immunodeficiency in 22q11DS is caused by thymus hypoplasia, and it is logical to conclude that the autoantibodies arise from loss of central tolerance, due to impaired selection processes in the thymus. The antigens that were demonstrated to be reactive in our study were, however, not representative of tissue-restricted antigens that are known to be presented by self-MHCs on mTECs in the thymus during negative selection (169). Furthermore, the autoimmune manifestations in 22q11DS are not equivalent to the manifestations in

patients with impaired negative selection in the thymus, such as in APS1 (207). Therefore, it seems more likely that the autoantibodies develop as a result of loss of peripheral tolerance, driven by T-cell lymphopenia. In T-lymphopenia, homeostasis of the T-lymphocyte compartment is mainly mediated by peripheral proliferation (38). Proliferation, induced by lymphopenia, has been associated with autoimmunity in both murine models, and in human disorders, such as Omenn syndrome and Wiskott-Aldrich syndrome (38). T lymphocytes with low affinity for self-antigen-MHC complexes are thought to undergo to slow homeostatic proliferation, mediated by IL-7 and IL-15, whereas T cells that bind with high affinity to self-antigen-MHC complexes can proliferate faster under inflammatory conditions, driven by cytokines such as IL-6 (38). Regulatory T cells play a role in controlling homeostatic mechanisms, and their reduced numbers or impaired suppressive function can contribute to autoimmunity (38). In summary, it is possible that a multi-step process leads to the development of autoimmunity in 22q11DS is: 1) impaired thymic functions leads to T-cell lymphopenia; 2) the lymphopenia induces proliferation that favors self-reactive T cells; 3) frequent infections create the conditions particularly for T cells with high affinity for self-antigen-MHC complexes; and 4) the reduced number of regulatory T cells impairs their ability to control the homeostatic mechanisms, resulting in autoimmunity.

Our study was not designed to determine whether the identified autoantibodies drive pathogenic mechanisms. Even so, the increased frequency of autoantibodies in the 22q11Low group may indicate autoimmune predisposition and immune dysregulation, which might, in time, have clinical consequences. A register-based study that included more than 2,000 patients with various inborn errors of immunity showed that early onset of autoimmunity, often in the form of ITP and/or AIHA, implied a poor prognosis, especially for patients with a T-cell deficiency (267). In combined immunodeficiencies and in CVID, early onset autoimmune manifestations are associated with increased risks to develop severe complications, such as granulomatous-lymphocytic interstitial lung disease (GLILD), which in turn is associated with premature death (268). There are still no large studies, only a few case reports, regarding immune complications in adults with 22q11DS. There are four case reports non-malignant lymphoproliferation as a complication to 22q11DS, either in the form GLILD or lymphoproliferation affecting the liver: and in all cases the lymphoproliferation was preceded by autoimmune hematologic manifestations in childhood (67, 269-271).

Malignant lymphoproliferation (as in lymphoma), also in these cases preceded by ITP, has been described in patients with 22q11DS (78-83). New studies are needed to clarify if early onset autoimmune manifestations and/or the presence of autoantibodies in 22q11DS entail an increased risk of future immune-dysregulatory complications, as is the case for other forms of inborn errors of immunity (267). Furthermore, long-term follow-up studies of the TREC screening programs that include high numbers of individuals with 22q11DS are needed to determine whether low numbers of TRECs at birth confer an increased risk of manifest autoimmune disease. The international follow-up guidelines for pediatric patients with 22q11DS include recommendations for repeated immunologic assessments and follow-up by a pediatric immunologist (24). By comparison, the follow-up guidelines for adult patients state that the clinical demand for immunologic follow-up is weak, and they recommended that such follow-up should be limited to those with opportunistic infections or hypogammaglobulinemia (25). Given the poor prognosis for early onset autoimmunity in other inborn errors of immunity, and the lack of evidence in cases of 22q11DS, it is reasonable to consider increased surveillance also for adults. This includes follow-up by an immunologist who is familiar with immune-dysregulatory complications, for those 22q11DS patients who have early onset autoimmune manifestations.

The striking overlap in reactive antigens between the 22q11Low and 22q11Normal groups demonstrated in **Paper III** is interesting, and many of these overlapping antigens were verified as autoreactive when we applied a different method (the bead array). Further studies of these antigens are desirable, as they might provide insight into the mechanisms that drive autoimmunity in 22q11DS, which in turn might pave the way for new treatments. Furthermore, it is possible that some of these autoantibodies have pathogenic properties, and one speculation is that some of the phenotypic heterogeneity seen in 22q11DS could be explained by autoimmunity. There is, for example, growing evidence that a dysregulated immune system has a role in the pathogenesis of schizophrenia, which is clearly over represented in 22q11DS (272, 273), and a few studies have indicated immune dysregulation or inflammation in association with psychosis in 22q11DS (272, 274). Autoantibodies are easy to monitor and often appear before the onset of autoimmune symptoms (275). It is possible that future studies will identify specific autoantibodies that can be used as biomarkers for immune dysregulation in 22q11DS. Such biomarkers would help to stratify 22q11DS patients who would benefit from immunologic follow-up.

6 CONCLUSION

This study shows that a subpopulation of infants with 22q11DS are identified in TREC newborn screening programs. The number of infants with 22q11DS identified depends on the cutoff applied in the screening program. Low numbers of TRECs at birth in individuals with 22q11DS were associated with a more evident limitation of thymus output and signs of premature immune senescence at follow-up. Furthermore, low numbers of TRECs at birth were linked to an increased frequency of autoantibodies at follow-up. Even so, the two groups of 22q11DS individuals in the study showed a clear overlap of autoantibody specificities. It is possible that some of these autoantibodies are specific for the syndrome. Considering the young age of the individuals in the study at follow-up, and that immunologic aberrations may progress with time, risks to acquire autoimmune disease and other immune-dysregulatory disorders will possibly increase with older age. Until there is further evidence on the clinical implications, our findings support the need for long-term immunologic monitoring of individuals with 22q11DS and low TREC numbers at birth. Such monitoring can form a basis for safe decisions regarding vaccination with live strains of virus or bacteria, and regarding the administration of blood transfusions and to take action to prevent infections. During adolescence and adulthood, increased surveillance for autoimmune and immune-dysregulatory complications is warranted.

7 FUTURE PERSPECTIVES

The fact that 22q11DS is a relatively common condition and is associated with a considerable burden of disease for many of the affected individuals means that this syndrome warrants further investigation. The heterogeneous clinical presentation makes diagnosis based on clinical findings difficult, which is one reason why the condition is still under-recognized. The heterogeneity also increases the demand for individualized care, which often involves multidisciplinary teams and comes with high costs (26). Consequently, biomarkers to guide risk stratification, which could serve as the basis for individualized follow-up schemes, are needed. Several studies have confirmed that low numbers of T cells are linked to autoimmunity in 22q11DS. Our research shows that low numbers of TRECs at birth in individuals with 22q11DS are associated with long-term aberrations of the immune system. Further clinical trials and multicenter collaborations are needed to clarify whether these aberrations are linked to clinical consequences, such as autoimmunity. By merging immunological and clinical data from several 22q11DS cohorts, sufficient power could be attained to allow for multidimensional analytics, identifying new patterns among biomarkers that can distinguish clinical entities. Overall, knowledge regarding the immune complications in adults with 22q11DS is sparse and needs more research. Autoantibodies are easy to monitor and are thus suitable as biomarkers. Based on our findings, there may be autoantibodies that are specific to 22q11DS. Furthermore, our findings indicate that autoantibodies have the potential to serve as biomarkers for an immune-dysregulatory state in 22q11DS. Our future research will be aimed at studying autoantibodies in 22q11DS in more detail in larger cohorts. It is also possible that autoantibodies contribute to disease manifestations in 22q11DS, which opens up an opportunity for targeted treatments in the future.

Since the first cases of congenital athymia were described in the 1960s, it has contributed to our current understanding of the role of the thymus in cellular immunity (4). Although much knowledge has been gained on the complex immune system since then, many aspects of the mechanisms leading to immune-related diseases remain to be unraveled. Studies of 22q11DS may still serve as a model, to illustrate the role of the thymus in the development of common health problems, such as increased susceptibility to infections, autoimmunity and cancer.

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